Current Topics in Behavioral Neurosciences 7

Jim J. Hagan *Editor*

Molecular and Functional Models in Neuropsychiatry

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Current Topics in Behavioral Neurosciences

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Jim J. Hagan
Editor

Molecular and Functional Models in Neuropsychiatry

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For Carole, Luke and Kate

Preface

Despite decades of research, common neuropsychiatric diseases remain enigmatic and debilitating disorders which are associated with significant human and economic costs. For example, autism and attention-deficit disorder blight the lives of the young, and schizophrenia, with its profound functional impairments, often impacts in early adulthood with devastating lifelong consequences. Major depressive disorder affects 5–9% of women and 2–3% of men and, according to the World Health Organization (WHO), about 4% of the world's population suffers from some form of drug abuse disorder.

The development of more effective treatments for these and other neuropsychiatric disorders requires scientific progress on a broad front. Animal models have a vital role to play in advancing the field. When deployed in conjunction with detailed study of these diseases in man, they bring the power to make controlled experimental interventions, which allow the functional consequences of genetic variations and polymorphisms to be understood in terms of their cellular systems and behavioural effects. Further, they provide a means by which complex cognitive and behavioural phenomena may be dissected and understood. Finally, they provide a bridge to understanding the effects of drugs on the functioning of the central nervous system, thereby improving our understanding of the actions of those drugs in man.

This volume discusses some of the latest and most exciting advances. The selection of topics eschews the conventional approach of organizing material by discipline, focusing instead on more eclectic, multidisciplinary approaches. It reflects a personal perspective of those areas in which exciting and important new developments are taking place. These span the established areas of study, reflecting both technical and theoretical advances, but also encompass emergent areas such as the use of MRI in the study of systems responses, epigenetic regulation and gene/environment interactions, all topics which will surely play an increasing role in the scientific discourse related to neuropsychiatric diseases.

It is over 60 years since Pauling (Pauling et al. 1949) elucidated the notion of molecular medicine in the context of sickle cell anaemia. The coming decades must see the concept firmly embedded in the practice of neuropsychiatric medicine if the

much needed improvements in therapy are to be delivered. The work discussed in this volume shows some of the ways in which this vision is being realized.

I am grateful to the authors, all leaders in their fields, who so willingly devoted their time, energy and expertise to share their research perspectives and produce a volume which I hope will inform and excite students and experts alike.

February 2011 Jim J. Hagan

Reference

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Contents

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Mouse Mutagenesis and Disease Models for Neuropsychiatric Disorders

Yoichi Gondo, Takuya Murata, Shigeru Makino, Ryutaro Fukumura, and Yuichi Ishitsuka

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Abstract In this chapter, mutant mouse resources which have been developed by classical genetics as well as by modern large-scale mutagenesis projects are summarized. Various spontaneous and induced mouse mutations have been archived since the rediscovery of Mendel's genetics in 1900. Moreover, genome-wide, large-scale mutagenesis efforts have recently been expanding the available mutant mouse resources. Forward genetics projects using ENU mutagenesis in the mouse were started in the mid-1990s. The widespread adoption of reverse genetics, using knockouts and conditional mutagenesis based on gene-targeting technology, followed. ENU mutagenesis has now evolved to provide a further resource for reverse genetics, with multiple point mutations in a single gene and this new approach is described. Researchers now have various options to obtain mutant mice: point mutations, transgenic mouse strains, and constitutional or conditional knockout mice. The established mutant strains have already contributed to modeling human diseases by elucidating the underlying molecular mechanisms as well as by providing preclinical applications. Examples of mutant mice, focusing on neurological and behavioral models for human diseases, are reviewed. Human diseases caused by a single gene or a small number of major genes have been well modeled by corresponding mutant mice. Current evidence suggests that quantitative traits based on polygenes are likely to be associated with a range of psychiatric diseases, and these are now coming within the range of modeling by mouse mutagenesis.

Keywords ENU · Forward genetics · Functional genomics · Model mouse · Mutagenesis · Neurological mutation · Reverse genetics

Abbreviations

AD	Alzheimer's disease
APP	Amyloid precursor protein
CD	2-Hydroxypropyl-b-cyclodextrin
CNS	Central nervous system
DA	Dopamine
DAAO	D-amino acid oxidase
EA	Episodic ataxia
EMMA	European Mutant Mouse Archive
ENU	*N*-ethyl-*N*-nitrosourea
EUCOMM	European Conditional Mouse Mutagenesis
FST	Forced swim test
FXTAS	Fragile X tremor/ataxia syndrome
G0, G1, G2	Generation-0, Generation-1, Generation-2
GAG	Glycosaminoglycan
GSL	Glycosphingolipid
HD	Huntington's disease

HRM	High-resolution melting
IMSR	International Mouse Strain Resource
KO	Knockout
KOMP	Knockout mouse mutagenesis project
L100P	Leu100Pro
LI	Latent inhibition
MEB	Muscle–eye–brain disease
MPS	Mucopolysaccharidosis
NMDAR	*N*-methyl-D-aspartate receptor
NorCOMM	North American Conditional Mouse Mutagenesis
NPC	Niemann–Pick C disease
NSAID	Nonsteroidal anti-inflammatory drug
OMIM	Online Mendelian inheritance in man
PD	Parkinson's disease
PPI	Prepulse inhibition
PS	Presenilin
Q31L	Glu31Leu
RIKEN BRC	RIKEN BioResource Center
SCA	Spinocerebellar ataxia
TAF	TBP-associated factor
TBP	TATA-binding protein
TFTC	TBP-free TAF-containing complex
TGCE	Temperature gradient capillary electrophoresis

1 Introduction

Classically, spontaneous mutations or polymorphic alleles were the only resource available for mouse genetics. Fancy mice carrying various visible mutations provided a good resource for genetic studies. Eye and coat-color mutations of *agouti, brown, albino, dilute*, and *pink-eyed dilution* were originally designated as *A*, *B*, *C*, *D*, and *E* loci by indicating their mutant alleles with lowercase letters. All the mutant allele names (*a*, *b*, *c*, and *d*) still stand even now except for the *pink-eyed dilution* that has been renamed as *p*. These loci and mutant alleles were contributed to advance the study of Mendelian inheritance in the animal kingdom. The very first genetic linkage in a mammal was indeed discovered between albino *c* and pink-eyed dilution *e* (or *p* at present) loci in 1915 (Haldane et al. 1915). At present, this linkage group is mapped on chromosome 7. Efficient mutagens such as X-ray and various chemical mutagens then accelerated the production of mutant alleles genome-wide. The mutagenic activity of X-rays was originally discovered in the mouse (Little and Bagg 1923) although the validation of Mendelian inheritance of the X-ray-induced mutation was first accomplished in *Drosophila melanogaster* (Muller 1927).

Mouse genetics have not only pioneered the field of classical genetics but are also on the frontier to develop modern genetics and functional genomics.

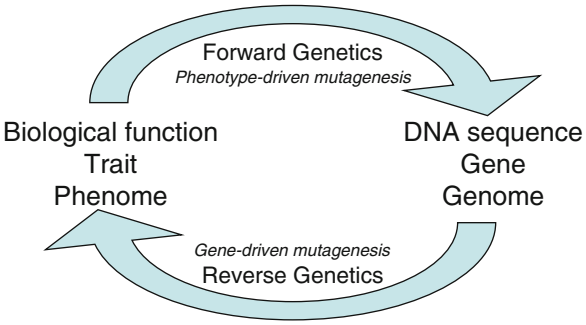

Fig. 1 Two basic schemes of mutagenesis for functional genomics. To conduct forward genetics, the screening of a key mutant phenotype is crucial. Then, genetic mapping and positional cloning identify the responsible mutation in the DNA sequence. In reverse genetics, the gene-targeting technology introduces a mutation to a particular gene first, and then the embryonic engineering system produces live mice carrying the mutation and allows the functional analysis of the gene

For instance, in 1982 the giant mouse was constructed using transgenic mouse technology (Palmiter et al. 1982). Then, combining embryonic stem cells and gene-targeting technology, the knockout (KO) mouse also became available (Thomas et al. 1986; Doetschman et al. 1988; Robertson et al. 1986). The conventional transgenic and KO mouse methods allow explorations of loss and gain of functions, respectively, associated with particular DNA sequence. The KO mouse method also made it possible to conduct site-directed mutagenesis for the first time in mammals. As shown in Fig. 1, classic mutagenesis is now called "forward genetics" with a phenotype-driven approach to elucidate the gene function. On the other hand, the KO mouse approach is termed "reverse genetics" based on its gene-driven approach.

Systematic repositories provide an essential starting point for researchers seeking mouse models. The first step for investigators wishing to develop new models may be to check the International Mouse Strain Resource (IMSR; http://www.findmice.org/) and other mutant mouse resources such as Jackson Laboratory (http://www.informatics.jax.org/), RIKEN BioResource Center (RIKEN BRC; http://www.brc.riken.jp/lab/animal/), and the European Mutant Mouse Archive (EMMA; http://www.emmanet.org/). If appropriate mutant mice are unavailable, a variety of methods are available to construct new models. In this chapter, the various construction methods of mutant mice are reviewed. Some examples of established mutant mice are then discussed, particularly focusing on models of neurological disorders.

2 Forward Genetics with *N*-Ethyl-*N*-Nitrosourea

Genetics directly associates genes with traits. Classically, a mutant strain is established by its heritable trait first, and then the causative gene is positionally cloned to understand the biological function (phenotype) at the molecular level (DNA sequence and gene expression). In this forward genetic approach, a mutant mouse

strain may be obtained from a preexisting mutation in a natural population as polymorphism. Alternatively mutant strains are generated using mutagenesis. In either case, a large number of mice must be screened to obtain the necessary mutations.

2.1 Phenotype-Driven Mutagenesis

Among the various mutagens discovered and studied in the mouse, *N*-ethyl-*N*-nitrosourea (ENU) was found to be one of the most potent (Russell et al. 1979, 1982; Hitotsumachi et al. 1985). X-rays cause large deletions, often exceeding a mega base of base pairs (Lyon et al. 1992). On the other hand, ENU induces mostly, if not exclusively, base substitutions (Noveroske et al. 2000). These mutagens randomly induce mutations genome-wide, and a phenotype-driven approach (forward genetics) is usually necessary to identify causative gene by positional cloning.

The positional cloning of human genetic diseases started in late 1980s, followed by initiation of the human genome sequencing project. The importance of developing model animals had been anticipated. In particular, mutant mice were expected to be one of the best model animals since the mouse is only the mammalian species in which the various embryonic and genetic engineering technologies have been established, has a short generation time, and is small in size. As a result, it has been one of the main species used in genetic studies. In the mouse, numerous genetic tools have also been furnished with many inbred strains and genetic markers. To effectively produce human disease models, phenotype-driven mouse mutagenesis studies with ENU have been carried out on a large scale from the mid-1990s (reviewed by Gondo 2008). The basic scheme of ENU mouse mutagenesis is depicted in Fig. 2.

In early 1990s, Dove and his colleagues had successfully developed a human disease model using the ENU approach (Moser et al. 1990; Su et al. 1992). They had found a mutant line that modeled human familial polyposis and positionally identified an ENU-induced mutation in the responsible gene, Apc (Moser et al. 1990; Su et al. 1992). Takahashi's group had also vindicated the power of ENU mutagenesis by discovering a novel gene, *clock*, by the phenotype-driven approach (Vitaterna et al. 1994; King et al. 1997). This was the first identification of a gene controlling circadian rhythms in a mammalian species. Using running wheels to continuously monitor locomotor activity, Vitaterna et al. (1994) discovered a dominant mutant founder mouse which exhibited a circadian period approximately 1 h longer than the 23.7-h period found in wild-type controls. These pioneering studies gave the rationale and incentive to conduct ENU mouse mutagenesis on a large scale, genome-wide. The overall scheme for an ENU mutagenesis study in the mouse is summarized in Fig. 2. The generation-1 (G1) mice, which exhibit some phenotypic anomaly compared to the littermates, are the mutant candidates. When the characteristic phenotype identified in G1 is inherited in G2 offspring in a Mendelian fashion, the G1 strain is established as a new mutant line.

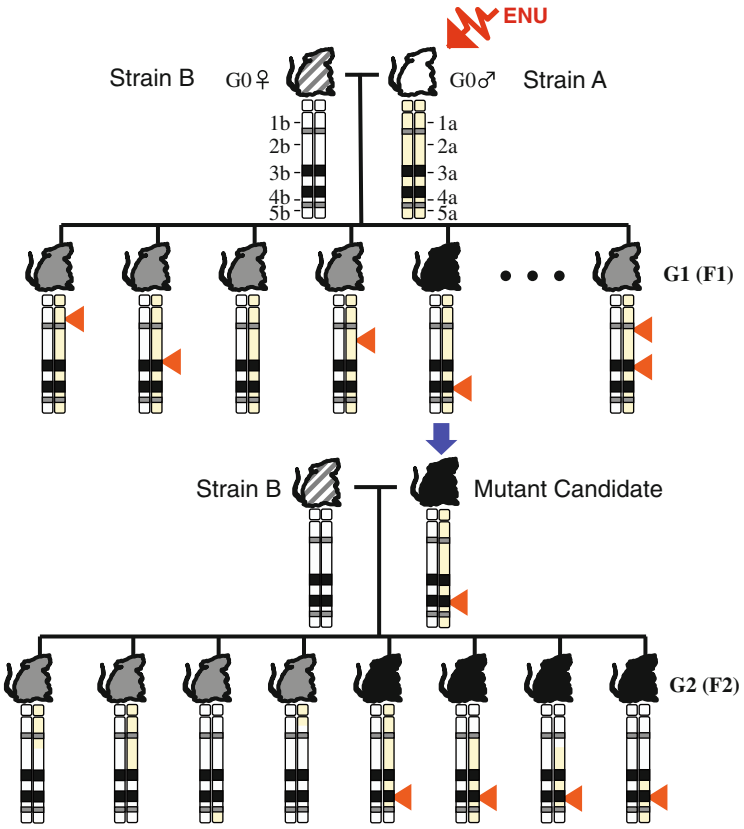

Fig. 2 Large-scale mouse mutagenesis with ENU in the mouse. A large number of Generation-1 (G1) mice are produced from the ENU-treated G0 mice. G1 traits are subjected to phenotypic screening. Mutant candidate G1 is then mated to confirm the inheritance with respect to the identified trait. When two different inbred strains are used in G0 parents, this mating scheme also directly provides the information for the mapping of the established mutant in G2. The tightly linked molecular markers provide the genetic mapping of the mutation in the mouse genome. In this schematic chromosome in G2 mice, the marker for locus **4** in strain A (**4a**) is tightly linked to the mutant phenotype ("*filled*" trait); thus the causative gene (*triangle*) is very close to locus **4**. See Sect. 2.2 for details. It is noteworthy that completely recessive mutations cannot be identified in this scheme

2.2 Gene Mapping and Positional Cloning

Forward genetics with ENU efficiently provides model mice for human diseases. An appropriate phenotyping platform, based on diagnostic systems of human diseases, allows the establishment of mutant strains knowing neither the causative genes nor the molecular pathways. Rather, mutant strains are established by the identification of some phenotype(s) similar to the targeted human disease. The pioneering work described in the previous section focused on a particular trait

related to either tumorigenesis in the intestine (Moser et al. 1990; Su et al. 1992) or circadian rhythms (Vitaterna et al. 1994; King et al. 1997). The subsequent large-scale ENU mouse mutagenesis projects have conducted more comprehensive phenotyping to established model mice more efficiently using, for example, a battery of morphological, sensory, and behavioral measures known as SHIRPA (Rogers et al. 2001), which incorporates hematological and biochemical analyses of blood, blood pressure measurement, and/or funduscopy as part of a comprehensive phenotyping battery.

Based on the indentified heritable trait, the causative gene is genetically mapped and positionally cloned. As shown in Fig. 2, a G1 mouse carrying a mutant trait in an F1 genetic background is mated to the strain **B**. At first, by analyzing ~50 F2 offspring (=G2) with ~100 genetic markers between strain **A** and **B**, the causative gene is roughly mapped in an order of 5–10 cM of the genetic distance between the mutation and markers. After the rough mapping, the fine mapping analysis with more genetic markers in the roughly mapped region and with 400 or more progeny from the backcross narrows down the mutant locus in the mouse chromosome. In these genetic mapping studies, it is necessary to produce 500 or more of the F2 (G2) mice to pinpoint the candidate region within approximately 1 cM. Naturally, denser genetic markers provide less experimental deviations as well as more concrete landmarks to conduct the fine physical mapping. After narrowing the genetic distance between the mutation and closest marker(s) down to ~1 cM or less, the physical mapping is conducted as follows. Genomic DNA clones covering all the closest genetic marker(s) corresponding to the genetic mapping region are isolated from a genomic DNA library prepared from the mutant mice. In the mouse genome, 1 cM corresponds to roughly 2 Mb. Thus, even if the genetic mapping narrows the mutated genomic sequence down to 1 cM, the genomic clones should cover, at least, a 2 Mb region. The resolution of the fine mapping is in the order of a megabase pair. On the other hand, the standard size of a cloned genomic DNA fragment even in the BAC library is an order of 100 kb. This tenfold gap between the genetic mapping and the size of molecular clones renders positional cloning time-consuming and painstaking.

2.3 Features of Phenotype-Driven ENU Mouse Mutagenesis

Large-scale ENU mouse mutagenesis does not require prior knowledge of genes and genomic sequences as shown above. It is a classical forward genetics that necessarily requires neither any recombinant DNA nor advanced embryonic engineering technologies but does require a large mouse facility and high-throughput phenotyping platforms. Its comprehensiveness is basically dependent on how many G1 mice are screened and how extensively their phenotypes are accessed. In a large-scale project, usually thousands of G1 mice are systematically screened by phenotyping their size, weight, color, morphology, behavior, neuromuscular coordination, vision, hearing, hematology, clinical biochemistry, histopathology, and so on (e.g., German Research

Center for Environmental Health ENU mutagenesis, http://www.helmholtz-muenchen.de/en/ieg/group-functional-genetics/enu-screen/; MRC, http://www.har.mrc.ac.uk/research/mutagenesis/phenotype_driven_screens.html; RIKEN, http://www.brc.riken.go.jp/lab/gsc/mouse/AboutUs/screening.htm). However, there are some drawbacks; for example, the approach is very effective for dominant but not for recessive mutant phenotypes. Furthermore, identified mutant phenotype(s) may not always be inherited in a simple Mendelian fashion. Some identified mutant mice may also suffer sterility or even lethality depending on the mutant phenotype, and it is too late to preserve such mutant strains after the onset of fatal phenotypes, since it is a dominant phenotype-driven mutagenesis and the G1 founder mouse is only the resource at the time of phenotypic screening. To maintain such disease models, all the G1 sperm samples are surgically obtained and cryopreserved in liquid nitrogen semipermanently before the onset of the fatal phenotypes (Inoue et al. 2004). This frozen sperm archive has provided the key resource for the next-generation gene targeting in the mouse (see Sect. 4.1).

3 Reverse Genetics with Gene Targeting

The draft human genome project to sequence all human genomic DNA was completed in 2001 (International Human Genome Sequencing Consortium 2001). The completion of the human genome sequencing project showed, to the surprise of the research community, that the human genome potentially coded less than 30,000 genes in the entire human genome, whereas it had been previously estimated to code for over 100,000 genes (Chikaraishi et al. 1978). Immediately following the whole human genome sequencing, the mouse became the second mammalian species to have its genomic DNA fully sequenced (Mouse Genome Sequencing Consortium 2002). The completion of human and mouse genome projects spurred the initiation of large-scale mouse mutagenesis efforts to conduct gene targeting or gene KO studies for all of the mouse genes. The downward revision of the estimated number of genes in the human and mouse genomes improved the feasibility of these projects and improved the chances of successful completion. The coding sequences in the human and mouse genomes were estimated to be only 1–2%. In 2003, mouse researchers gathered at the Banbury Center at the Cold Spring Harbor Laboratory, USA, and proposed an international mouse KO/conditional mutagenesis projects (Austin et al. 2004; Auwerx et al. 2004) and led, in 2006, to a 5-year international collaborative effort with three major projects; the Knockout Mouse Project (KOMP) in the USA, the European Conditional Mouse Mutagenesis (EUCOMM) project in Europe, and the North American Conditional Mouse Mutagenesis (NorCOMM) in Canada. To coordinate the projects, the International Mouse Knockout Consortium was also organized (International Mouse Knockout Consortium 2007). The basic scheme of gene targeting, KO/conditional mutagenesis, and the features of the International Mouse Knockout Consortium are described in the following section.

3.1 Gene Targeting

In 2007, the Nobel Prize in physiology or medicine was awarded to Drs. M. R. Capecchi and O. Smithies for gene targeting and Dr. M. J. Evans for ES cell development, which made it possible for the first time to conduct site-directed mutagenesis in the mouse. Briefly, a targeting vector containing genomic DNA fragments of the targeting gene that is disrupted by a drug resistant gene, such as the G418-resistant gene, is introduced into mouse ES cells. The rare event of homologous recombination between the targeting vector and host genomic DNA substitutes the corresponding endogenous genomic sequence in the target gene for the disrupted sequence in the targeting vector. After establishing the homologous-recombinant ES cell line, live mice carrying the disrupted allele in the targeted gene may be generated by germline transmission through germline chimeric mice. The approach primarily disrupts the targeted gene providing a null-type allele. KO mice often result in recessive lethality during embryogenesis due to disruption of the essential functions of the target gene during early development. Such developmentally essential genes may often exert different functions in adulthood or may even be dispensable in the adult, depending on the tissue type and/or phase in development phase (Duyao et al. 1995; Nasir et al. 1995). To elucidate the functions gene throughout in later developmental stages, it is therefore necessary to develop mutant mice in which the essential function in the early embryogenesis remains intact and deletion is targeted to the tissue and/or developmental time to be disrupted. An alternative approach is to produce a hypomorph-type mutation rather than a null-type by knock-in targeting (see Sect. 7).

3.2 Conditional KO Mouse Mutagenesis

The Cre–loxP system has widely been used to maintain expression of an essential gene during embryogenesis and to turn it off in a particular tissue and/or at a specific developmental time (for review, see Yu and Bradley 2001). The Cre enzyme specifically and efficiently mediates homologous recombination between two copies of the loxP sequence. When two copies of loxP reside in the same DNA strand with a head-to-tail orientation, the intervening sequence between the two loxP is excised out by the Cre recombinase. A basic procedure is as follows. In one gene-targeting strain, two loxP sequences and the G418 gene are placed in two introns by the gene-targeting method without disturbing the expression of the target gene. Independently, a Cre-expressing transgenic mouse strain is generated with the expression of Cre controlled by an appropriate promoter, so that it is expressed in a specific tissue or at a specific developmental time in an inducible manner, depending on where and when the target gene is to be knocked out. Finally, in the offspring obtained by mating the two strains, the Cre expression conditionally disrupts the target gene in a manner determined by the functional characteristics of the promoter of the Cre recombinase.

A hypomorph-type mutation is constructed using a similar strategy to the Cre–loxP conditional mutagenesis described above. A point mutation(s) instead of loxP sequences is introduced into the critical coding exon. The constructed knock-in mouse thus carries, for instance, a single amino acid substitution and is a suitable approach for evaluating human diseases associated with identified SNPs.

3.3 Features of the International KO Mouse Project

The International Mouse Knockout Consortium (2007) aims to collaboratively establish targeted ES cell strains covering all the mouse genes. So that the research community is able to freely obtain KO mice for any gene, thus avoiding redundant efforts to target the same genes. In addition, the genetic background of ES cell lines, targeting vectors, and constructed mutant mice may be standardized. Thus, it also becomes possible to directly compare the results of analyses among various researchers and laboratories. The targeting vector is designed to have a "KO first and conditional ready" structure, so that users may apply the established mutant stain for the conditional disruption of the target gene. This international effort tremendously reduces the time, cost, and manpower necessary to construct every mouse gene to be knocked out. However, there are some drawbacks. Users usually have to derive live mutant mice from the established ES cell line(s), and various transgenic strains that drive the Cre expression under variety of promoters must be independently constructed to fully utilize the conditional mutagenesis option. It must be noted that the KO mouse project aims to disrupt only the protein-coding sequences that constitute about 1–2% of the mouse genome. However, mutations and SNPs in noncoding sequences often give rise to altered biological functions and may be responsible for some human diseases (Emison et al. 2010). Recently, it has also been proposed to elucidate the biological functions of noncoding RNAs (Mattick 2009). The functional analysis of noncoding sequences remains to be elucidated by other approaches.

4 Next-Generation Gene Targeting with ENU

As shown in Sect. 3.2, point mutations constructed by knock-in mutagenesis provide effective tools to elucidate the fine biological functions of target genes at the level of each base pair. The conventional gene targeting method is, however, not efficient enough to develop a variety of point mutations for even a single gene. A new gene-targeting system to provide various point mutations in any target genes (and any target genomic sequences including noncoding sequences) has been developed, paradoxically using ENU mutagenesis. As described in Sect. 2, ENU randomly induces point mutations without bias for particular base pairs, and when a large enough collection of ENU-induced mutations are collected and archived, any target genes or genomic sequences may contain sufficient allelic point mutations to

enable their fine biological functions to be elucidated. The new reverse genetics, ENU-based gene-driven mutagenesis, has become possible by archiving tens of thousands of G1 mutant mouse strains as frozen sperm (Fig. 2) with high-throughput mutation discovery systems. This "next-generation" gene-targeting approach provides an important additional mouse mutagenesis system to the research community (Gondo 2008; Gondo et al. 2009). The schematic flow of the ENU-based gene-driven mutagenesis is shown in Fig. 3.

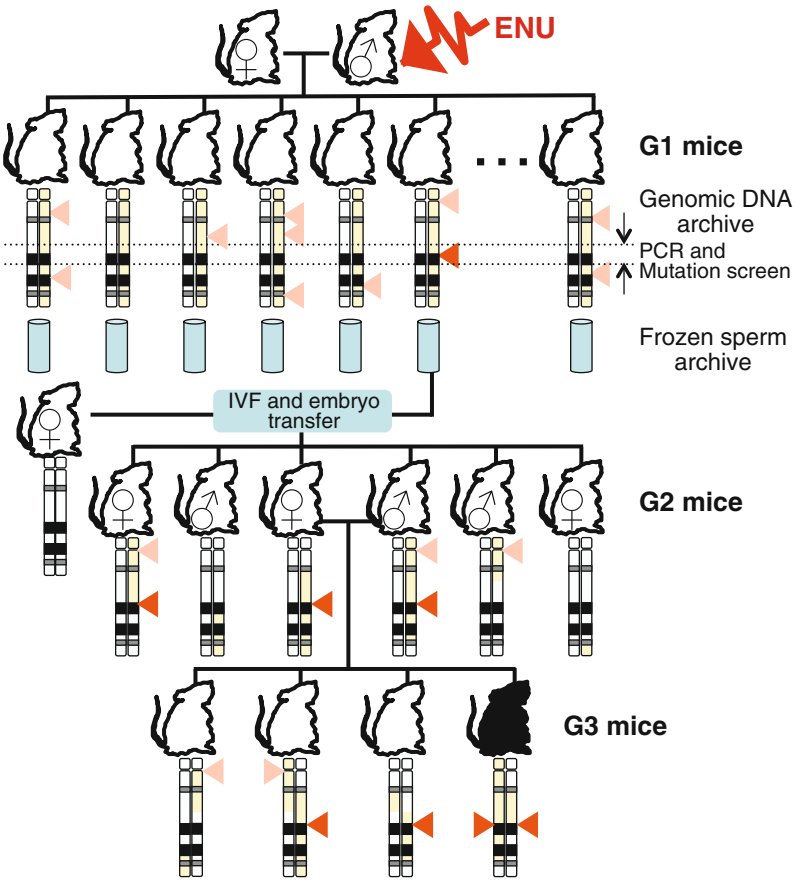

Fig. 3 Next-generation gene targeting: ENU-based gene-driven mutagenesis. ENU mutant mouse library consists of dual archives of G1 genomic DNA and sperm samples. PCR primers are designed to amplify the target gene. The PCR products are then subjected to a high-throughput mutation discovery system. The G1 strains, which are found to carry an ENU-induced mutation (*filled triangle*) in the target gene, are revived by in vitro fertilization (IVF) and embryo transfer technologies. G2 progeny are genotyped and a heterozygous pair is mated to produce G3 mice, one quarter of which are expected to become homozygous for the discovered mutations. Since a G1 mouse carries 3,000 mutations, G3 mice still have many unidentified ENU-induced mutations (*shaded triangles*)

4.1 Mutant Mouse Library: G1 Mouse Archives by ENU Mutagenesis

To make the ENU-based gene-driven mutagenesis possible, it was necessary to first archive sufficient numbers of ENU-induced point mutations. As described in Sect. 2.3, G1 mice carrying ENU-induced mutations have often been archived as frozen sperm, providing the resource for the new reverse genetics. Summarizing the pioneering analyses of ENU-induced mutations in the G1 archive, it was found that each G1 mouse carried roughly 3,000 base substitutions (reviewed by Gondo et al. 2009). For instance, at RIKEN, approximately 10,000 G1 mice have been archived, which implies that 3×10^7 point mutations are ready for functional analyses. Considering that the size of the mouse genome is 3×10^9 bp, on average we may find a point mutation every 100 bp across the genome. Hence, it is large enough to be used as a mutant mouse library. To screen for ENU-induced mutations in the target gene, the genomic DNA from each G1 mouse has also been archived. The dual archives of G1 sperm and genomic DNA form the mutant mouse library resource needed for the next-generation gene targeting. It is noteworthy that only half of the G1 mice produced so far are currently archived as mutant mouse libraries, since only the G1 males are preserved as frozen sperm. The establishment of an efficient and reliable ovary cryopreservation method should, making the G1 females available for the library, contribute significantly to the construction of a complete mutant mouse library, Alternatively, cryopreservation of fertilized G1-oocytes and/or clone mouse construction from G1 female cells with a quick and cost-effective method may also make G1 females available for the construction of the library.

4.2 High-Throughput Mutation Discovery Systems

As shown in Fig. 3, PCR amplification of the target gene has been the first step in finding ENU-induced mutations in target sequences. To identify the ENU-induced mutation(s) in the amplified fragment, direct DNA sequencing with the Sanger method would be the simplest and most straightforward method. However, each G1 genome has 3,000 mutations in 3×10^9 bp of the genome, implying that to find one ENU-induced mutation, 1 Mb ($=3 \times 10^9$ bp divided by 3,000 mutations) of G1 genomic DNA must be sequenced. Thus, it has not been practical to screen for ENU-induced mutations by the direct DNA sequencing method. The heteroduplex detection methods have been found to be a very effective and practical method to discover the ENU-induced mutations in the G1 mutant mouse library. As shown in Fig. 2, all the ENU-induced mutations in the G1 mice are heterozygous. The heteroduplex formation and representative detection systems are schematically drawn in Fig. 4. After the PCR amplification of target sequences, the PCR products are denatured and then annealed back again. When an ENU-induced mutation

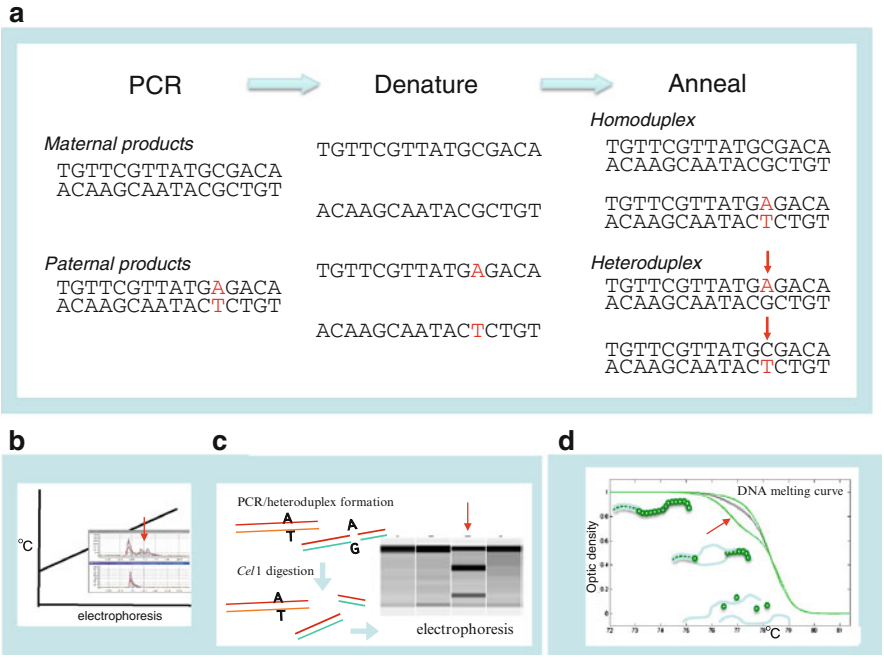

Fig. 4 Heteroduplex formation and detection of ENU-induced point mutations. (**a**) All the G1 mice heterozygously carry the ENU-induced mutation. The PCR amplification of the target sequence faithfully replicates the maternal and paternal sequences. The heteroduplex and homoduplex double-stranded DNA fragments are produced with equal molar ratio by denaturation and annealing of such PCR products. (**b**)–(**d**) Samples containing heteroduplex fragments are then efficiently detected by several methods. (**b**) Temperature gradient capillary electrophoresis (TGCE) separates the heteroduplex from homoduplex by the mobility change of the electrophoresis due to the T_m difference between heteroduplex and homoduplex. (**c**) TILLING/Cel1 digestion method. The Cel1 endonuclease effectively cleaves a single-base-pair mismatch(es) in the double-stranded DNA. The heteroduplex sample treated with Cel1 therefore exhibits two cleaved bands in the electrophoresis, while the homoduplex shows the single band of the intact size. (**d**) High-resolution melting (HRM) uses the optical density shift from the double-strand to single-stranded conformation change of DNA fragments. When the temperature increases, the heteroduplex fragments dissociate to single-stranded DNA faster than the homoduplex fragments. Accordingly, the reduction in the optic density of the heteroduplex is faster than that of homoduplex. The *red arrows* in (**b**), (**c**), and (**d**) indicate the signals of heteroduplex in each detection system

heterozygously exists in a G1 mouse, half of the PCR products contain the mutation. Such PCR products form heteroduplex after reannealing (Fig. 4a). Temperature gradient capillary electrophoresis (TGCE) (Gao and Yeung 2000; Li et al. 2002; Murphy et al. 2003), TILLING/Cel1 digestion (Oleykowski et al. 1998; Till et al. 2003), and high-resolution melting (HRM) (Wittwer et al. 2003; Bennett et al. 2003) are sensitive and reproducible heteroduplex detection methods (see Fig. 4b–d, respectively). Based on this principle, heteroduplex detection reveals which G1 mice carry an ENU-induced mutation in the target PCR products. After the primary

screening by heteroduplex detection, only the PCR products containing the heteroduplex are subjected to direct DNA sequencing to identify the sites and types of ENU-induced base substitutions.

4.3 Features of ENU-Based Gene-Driven Mutagenesis

ENU-induced mutations have indeed been found randomly in the whole genome (Sakuraba et al. 2005). Mutation "cold" or "hot" spots have not been identified. Therefore, it is statistically possible to estimate how many mutations would be found by screening the target genes. For instance, the RIKEN mutant mouse library encompasses ~3×10^7 mutations. The coding sequences are at least 1% in which 30,000 genes are coded at most. Therefore, more than 3×10^5 ENU-induced mutations reside in the coding sequences; namely, ten mutations reside in each gene on average. It also has been estimated that 60% and 10% of ENU-induced mutations in the protein-coding sequences are missense and KO-type mutations, respectively (Sakuraba et al. 2005). Here, KO-type mutations are defined as nonsense mutations or splicing mutations which cause drastic peptide changes or even have no mRNA products due to nonsense mediated decay. In summary, the RIKEN mutant mouse library itself is able to provide 1.8×10^5 missense and ~3×10^4 KO-type mutations. The RIKEN mutant mouse library has been open to the research community since 2002 and is available by accessing http://www.brc.riken.go.jp/lab/mutants/genedriven.htm. Briefly, users may simply design PCR primers for their target genes and obtain allelic series of mutant strains for functional analyses. As shown in Fig. 3, it is particularly a powerful way to analyze the recessive effect of a single-base pair in the G3 mice. One of the drawbacks, however, is that any X-linked genes cannot be targeted, since X chromosomes carrying ENU-induced mutations in G0 are not transmitted to G1 males (see Fig. 3). With respect to the targeting of X-linked genes, the development of the G1 females in the mutant mouse library as described in the previous section is also the key. The G1 mice also carry many ENU-induced mutations in addition to the discovered mutations, and to analyze the effect of the discovered mutation only, more than six generations of backcrosses are usually conducted to eliminate other mutations. In turn, modifiers, or gene–gene interactions, may be identified among 3,000 genes in a G1 mouse.

5 Examples of Classic Neurological Mutant Mouse Strains

Large-scale mouse mutagenesis projects have been accelerating the development of mutant mouse strains. In the best summary available, Lyon et al. (1996) listed 127 mutant loci in the "neurological and neuromuscular" category. Nineteen additional loci were also added in the "other behavioral" category. In this section, some

representative classic neurological and/or behavioral mutations established by forward genetics are reviewed.

5.1 shi, *Shiverer*

This classic recessive spontaneous mutant exhibits generalized tremor when disturbed from day 12 after birth and increases in severity with age (http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=alleleDetail&key=264). The identification of the gene of *shi* was one of the pioneering works of positional cloning in mammals. Roach et al. (1983) found that the myelin basic protein (Mbp) was responsible for *shi*, using Southern hybridization methods with a rat Mbp cDNA clone as a probe. Kimura et al. (1985) identified that the *shi* had a large deletion from intron 1 to exon 1 of the *Mbp* gene in the mouse. The lack of myelin resulted in the shivering phenotype. It is a loss-of-function type mutation by a large spontaneous deletion.

5.2 pcd, *Purkinje Cell Degeneration*

In 1976, an autosomal recessive mutation, *pcd*, was identified by its moderate ataxia with rapid degeneration of nearly all Purkinje cells in the cerebellum beginning at 15–18 days (Mullen et al. 1976). They also found a pleiotropic phenotype of male sterility in *pcd* homozygotes. In 2002, the *Nna1* gene (*Agtpbp1*; *ATP/GTP binding protein 1*) was identified from the *pcd* locus (Fernandez-Gonzalez et al. 2002) by positional cloning. By fine mapping of the *pcd* region in the mouse genome and syntenic analysis with the human genome, they nominated the *Nna1* gene as a candidate. Then, they conducted northern blot analysis showing much reduced or altered expression of *Nna1* mRNA of all the three tested *pcd* alleles (pcd^{1J}, pcd^{2J}, and pcd^{3J}). The difference in phenotypic severity among the three alleles was reflected in the mRNA expression. The genomic sequences of pcd^{2J} and pcd^{3J} had a ~12.2-kb deletion between introns 5 and 8 and an ~7.8-kb insertion in intron 13, respectively. The entire coding sequences of pcd^{1J} were the same as wild type, and thus they suggested a regulatory mutation in the pcd^{1J} allele. The expression of the wild-type cDNA in the *pcd* homozygotes rescued the *pcd* phenotypes by the transgenic mouse method (Wang et al. 2006), vindicating the finding that the *Nna1* mutation alone is solely responsible for the *pcd* phenotype.

5.3 d, *Dilute*

This very old recessive coat-color mutation originated from fancy mice was not primarily classified in the neurological category (Lyon et al. 1996). However, some

d alleles have been well known by their pleiotropic traits encompassing a neurological disorder and/or lethality. The molecular cloning of the old *d* allele revealed that the mutation was caused by the insertion of an ecotropic murine leukemia virus (called Emv3) into the *Myo5a* (myosin VA) gene (Jenkins et al. 1981, 1982; Mercer et al. 1991). The *Myo5a* mutation hampered the formation of dendritic processes in the melanocyte, causing the melanin granules to clump around the nucleus of the melanocyte and give rise to the coat-color variation.

The neurological anomaly found in some *d* alleles was implicated in the "tail region mutation" of the *Myo5a* gene (Huang et al. 1998). Among ten viable *d* alleles collected from spontaneous, radiation, and ENU mutations, they found that three alleles had not only a lightened coat color but also neurological impairment. Their study strongly vindicated the importance of the functional analysis of the gene by various allelic series. It is a good example of how studying only one mutation allele may overlook and dismiss other significant functions of the gene.

5.4 p, *Pink-Eyed Dilution*

Another very old coat-color mutation derived from various fancy mice, *p*, is the *Oca2* gene, which is the homolog for human oculocutaneous albinism type II (http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=markerDetail&key=12123). Some X-ray-induced *p* alleles exhibited pleiotropic phenotypes in addition to the albino eye and coat colors; for instance, neurological, cleft palate, reproductive, or endocrine disorders were concomitantly found in some X-ray-induced mutations (summarized by Lyon et al. 1996). Some pleiotropic phenotypes belong to different complementary groups, suggesting that the X-ray deleted a large fragment encompassing not only the *p* gene but also a flanking gene(s). Brilliant et al. (1991) conducted the molecular cloning of *p* without mapping as follows. They focused on one of the *p* alleles, p^{un}, since it spontaneously reverts to the wild type with a very high frequency. Using a part of IAP, a retroposon-like mobile element, as a probe they conducted a large southern blot analysis and found a uniquely enhanced 2.9-kb signal only in the p^{un} genome compared to the wild or revertant genomes. Based on the molecular cloning of the 2.9-kb fragment, the structure and mechanisms of the high reversion rate of the p^{un} allele were revealed as a large direct duplication and an unequal homologous recombination between the direct repeat, respectively (Gondo et al. 1993). The 2.9-kb fragment also allowed the cloning of the full-length cDNA clone (Gardner et al. 1992) as well as flanking molecular clones to the *p* locus (Lyon et al. 1992). Based on the molecular analysis of several mega base pairs of genomic region in and around the *p* locus, several new neurological loci were implicated (Lyon et al. 1992). It has lead to the molecular identification of new loci and genes close to the *p* locus; e.g., the $GABA_A$ gene (Nakatsu et al. 1993) and the Prader–Willi chromosomal region (Nakatsu et al. 1992; Gardner et al. 1992).

6 Mouse Models for Human Neurological Diseases

Advances in DNA-cloning and genomic-engineering technologies have enabled identification of the causative genes for human genetic diseases by positional cloning since mid-1980s. The completion of human genome sequencing has further enhanced the identification of various genetic risk factors in the human genome. The HapMap Project (International HapMap Consortium 2003; http://hapmap.ncbi.nlm.nih.gov/) was the first large-scale survey of causative genes and genetic risk factors for human diseases. The identified genes and loci are summarized in Online Mendelian Inheritance of Man (OMIM; http://www.ncbi.nlm.nih.gov/omim/). To validate and elucidate the molecular cause and function of the candidate genes, various mouse models have been developed.

6.1 Neurodegenerative Diseases

The term neurodegenerative disease encompasses Parkinson's disease (PD), Alzheimer's disease (AD), and other disorders characterized by progressive neuronal cell death. They are usually caused by heterogeneous genetic mutations, and many candidate genes have been identified by genetic analyses of the patients' families (e.g., AD, OMIM #104300; PD, OMIM #168600). Mouse models have been generated by reverse genetics.

Parkinson's disease (PD) is one of three main classes of basal ganglia disorders (the others being Huntington's disease and the dystonias) and affects approximately 1% of the population over age 50. The clinical diagnosis is severe motor disturbances characterized mainly by the resting tremor accompanied by bradykinesia, muscular rigidity, and postural instability. Neuronal degeneration is found in substantia nigra, locus ceruleus, nucleus basalis, hypothalamus, cerebral cortex, cranial nerve motor nuclei, and the central and peripheral portion of the autonomic nervous system. Mouse models have been constructed based on the two different approaches. One approach is to focus on the development and maintenance of the dopaminergic (DAergic) neurons, because DAergic neurons are severely affected in most of the PD patients. Based on this approach, the KO mouse of a nuclear receptor superfamily gene, *Nr4a2* (*Nurr1*), seems to be a good model for PD because aged heterozygotes exhibited loss of DAergic neurons, leading to the progressive loss of DAergic functions. These animals show decreased rotarod and locomotor performance, suggesting a motor impairment that is analogous to Parkinsonian deficits (Jiang et al. 2005). Allelic variants for the human *NR4A2* gene, such as a −291 deletion of T and a −245 substitution from T to G, were reported in the familial PD patients (Le et al. 2002).

The other approach is derived from the neuropathological observations that most PD patients present intracellular eosinophillic inclusions, known as Lewy bodies, consisting of ubiquitin, α-synuclein, and other components. It is noteworthy that

inclusion bodies have been observed in other neurological disorders as well, such as AD and Huntington's disease (HD). The double KO mouse strain for the ubiquitin pathway genes, *Uch-L1* and *Uch-L3*, was developed (Kurihara et al. 2001). Double homozygous KO mice for *Uch-L1*^{gad} and *Uch*$^{\Delta 3\text{-}1}$ displayed earlier lethality due to dysphasia. Axonal degeneration of the nucleus tractus solitarius, area postrema of the medulla, and gracile tract of the medulla and spinal cord were observed in these mice. Besides, degeneration of dorsal root ganglia cell bodies was detected. Allelic variants for the human *UCHL1* gene, such as Ile93Met and Ser18Tyr amino acid substitutions, were found in familial PD patients (Leroy et al. 1998). Regarding the association between PD and the ubiquitin pathway, many allelic variants for the *Parkin ubiquitin ligase* (*PARK2*) gene have been reported in PD patients (Nuytemans et al. 2010). *Park2* mutant mice (Itier et al. 2003) also showed a loss of catecholaminergic neurons in the locus ceruleus and an accompanying loss of norepinephrine in discrete regions of the central nervous system (CNS) but not accompanied by the loss of the nigrostriatal dopaminergic system that is characteristic of patients with Parkinson's disease. However, the level of dopamine transporter protein was reduced in the mutant, suggesting a decreased density of dopaminergic nerve terminals or other adaptative changes in the nigrostriatal dopamine system.

LRRK2 was identified as a causative gene of PD in the *PARK8* locus (Paisán-Ruíz et al. 2004), which contains 51 exons spanning 144 kb and encodes a 2,527 amino acid protein (~250-kDa) with a leucine-rich repeat, a kinase domain, an RAS domain, and a WD40 domain (Zimprich et al. 2004). Mouse models of KO and knock-in (Arg1441Gly, Arg144Cys) have been generated; however, no significant gross neuropathological abnormalities have yet been found (Li et al. 2009; Tong et al. 2009). In the case of *Caenorhabditis elegans* and *Drosophila* expressing mutant human LRRK2, degeneration of DA neurons and behavioral abnormalities were reported by Yao et al. (2010) and Liu et al. (2008), respectively. Recently, human Ala53Thr α-synuclein transgenic mice have been combined with a knockdown of *LRRK2* which has been reported to cause synergistic toxicity to neurons and accelerate the progression of α-synuclein-mediated neuropathology (Lin et al. 2009). The Ala53Thr α-synuclein transgenic mouse had originally been reported to develop a severe and complex motor impairment leading to paralysis and death, correlating with the formation of neuronal inclusions (Giasson et al. 2002).

Spinocerebellar ataxias (SCAs) and HD are typical triplet repeat diseases exhibiting neurodegeneration of particular neurons. SCAs refer to the autosomal dominant cerebellar degenerative disorders, which are defined as cerebellar ataxias with variable involvement of the brainstem and spinal cord. The clinical features of the disorders are caused by degeneration of the cerebellum and its afferent and efferent connections. Clinical and later molecular diagnosis by chromosomal mapping have identified and classified several SCAs. Some of the SCA subtypes, such as SCA1, 2, 3, 6, 7, and 17, were shown to result from the expansion of the CAG repeat expansions on the specific locus on the different autosome. The causative genes were named as *Ataxin* (*ATXN*)- 1, 2, 3, 6, 7, and 17, respectively, although there are no structurally common properties among these proteins. Like HD, the CAG triplet

encodes a polyglutamine stretch. In the case of SCA1, the mutant ataxin-1 protein predominantly caused degeneration of cerebellar Purkinje cells and neurons within the brain stem and spinal cord, producing a progressive ataxia and bulbar dysfunction. Of these subtypes, Atxn1, 7, and 17 have been reported as SCA model mice (Watase et al. 2002; Helmlinger et al. 2006; La Spada et al. 2001).

The knock-in compound-heterozygous mutant mice of the *Atxn1* (= *Sca1*) gene, $Sca1^{154Q/2Q}$ showed a severe SCA1-like neurological phenotype (Watase et al. 2002). $Sca1^{154Q/2Q}$ mice developed a complex, slowly progressive neurodegeneration resembling that seen in SCA1 patients. Although general function of Ataxin-1 (ATXN1) protein has not been fully revealed, ATXN7 acts as a subunit of TFTC, the TATA-binding protein (TBP)-free TBP-associated factor (TAF)-containing complex. Both knock-in (Helmlinger et al. 2006) and transgenic mice (La Spada et al. 2001) of the *Atxn7* (=*Sca7*) gene have been reported. For example, $Sca7^{266Q/5Q}$ mice (Helmlinger et al. 2006) showed the salient features of infantile SCA7, such as selective neuronal vulnerability and the functional consequences of the mutant protein on the retina, cerebellum, and hippocampus. ATXN17 encodes TBP, which is also one of the basal transcription factors. The transgenic mice, in which TBP-71Q and TBP-105Q were expressed under the mouse prion promoter, were generated (Friedman et al. 2007). While both transgenic lines exhibited a neurodegenerative phenotype with aggregated mutant proteins inside of the nucleus, TBP-105Q exhibited a more severe phenotype than TBE-71Q.

Alzheimer's disease is characterized by progressive impairments in memory, behavior, language, and visuospatial skills. Hallmark pathologies within vulnerable brain regions include extracellular-amyloid deposits, intracellular neurofibrillary tangles, synaptic loss, and neuronal cell loss. The most prominent mouse models for this disorder model abnormalities of amyloid precursor protein (APP) and presenilin (PS) (reviewed by McGowan et al. 2006). Many transgenic mice have been generated and reported to develop amyloid plaque pathology. The Tg2576 model (Lys670Met/Asn671Leu) showed reduced spontaneous alternation performance in "Y"-maze testing, which was enhanced in a double transgenic mouse with mutated PS1 (Holcomb et al. 1998). TgCRND8 carrying mutated APP (Lys670Met/Asn671Leu, Val717Phe) was shown to have correlative memory deficits by Morris water-maze testing (Chishti et al. 2001). Recent progress and prospective have also been reviewed by Götz and Ittner (2008) and Ashe and Zahs (2010).

6.2 *Mental Retardation, Ataxia, and Other Neurological Disorders*

Fragile X syndrome and fragile X tremor/ataxia syndrome (FXTAS) are caused by the same gene, FMR1, with extended CGG repeats in the 5′-UTR. In patients with Fragile X syndrome, more than 200 units of CGG repeat are found. FXTAS appears in a subgroup of premutation carriers of fragile X syndrome with 55–200 CGG

units. The first mouse model was developed by targeting the *Fmr1* gene (Dutch-Belgian Fragile X Consortium 1994), as fragile X syndrome patients lacked FMR1 protein due to the silencing of the FMR1 gene by amplification of a CGG repeat and subsequent methylation of the promoter region. The *Fmr1* KO mice showed macroorchidism, learning deficits, and hyperactivity. A $(CGG)_{98}$ knock-in mouse model was generated to model FXTAS (Van Dam et al. 2005). The knock-in mutant mouse exhibited elevated Fmr1 mRNA levels and ubiquitin-positive intranuclear neuronal inclusions as described in the patients. FXTAS has the clinical features of progressive intention tremor and ataxia. They also assessed the $(CGG)_{98}$ knock-in mutant mice for cognitive, behavioral, and neuromotor performance at different ages. Visual–spatial memory and rotarod performance declined with age, and the older mice tended to avoid the center area during open field tests, potentially indicating the elevated anxiety. These age-related disturbances may reflect the progressive cognitive and behavioral difficulties observed in FXTAS patients.

Muscle–eye–brain (MEB) disease was mapped to 1p34-33, and *protein O-linked mannose beta1,2-N-acetylglucosaminyltransferase* (*POMGNT1*) was found to be a causative gene. The common clinical features of MEB patients are severe muscular dystrophy, ocular defects, and mental retardation. A gene-trapped mutant mouse was identified in which a retroviral vector was inserted into the second exon of the *Pomgnt1* gene (Liu et al. 2006). They found that a retroviral insertion in front of the initiation codon abolished the expression of the *Pomgnt1* mRNA, and the glycosylation of α-dystroglycan was also reduced. The *Pomgnt1* mutant mice were viable, exhibiting multiple developmental defects in muscle, eye, and brain, similar to the clinical features of MEB.

Episodic ataxia (EA) is an autosomal dominant channelopathy that manifests as attacks of imbalance and in-coordination (reviewed by Jen et al. 2007). A responsible gene was mapped to the potassium channel gene, *KCNA1*, and affected individuals are heterozygous. KO mice for the *Kcna1* gene displayed frequent spontaneous seizures that correlated on the cellular level with alterations in hippocampal excitability and nerve conduction (Smart et al. 1998). Later, a knock-in mouse carrying the Val408Ala amino acid substitution, which had been found in a patient's family on the *KCNA1* gene, was also constructed (Herson et al. 2003). In contrast to the *Kcna1* KO mice, the Val408Ala knock-in homozygous mice were embryonic lethal. The heterozygotes showed stress-induced loss of motor coordination that was ameliorated by acetazolamide, a carbonic anhydrase inhibitor that minimizes EA1 symptoms in human patients.

7 Mouse Models for Neurodegenerative Lysosomal Disorders

Lysosomal storage diseases are a group of inherited disorders caused by dysfunction of synthesis and processing of lysosomal enzymes (Beck 2007; Jakobkiewicz-Banecka et al. 2007). A lysosomal defect results in accumulation of undegraded substrates and leads to dysfunction of tissues and organs, including neurons.

Although most of these diseases are fatal, new therapies have been developed with recent intensive studies using mouse models.

7.1 Niemann–Pick C Disease

Niemann–Pick C (NPC) disease is a neurodegenerative lysosomal storage disorder characterized by an accumulation of lipids including cholesterol and glycosphingolipids (GSLs) within a cell. Clinically identical diseases, NPC type 1 and type 2, are caused by mutations in two different proteins: NPC1, a lysosomal–endosomal transmembrane glycoprotein, and NPC2, soluble lysosomal protein with cholesterol-binding properties, respectively. To address the functions of these two genes, the genetic interaction between *Npc1* and *Npc2* was studied using mouse models (Sleat et al. 2004). They generated an *NPC2* hypomorphic allele by gene targeting and crossed with a previously established *Npc1* spontaneous null allele (Loftus et al. 1997) to obtain *Npc1;Npc2* double KO mutants. The results showed that the phenotypes of *Npc1* and *Npc2* single mutants and an *NPC1;NPC2* double mutant are almost identical in terms of disease onset and progression.

Using model mice, pharmacological approaches to NPC have been studied. Recent biochemical work (Kwon et al. 2009) has shown that both Npc1 and Npc2 proteins function in concert to facilitate the exit of cholesterol from lysosomes to other sites in the cell. One of the possible treatment strategies for NPC is substrate deprivation therapy. To reduce the synthesis of the metabolic products GSLs, *N*-butyldeoxynojirimycin, which is an inhibitor of glucosylceramide synthase, a pivotal enzyme in the ganglioside synthesis pathway, was tested in the *Npc1* mutant mice (Zervas et al. 2001). In this experiment, treated animals showed delayed onset of neurological dysfunction, increased average life span, and reduced GSL accumulation, suggesting that inhibitors for GSL synthesis could have a similar ameliorating effect on the human disorder. More recently, a second therapeutic agent, 2-hydroxypropyl-b-cyclodextrin (CD), was tested in *Npc1* and *Npc2* mutant mice. The result demonstrated that treatment with CD delayed clinical disease onset, reduced intraneuronal storage and secondary markers of neurodegeneration, and significantly increased lifespan of both *Npc1* and *Npc2* mutant mice (Davidson et al. 2009).

In addition to the effective substrate deprivation therapy, potential second/adjunctive treatments were reported using model mice. In NPC-1-mutant cells, lysosomal calcium was substantially decreased as a result of sphingosine storage, which then resulted in accumulation of lipids including sphingosine and GSL in the acidic compartment of the cell (Lloyd-Evans et al. 2008). Taking advantage of this finding, it was demonstrated that elevation of cytosolic calcium with a pharmacological agent, curcumin, normalized NPC1 disease cellular phenotype and prolonged survival of the NPC mouse (Lloyd-Evans et al. 2008). In common with other neurodegenerative disease, brain inflammation is observed in the NPC1 mouse with activation of microglia before the neuronal degeneration (Baudry et al. 2003).

In order to evaluate the contribution of the inflammation to the pathology of NPC disease, nonsteroidal anti-inflammatory drugs (NSAIDs) were administrated to the NPC1 mouse (Smith et al. 2009). The results showed that NSAIDs significantly prolonged the lifespan and slowed the onset of clinical signs, indicating that anti-inflammatory therapy may be a useful adjunctive treatment.

7.2 Mucopolysaccharidosis

Mucopolysaccharidosis (MPS) is a lysosomal storage disease, which is characterized by an inability to degrade glycosaminoglycans (GAGs). Accumulation of undegraded GAGs in many tissues leads to severe symptoms including CNS deterioration. In this section, model mice for MPS II, MPS IIIA, and MPS IIIB are reviewed.

MPSII, also called Hunter syndrome, is an X-linked lysosomal storage disorder caused by a deficiency in the enzyme iduronate 2-sulfatase (IDS). MPSII is characterized by progressive somatic and neurological pathologies, including facial features, hepatosplenomegaly, skeletal deformities, severe retinal degeneration, hearing impairment, and mental impairment. A model mouse for MPSII was generated by targeting the *ids* gene (Muenzer et al. 2002). Hemizygous mice displayed a progressive accumulation of GAGs in many organs, skeleton deformities, and neuropathological defects. Furthermore, the affected mice performed poorly in open-field tests and showed a severely compromised walking pattern (Cardone et al. 2006). To design an efficient gene therapy approach for the treatment of MPSII disease, the adeno-associated virus vector carrying *ids* was administered to MPSII adult mice (Cardone et al. 2006) and pups (Polito and Cosma 2009). Treatment with this viral vector resulted in the rescue of GAG accumulation in visceral organs as well as the CNS. Remarkably, this CNS correction arose from the crossing of the blood–brain barrier by the IDS enzyme itself, indicating that early treatment of MPSII mice with adeno-associated virus carrying *ids* resulted in prolonged and high levels of circulating the IDS protein that could efficiently and simultaneously rescue both visceral and CNS defects.

MPS III, also called Sanfilippo syndrome, is a lysosomal storage disease, which is characterized by inability to degrade heparan sulfate, one of the GAGs. Accumulation of undegraded heparan sulfate in many tissues leads to severe symptoms including CNS deterioration. The causative gene for MPS IIIA is the *N-sulfoglucosamine sulfohydrolase* (*sulfamidase*, *Sgsh*) gene. A mouse model for MPSIIIA carried a point mutation in the *Sgsh* gene, which altered the corresponding amino acid from Asp to Asn (Bhattacharyya et al. 2001) and exhibited biochemical, pathological, and clinical features observed in MPSIIIA patients (Bhaumik et al. 1999). To find a potential therapy option for MPSIIIA, inhibition of GAG synthesis with rhodamine B, a nonspecific inhibitor for GAG biosynthetic pathway, was tested in the MPS IIIA model mice. Treatment of rhodamine B altered several clinical parameters of the disease pathology, including reduction in urinary GAG

excretion, total liver GAG, and lysosomal GAG content in liver and brain in MPS IIIA mice (Roberts et al. 2006). Furthermore, the rhodamine B treatment was demonstrated to improve the behavior in MPSIIIA mice (Roberts et al. 2007). Although substrate reduction therapy is an effective option, inhibition of GAG synthesis by rhodamine B is not specific. To overcome this problem, other potential treatments have been developed including transplantation of ES cell-derived glial precursor cells expressing sulfamidase (Robinson et al. 2010), *Sgsh* gene transfer by canine adenovirus vectors (Lau et al. 2010), and intracerebrospinal fluid injection of the sulfamidase protein (Hemsley et al. 2008, 2009) to the MPSIIIA model mice.

MPSIIIB is caused by the alpha-*N*-acetylglucosaminidase (*Naglu*) gene, which is a lysosomal enzyme required for the stepwise degradation of heparan sulfate. To generate a mouse model for MPSIIIB, the *Naglu* gene was disrupted by gene targeting (Li et al. 1999). This MPSIIIB model mouse showed massive accumulation of heparan sulfate as well as secondary changes in the activity of several other lysosomal enzymes and elevation of gangliosides in brain. In addition, alterations in circadian rhythm, activity level, motor function, vision, and hearing, which are similar to the human disease, were observed (Heldermon et al. 2007). Although MPSIIIB results in severe progressive neurological defects with high mortality, no treatment is currently available because of the difficulty in delivering therapeutic materials to globally affected tissue in the CNS. To develop a new treatment for this disease, the feasibility of gene-based therapy was addressed in the MPSIIIB mouse model. Vectors derived from adeno-associated virus coding for *Naglu* were transferred by injection into striatum (Cressant et al. 2004) or via an intravenous and an intracisternal injection following mannitol infusion (Fu et al. 2007) into the affected MPSIIIB mice. Improved behavior, efficient Naglu delivery to the brain, and the correction of lysosomal storage with prolonged lifespan suggested that adeno-associated virus injections might represent an effective treatment for MPSIIIB. A lentivirus Naglu vector also restored the normal enzyme activity throughout the large portion of the brain, and the treated animals showed a significantly improved behavioral performance (Di Domenico et al. 2009). Gene therapy with viral vectors is a promising treatment for lethal conditions such as MPS, but there are major issues for clinical application, including carcinogenesis following virus integration into the host genome, uncontrolled overexpression of the enzyme, and inappropriate immune responses to the introduced virus (Sands and Davidson 2006).

7.3 Other Neurodegenerative Lysosomal Storage Disorders

GM1 gangliosidosis is an incurable GSL lysosomal storage disease caused by a genetic deficiency in β-galactosidase (*β-gal*) and leads to the accumulation of ganglioside GM1 and its derivative GA1 in the CNS. A mouse model lacking a functional β-gal gene was generated by homologous recombination (Hahn et al. 1997; Matsuda et al. 1997). Abnormal accumulation of GM1-ganglioside was progressively observed in the mutant brain and tremor, ataxia and abnormal gait

become apparent in older mice, thus mimicking the pathological, biochemical, and clinical abnormalities of the human disease.

Tay-Sachs disease and Sandhoff disease are progressive neurodegenerative disorders characterized by an accumulation of GM2 gangliosides, particularly in neurons. These two diseases are clinically indistinguishable and caused by a mutation in the *alpha* and *beta* subunit of hexosaminidase (Hex), respectively. Targeted KO lines of the *Hexa* gene were independently generated by three groups (Yamanaka et al. 1994; Cohen-Tannoudji et al. 1995; Phaneuf et al. 1996). All three mutant lines exhibited consistent results; the ganglioside was accumulated in neurons as membranous cytoplasmic bodies characteristically found in the neurons of Tay-Sachs disease patients. However, the mutant mice showed no apparent defects in motor or memory function. On the other hand, targeted KO mice of the *Hexb* gene resulted in accumulation of both GM2 ganglioside and glycolipid GA2 throughout the CNS. Furthermore, *Hexb*-deficient mice developed a fatal neurodegenerative disease, with spasticity, muscle weakness, rigidity, tremor, and ataxia (Sango et al. 1995; Phaneuf et al. 1996).

To develop potential treatment methods for gangliosidosis, one of the used strategies is to enhance the lysosomal enzyme levels. This method is aimed to remove ganglioside accumulating within the lysosome and includes enzyme replacement, bone marrow transplantation, chemical chaperon therapy, and gene therapy (Lacorazza et al. 1996; Norflus et al. 1998; Matsuda et al. 2003; Takaura et al. 2003). Alternatively, substrate reduction therapy aims to reduce GSL synthesis to counterbalance the impaired rate of catabolism, using small compounds, which inhibits the GSL biosynthesis (Kasperzyk et al. 2005; Lee et al. 2007; Baek et al. 2008; Elliot-Smith et al. 2008).

8 Mouse Models for Neuropsychiatric Diseases

Psychiatric diseases such as schizophrenia, bipolar disorder, and autism spectrum disorders each have a prevalence of about 1% (see O'Tuathaigh et al. (2011); reviewed by Burmeister et al. 2008), and approximately 17% of the population experience major depressive disorder (see Krishnan and Nestler 2011). In addition to the striking population prevalence, psychiatric diseases often have extremely high heritability; e.g., schizophrenia, bipolar disorder, autism spectrum disorders, and major depressive disorder with 70–85%, 60–85%, 90%, and 40%, respectively (Burmeister et al. 2008). Thus, mutant mice might effectively provide good models for human psychiatric diseases. On the other hand, psychiatric diseases appear to be mostly quantitative traits governed by polygenes and current mouse mutagenesis approaches, particularly transgenic and KO/conditional approaches, usually manipulate only one gene at a time. In this section, recent examples of mouse *Disc1* and *Srr* mutations are reviewed with respect to human psychiatric disease models (discussed in O'Tuathaigh et al. 2011).

8.1 Disrupted in Schizophrenia 1 *Gene*

Genetic epidemiological analyses from family and twin studies have consistently implicated genetic factors in the development of schizophrenia, although Mendelian heredity has not been confirmed (Thaker and Carpenter 2001). Therefore, Schizophrenia is a common and genetically complex psychiatric disorder. Indeed, summarizing intensive genetic association studies with schizophrenia, the Online Mendelian Inheritance in Man (OMIM; http://www.ncbi.nlm.nih.gov/omim) has listed 35 candidate genes as genetic risk factors from 25 genomic regions in 13 human chromosomes.

Among them, the *disrupted in schizophrenia 1* (*DISC1*) was initially identified at translocation (1;11)(q42.1;q14.3) that was linked to major mental illness, including schizophrenia, depression, and bipolar disorder, in a large Scottish family (St Clair et al. 1990). The *DISC1* gene was then positionally cloned in human (Millar et al. 2000), and subsequently the mouse homolog, *Disc1*, was also cloned (Ma et al. 2002). The positional cloning of the human *DISC1* gene revealed that the full length of the DISC1 consisted of 854 amino acid residues, and the Scottish translocation truncated DISC1 after the 597th amino acid residue (Millar et al. 2000). DISC1, which has been found to interact with over 50 proteins, was expressed in multiple isoforms in a cell-type-specific manner (reviewed by Porteous and Millar 2006).

A 25-bp deletion of the mouse *Disc1* gene was found originally in the 129S6/SvEv inbred strain (Koike et al. 2006) and later in all the tested 129 derived inbred strains (Clapcote and Roder 2006). This deletion caused a frameshift mutation, resulting in 13 novel amino acids followed by a premature stop codon in exon 7. Neither full-length nor the predicted partial N-terminal protein was detected in brain, and thus the deletion allele seemed to be null type (Koike et al. 2006). They also found that both heterozygotes and homozygotes for the deletion allele exhibited an impaired working memory performance in the C57BL/6 genetic background, suggesting haploinsufficiency. However, one of the typical phenotypes of schizophrenia, the reduction in prepulse inhibition (PPI) was not detected even in homozygotes (Koike et al. 2006).

Two independent missense mutant strains were discovered in exon 2 of the *Disc1* gene from the RIKEN mutant mouse library (Clapcote et al. 2007; also Sect. 4). $Disc1^{Rgsc1393}$ and $Disc1^{Rgsc1290}$ had an amino acid substitution of Glu31-Leu (Q31L) and Leu100Pro (L100P) in the mouse Disc1 protein, respectively. Exon 2 of the *Disc1* gene encoded the interaction domain with PDE4B, suggesting that the interaction between Disc1 and PDE4B was a genetic factor contributing to the development of psychiatric disorders mediated through cAMP signaling (Millar et al. 2005). Using G1 cryopreserved sperm, the identified *Disc1* mutant strains were revived and backcrossed to the C57BL/6 strain to eliminate other ENU-induced mutations. At the N6 backcross generation, heterozygotes were intercrossed to generate each genotype of +/+, Q31L/+, Q31L/Q31L, L100P/+, L100P/L100P, and Q31L/L100P. These mice were subjected to various behavioral tests with or without antipsychotic drugs and antidepressants. In addition, anatomical

and biochemical studies were conducted for each genotype. In summary, two mutations exhibited quite distinctive characteristics. The L100P allele gave rise to dominant schizophrenia-like phenotype, while the Q31L allele showed rather recessive depression-like behavior (Clapcote et al. 2007). For instance, both the homozygous and heterozygous L100P mice showed lower PPI. Antipsychotic treatments with clozapine and haloperidol restored their PPI phenotype. Latent inhibition (LI) tests and working memory tests also detected the schizophrenic phenotypes in the L100P mice. The Q31L homozygous mice also showed mild reduction in PPI but did not respond to antipsychotic drugs. Rather, depression-like behaviors were more prominent in Q31L homozygous mice as suggested by findings in the forced swim test (FST), social interaction test, and by their reward responsiveness (Clapcote et al. 2007). An antidepressant, bupropion, reversed the depressive traits in the Q31L mice.

A dominant-negative *Disc1* transgenic mouse was also constructed (Hikida et al. 2007). The translocation (1;11)(q42.1;q14.3) found in the Scottish family indicated that the mental illness was inherited in a dominant fashion. Originally a haploinsufficiency model was proposed (reviewed by Porteous and Millar 2006), but a dominant-negative function of the truncated DISC1 protein was also shown in the cell culture system (Kamiya et al. 2005). To test the dominant-negative effect of the truncated DISC1 in vivo, transgenic mice expressing the N-terminal 597 amino acid residues of the human DISC1 were constructed with the αCaMKII promoter (Hikida et al. 2007). They found neurodevelopmental anomalies as reported in schizophrenia patients, and they also found that the transgenic mice exhibited several behavioral abnormalities such as hyperactivity, disturbance in sensorimotor gating, olfactory-associated behavior, and depression-like deficits (anhedonia) (Hikida et al. 2007).

8.2 Serine Racemace *Gene*

N-methyl-D-aspartate receptors (NMDAR) may be involved in the pathophysiology of schizophrenia, implicated by the association of the reduction in NMDAR-mediated signaling pathways, particularly D-serine deficiency, with the disorder (Coyle 2006). The level of D-serine in vivo is regulated by the synthetic enzyme, serine racemace (*srr*; Wolosker et al. 1999a, b), and the degradatory enzyme, D-amino acid oxidase (DAAO; Mothet et al. 2000). Genetic studies in human populations showed that SNPs found in the *srr* and DAAO genes are associated with schizophrenia (Chumakov et al. 2002; Schumacher et al. 2004; Morita et al. 2007). To investigate the molecular association of the *srr* gene, several mutant mice for the *srr* gene have been developed (Basu et al. 2009; Labrie et al. 2009).

The KO mouse for the *srr* gene was constructed by Basu et al. (2009). They first confirmed that the *srr* KO mice expressed neither protein products nor enzymatic activities. The cortical D-serine levels in the +/+, heterozygotes, and homozygotes were 3.6, 2.5, and 0.4 μmoles/g protein, respectively. Electrophysiological studies

showed impaired glutamatergic neurotransmission and attenuated synaptic plasticity in the *srr* homozygous KO mice compared to that of wild type. Finally, the battery of behavioral tests revealed mild hyperactivity, impaired spatial memory, and a subtle elevation of anxiety. These findings are consistent with schizophrenia traits. The PPI test, however, did not detect any differences between the KO homozygotes and the wild type (Basu et al. 2009).

The next-generation gene-targeting system with ENU mutagenesis (Sect. 4) independently provided allelic series of *srr* mutant mice (Labrie et al. 2009). Eight mutant mouse strains carrying a base substitution in the *srr* gene were identified in the RIKEN mutant mouse library (all the *srr* mutant strains are available from RIKEN BioResource Center; http://www.brc.riken.go.jp/lab/mutants/jp/genedriven.htm). One of the eight strains had a nonsense mutation of Tyr269Stop in the last exon of the *srr* gene, predicting a truncated *srr* product. The Tyr269Stop mice were revived from frozen sperm and subjected to biochemical, behavioral, and pharmacological studies (Labrie et al. 2009). In the Tyr269Stop homozygotes, the transcription of *srr* mRNA was reduced ~50% of that of wild type. Western blotting analysis exhibited no *srr* protein products including the predicted truncated counterpart. No *srr* enzymatic activities were detected in the brain of Tyr269Stop homozygotes. Thus, the next-generation gene targeting with ENU mutagenesis indeed provides an authentic null allele that is equivalent to the KO allele. The Tyr269Stop mutant mice exhibited schizophrenia-like behaviors, for instance, reduced sociability, impaired object recognition, spatial learning and memory deficits, and impaired PPI. These behaviors were restored by clozapine, an antipsychotic drug, and by D-serine for which some preliminary findings suggest antipsychotic activity (Heresco-Levy et al. 2005; Coyle 2006). A striking difference was the finding of the PPI impairment in Tyr269Stop null mutant mice (Labrie et al. 2009) that was not observed in the above-described KO mice (Basu et al. 2009), although both of them were analyzed in the same genetic background of C57BL/6. The schizophrenia-like features seem to be more prominent in the Tyr269Stop *srr* mice than in the conventional *srr* KO mice, a difference that requires further elucidation.

9 Concluding Remarks

Various mutant mouse resources and mutagenesis systems are reviewed in this chapter. Many ENU-induced mutant mouse strains established by the phenotype-driven approach are currently undergoing positional cloning to identify the causative genes. The next-generation resequencing system should enhance the pace of positional cloning. The phenotype-driven approaches may also be combined with sensitized screening to detect modifiers, namely gene–gene interactions or epistatic interactions (see Bountra et al. 2011).

Conditional-ready KO mice for every mouse gene will be available as ES cell lines in a year or so by the international collaborative efforts (Sect. 3). In these

efforts, the KO/conditional mice are constructed in the standardized C57BL/6N genetic background, therefore eliminating the potentially confounding effects of segregating genetic factors in different genetic backgrounds. To effectively use the conditional resource, however, the batteries of Cre–zoo transgenic strains must be developed (Sect. 3).

Next-generation gene-targeting generates allelic series of ENU-induced point mutations of the target gene and is already available to the research community (Sect. 4). In addition, all the ENU-induced mutations in the mutant mouse library will be cataloged and analyzed using next-generation resequencing technology. The power of such multiple mutant alleles in the target gene was shown in the case for the *dilute* gene (Sect. 5.3) as well as for the *Disc1* gene (Sect. 8.1).

The next challenge for modeling human diseases is to develop mutant mice exhibiting a quantitative trait by combining multiple mutations. The effects of individual genetic risk factors in human psychiatric diseases are very weak (Pulver 2000), although overall heritability is very high and the diagnoses often overlap each other (Burmeister et al. 2008). Thus, psychiatric diseases appear to be typical quantitative traits governed by many weak polygenes with pleiotropy. KO/conditional mice have provided powerful models to elucidate human diseases with monogenic traits and those caused by several major genes by constructing double- or triple KO mice. ENU mutant mice and various inbred strains should give rise to model systems to dissect more complicated quantitative traits and genetic factors that are associated with human diseases.

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Drosophila as a Model Organism for the Study of Neuropsychiatric Disorders

Cahir J. O'Kane

Contents

Abstract The fruitfly *Drosophila* offers a model system in which powerful genetic tools can be applied to understanding the neurobiological bases of a range of complex behaviors. The *Drosophila* and human lineages diverged several hundred million years ago, and despite their obvious differences, flies and humans share many fundamental cellular and neurobiological processes. The similarities include fundamental mechanisms of neuronal signaling, a conserved underlying brain architecture and the main classes of neurotransmitter system. *Drosophila* also have a sophisticated behavioral repertoire that includes extensive abilities to adapt to experience and other circumstances, and is therefore susceptible to the same kinds of insults that can cause neuropsychiatric disorders in humans. Given the different physiologies, lifestyles, and cognitive abilities of flies and humans,

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many higher order behavioral features of the human disorders cannot be modeled readily in flies. However, an increasing understanding of the genetics of human neuropsychiatric disorders is suggesting parallels with underlying neurobiological mechanisms in flies, thus providing important insights into the possible mechanisms of these poorly understood disorders.

Keywords Addiction · Aggression · Autism · Courtship · Dopamine · *Drosophila* · Learning · Memory · Neuropeptides · Schizophrenia · Serotonin · Sleep

Abbreviations

ARM	Anesthesia-resistant memory
BBB	Blood–brain barrier
cAMP	Cyclic AMP
DAT	Dopamine transporter
GABA	Gamma-aminobutyric acid
LNs	Lateral neurons
NMJ	Neuromuscular junction
NPF	Neuropeptide F
NPY	Neuropeptide Y
PACAP	Pituitary adenylate cyclase-activating protein
PKA	cAMP-dependent protein kinase
SERT	Serotonin transporter
SSRI	Selective serotonin reuptake inhibitor
VMAT	Vesicular monoamine transporter

1 Introduction

Given the complexity and sophistication of behavioral phenotypes in neuropsychiatric disorders, can an apparently much simpler organism such as *Drosophila* make a useful contribution to our understanding of these disorders? At a number of levels from behavioral to molecular, the answer is certainly yes, if used appropriately. Flies and humans have found different solutions to the evolutionary struggle for survival, but their shared descent from a last common ancestor that already had a complex nervous system has left their brains and behavior with many traits in common. These shared traits include the main neurotransmitter systems, gross subdivisions of the brain along the body axis into forebrain, midbrain and hindbrain and shared behavioral traits including learning and many forms of behavioral and synaptic plasticity, circadian behavior, and some level of social behaviors. Flies offer many strengths for experimental approaches that are impossible in humans

and still difficult in other vertebrates, including their sophisticated genetics, ease of many cell biological manipulations, less gene redundancy and a very powerful toolbox of transgenic methods for neuronal circuit analysis – and for these reasons are continuing to play a pioneering role in genetic approaches to behavior. Set against this is their greater evolutionary distance from humans than vertebrate model organisms, which can manifest as lack of some key genes, lack or change of gene functions, and more divergent behavior. But as in any challenge, the key to success is to choose your weapon and exploit the best strengths of each system. For *Drosophila*, this can mean either studying a relevant defined behavior such as learning, circadian behavior, or responses to addictive substances, or increasingly, using *Drosophila* to study the cellular roles of gene products implicated by genetic mapping or genome-wide association studies in the human conditions.

2 The Architecture of the *Drosophila* Brain and Its Neurotransmitter Systems

2.1 The Fly Brain

Before looking at fly behavior and what can go wrong with it, how is the *Drosophila* brain that mediates this behavior built? The basic building blocks of the brain, neurons and glia, are found in both flies and mammals. Neurons show almost all the functional and molecular features of mammalian neurons: axons with their transport machinery, pumps, and voltage-gated channels that underlie action potential transmission, presynaptic terminals with all the machinery for synaptic vesicle release and recycling, dendrites, postsynapses with localized receptor fields and active zones. Glial cells are found intimately associated with neurons, for example often surrounding defined axon bundles and forming a blood–brain barrier (BBB) (Freeman and Doherty 2006).

With only around 10^5 neurons, the adult *Drosophila* nervous system has about one millionth as many neurons as humans have. Nevertheless, this nervous system still allows a remarkably complex behavioral repertoire, with new surprises about its capabilities continually appearing. It consists of a bilaterally symmetrical brain, which is joined to a ventral nerve cord that innervates the thorax and abdomen. Headless flies that retain only their nerve cord are capable of complex reflexive behavior, including grooming, and righting of the body if it is inverted (e.g., Ashton et al. 2001). Similarly to mammals, a segmentally repeated organization is most obvious in the nerve cord, but ontogeny and localized gene expression also show a division of the developing brain region into a protocerebrum, deutocerebrum, and tritocerebrum, which appear broadly evolutionarily homologous to the forebrain, midbrain, and hindbrain regions of vertebrates (Reichert 2005). At a more detailed level homologies between fly and mammalian brain regions are less obvious, because of the evolution of brain organizations that are adapted to their respective lifestyles.

However, molecular "evo–devo" analysis of a wider phylogenetic range of animals, including some with lifestyles less divergent from the last common bilaterian ancestor, is likely to reveal additional homologies, for example, the recent evidence for homology between the main neurosecretory regions of invertebrate brains and the hypothalamus–pituitary system of vertebrates (Tessmar-Raible 2007).

2.2 Neurotransmitter Systems

The similarities between fly and human nervous systems extend also to the main neurotransmitter systems and channels (Littleton and Ganetzky 2000), which are the targets of many pharmacological interventions relevant to neuropsychiatric conditions.

Acetylcholine is the main excitatory neurotransmitter in the *Drosophila* central nervous system (CNS), in contrast to the more limited role in the mammalian CNS. Like mammals, flies also have choline acetyltransferase, acetylcholinesterase, a vesicular acetylcholine transporter, and nicotinic (ionotropic) and muscarinic (G-protein-coupled) acetylcholine receptors that respond to nicotinic and muscarinic ligands, respectively (e.g., Reaper et al. 1998; Campusano et al. 2007). Glutamate is the excitatory neurotransmitter at the *Drosophila* neuromuscular junction (NMJ), but has a more limited role in the *Drosophila* than in the mammalian CNS. As expected, *Drosophila* have a vesicular glutamate transporter, a glutamate uptake transporter in glial cells (Rival et al. 2004), a range of ionotropic glutamate receptors (Littleton and Ganetzky 2000) that respond to some of the same ligands as mammalian receptors, including *N*-methyl D-aspartate (NMDA) receptors (Cattaert and Birman 2001). Flies also have metabotropic (G-protein-coupled) glutamate receptors that respond to mammalian receptor ligands (e.g., Zhang et al. 1999; Sinakevitch et al. 2010). As in vertebrates, GABA (gamma-aminobutyric acid) is the principal inhibitory neurotransmitter in flies, found widely throughout the brain. Flies have ionotropic GABA-A receptors, G-protein-coupled GABA-B receptors, and a vesicular GABA transporter (Enell et al. 2007). The pharmacology of these is broadly similar but not identical to that of vertebrate receptors. *Drosophila* GABA-A receptors are generally sensitive to inhibition by picrotoxin and potentiation by benzodiazepines (Buckingham et al. 2005; Wilson and Laurent 2005; Tanaka et al. 2009), but are inhibited only weakly by bicuculline (Buckingham et al. 2005). *Drosophila* GABA-B receptors are sensitive to at least one mammalian antagonist CGP54626 (Wilson and Laurent 2005).

Monoamine neurotransmitters have a variety of important roles in both *Drosophila* and vertebrates, mediated by G-protein-coupled receptors specific for each neurotransmitter. The neurotransmitter phenotype of these neurons is determined by the enzymes required to synthesize each transmitter, and by the vesicular monoamine transporter (VMAT) (Greer et al. 2005). The main types are:

Adrenergic: Octopamine and tyramine are the closest equivalents in *Drosophila* to the adrenergic neurotransmitters such as epinephrine and norepinephrine, based on

their pharmacological properties and homology between *Drosophila* octopamine receptors and vertebrate beta-adrenergic receptors (reviewed by Roeder 2005).

Dopaminergic: In humans, the dopaminergic system is the target of many addictive substances. *Drosophila* also has a dopaminergic system, comprising over a hundred neurons spread over some 15 clusters per adult brain hemisphere (Mao and Davis 2009, and references therein). The pharmacology of the *Drosophila* system is sufficiently conserved to see effects of substances including cocaine (see Sect. 3.4 below). While this makes *Drosophila* a good model for some features of addiction and dopaminergic function, the behavioral roles of dopaminergic neurons will be specific to the circuits that they belong to. For example, addictive substances often work partly by increasing the effects of dopamine in the dopaminergic mesolimbic system, which is associated with a general sensation of pleasure. On the other hand, in associative olfactory learning in flies, dopaminergic neurons that innervate the mushroom body signal an unpleasant aversive stimulus (see Sect. 3.2). Since the *Drosophila* brain has several clusters of dopaminergic neurons, it is entirely possible that some of them mediate a pleasurable stimulus, but the functions of most dopaminergic neurons in flies are not sufficiently well understood to answer this question.

Serotonergic: The human serotonergic system plays an important role in many aspects of mood regulation, affecting aggression, anxiety and depression. It is a target of a number of antidepressant therapeutics, including tricyclic antidepressants (e.g., imipramine) and selective serotonin reuptake inhibitors (SSRIs, e.g., Fluoxetine/Prozac). These potentiate the effect of serotonin by inhibiting the plasma membrane serotonin transporter (SERT), although tricyclics also target the adrenergic transporter. *Drosophila* also has a serotonergic system, with around 40 serotonergic neurons per brain hemisphere (Sitaraman et al. 2008), and a SERT that shows broadly similar but not identical pharmacological properties to human SERT (Demchyshyn et al. 1994; Petersen and DeFelice 1999). It has roles in a number of behaviors including diet (Vargas et al. 2010), sleep (Sect. 3.3) and aggression (Sect. 3.5).

Histaminergic: Histamine is the neurotransmitter used by a number of *Drosophila* neurons including photoreceptors, and is also transported by Drosophila VMAT (Romero-Calderón et al. 2006).

One important neurotransmitter system of psychiatric importance, whose effects *Drosophila* cannot model, is the cannabinoids. Initial pharmacological and sequence analysis suggested no members of the CB1 or CB2 cannabinoid receptor families in flies (e.g., McPartland et al. 2001). Although more sensitive searches subsequently identified a distantly related homolog in flies (McPartland et al. 2006), this has turned out to be an adenosine receptor (Dolezelova et al. 2007; Wu et al. 2009) and the original conclusions that flies lack cannabinoid receptors still stand. *Drosophila* has members of at least 15 classes of vertebrate neuropeptide receptors. Those of potential relevance to neuropsychiatric disorders include galanin, oxytocin/vasopressin, tachykinins, neuropeptide Y (NPY), thyrotropin-releasing hormone (TRH), bombesin/GRP, nociceptin, gastrin/cholecystokinin (Hewes and Taghert 2001; Hauser et al. 2006; Nässel and Homberg 2006). Many of these are

found in *Drosophila* interneurons and may therefore directly influence behavior. Known examples include Amnesiac, a neuropeptide similar to mammalian pituitary adenylate cyclase-activating protein (PACAP), involved in both olfactory memory and sleep (DeZazzo et al. 1999; Liu et al. 2008; Sects. 3.2 and 3.3); a pigment-dispersing factor (PDF) expressed in the lateral neurons (LNs) that regulates sleep and circadian rhythms (Sect. 3.3), and NPY, involved in behaviors including alcohol tolerance and aggression (Sects. 3.4 and 3.5).

3 Neuropsychiatric Behavioral Disorders in Flies?

3.1 Fly Behavior

At a most basic level, responses to sensory stimuli include attraction or repulsion to odors, locomotor responses to movement of single objects or the entire visual field, responses to mechanical or vibrational stimuli, and responses to noxious stimuli that appear to involve a perception of pain (Vosshall 2007). These behaviors appear largely reflexive (although are usually plastic to some degree) and have proved highly amenable to the sophisticated circuit analysis that *Drosophila* now offers (Luo et al. 2008; Olsen and Wilson 2008).

However, these responsive behaviors are not the main focus of this chapter. Fly behavior cannot readily be explained as a series of responses to sensory stimuli, any more than can human behavior; and it is the higher level control, modulation and motivation of behavior that is most relevant to neuropsychiatric disorders. We are gaining an increasingly sophisticated picture of the fly's behavioral repertoire, and are thus starting to define the parameters and phenomenology of such higher order behaviors including motivation, social behavior, and some of the features of addictive behavior (Vosshall 2007). Once the phenomenology is defined, a genetic approach allows the molecular and cellular and circuit mechanisms to be defined, as well as the mechanisms of any impairment caused by genetic or pharmacological manipulations. Spatial and temporal targeting of expression enables the study of transgenes such as wild-type learning genes, proteins that can either silence or activate neuronal activity (e.g., Sweeney et al. 1995; Luo et al. 2008; Sjulson and Miesenböck 2008).

3.2 Learning and Memory

A substantial capacity for learning and memory has been demonstrated in many tests. One of the best studied examples is olfactory associative learning, in which flies can learn to associate a specific odor with either punishment or a reward. The resulting memory can be dissected on the criteria of both molecules and circuitry into a number of phases, which range from short-term memory within

seconds or minutes, to long-term memory that lasts for days (Tully et al. 1994; Isabel et al. 2004). It requires the activity of neurons in a structure called the mushroom body, which is often compared to the mammalian hippocampus, although it is not clear whether there is any evolutionary homology between the structures. While our understanding of the mushroom body circuitry is still developing, targeted genetic manipulations suggest that punishment and reward are signaled by innervation of the presynaptic compartment of mushroom body neurons by dopaminergic and octopaminergic neurons, respectively (Schwaerzel et al. 2003; Riemensperger et al. 2005; Schroll et al. 2006). Association of a conditioned olfactory stimulus with punishment or reward would then ensue in the presynaptic compartment of small subsets of mushroom body neurons that receive both the olfactory stimulus and the punishment or reward stimulus. Retrieval would ensue when the same conditioned stimulus recurs, even in the absence of the unconditioned stimulus.

Much remains to be learned about the signaling pathways that mediate the different phases of learning and retrieval, but work in flies gave some of the earliest indications that cAMP signaling plays an important role, since mutations affecting the neuropeptide PACAP, the calcium-sensitive adenyl cyclase Rutabaga, the cAMP phosphodiesterase Dunce, protein kinase A, and the cAMP-responsive transcription factor CREB, all affect learning and memory (reviewed by McGuire et al. 2005). This function of the pathway appears widely conserved phylogenetically, since cAMP signaling is central to at least some forms of learning in organisms as diverse as the mollusc *Aplysia* and mammals (Hawkins 1984; Bourtchuladze et al. 1994; Silva et al. 1998). In humans, the role of the pathway in learning or memory disorders has not been well characterized, but inhibitors of cAMP phosphodiesterase show enhancing effects in many vertebrate memory paradigms and have therefore been suggested as cognitive enhancers (Rose et al. 2005). Furthermore, a human dunce ortholog PDE4B has been implicated as a risk factor in schizophrenia-like illness (see Sect. 4.1 below; O'Tuathaigh et al. 2011; Millar et al. 2005).

3.3 Sleep and Rhythms

Sleep disorders are a feature of many degenerative and neuropsychiatric conditions, including schizophrenia, bipolar disorder, and major depression (see Krishnan and Nestler 2011). The cause-and-effect relationships between sleep disturbance, circadian rhythmicity, and other disease symptoms are complex and remain largely to be dissected. For this reason among many others, there is a compelling need to understand the nature, regulation, and functions of sleep. Since flies and humans have very different brain architectures, it came as a great surprise – but perhaps not to those aware of the sophistication of fly behavior – that flies also have a resting behavioral state that resembles sleep. The name of *Drosophila* ("dew lover") itself reflects the elevated activity of individual flies around dawn and dusk, as if sleeping at night and taking siestas during the day. This circadian inactivity is suggestive

but insufficient to meet the criteria of sleep, but it shows additional characteristics that do: flies are less responsive to sensory stimulation during inactivity; "sleep deprivation" homeostatically evokes more short resting episodes and a "sleep rebound" to make good the deprivation; some of the same molecular expression markers of sleep and wakefulness are found in flies and mammals and *Drosophila* rest and activity can be modulated by stimulants and hypnotics (Hendricks et al. 2000; Shaw et al. 2000). In other parallels to mammals, wakefulness in flies is promoted by pharmacological or genetic stimulation of dopaminergic neurotransmission, and resting by its inhibition (Andretic et al. 2005; Kume et al. 2005). Serotonergic (Yuan et al. 2006) and GABAergic (Agosto et al. 2008; Parisky et al. 2008; Chung et al. 2009) neurotransmission both promote rest.

As with other biological processes, one cannot assume that the mechanisms of sleep are conserved in all respects between flies and mammals. Indeed sequence homology searches (data not shown) fail to detect one important mechanism for sleep control in flies, the orexin ligand–receptor pair (reviewed by Sakurai 2007). The absence of this important neuroendocrine component from flies appears to be due to a recent evolutionary loss from the fly lineage, since orexin receptor sequences are detected in other insects (data not shown). The other parallels between fly and mammalian sleep are possibly more ancient, and strong enough for flies to a compelling model to dissect the molecular and cellular mechanisms of sleep.

Several approaches are starting to yield rapid progress. Mutagenesis screens (see Gondo et al. 2011) have recently started to yield mutants of interest. One such screen has identified mutations in the voltage-gated K^+ channel *Shaker* as causing short sleep, associated with reduced lifespan, but retaining sleep homeostasis. Another screen has identified mutations that reduce recovery sleep after sleep deprivation that affect a glycosylphosphatidylinositol-anchored protein, Sleepless/Quiver (Koh et al. 2008). Interestingly, these mutations also reduce levels of Shaker channel and currents (Koh et al. 2008). Together, the strong effects of these mutations point toward membrane excitability as a key process in sleep. As in other areas of biology, the expectation is that mutant screens will continue to generate enough biological raw material for at least a generation of researchers in the field (Harbison and Sehgal 2008 and see Gondo et al. 2011).

The powerful targeted spatial and temporal expression technologies of *Drosophila* have identified a few specific brain regions or neurons in which molecules or pathways of interest can act. First, EGFR signaling in a group of neurosecretory neurons, the pars intercerebralis, promotes sleep. Intriguingly, this structure is proposed as evolutionarily related to the hypothalamus, central to the control of sleep in mammals (Foltenyi et al. 2007). Second, different subsets of mushroom body neurons (better known for its role in learning and memory) can either promote or antagonize sleep (Joiner et al. 2006). Third, the LNs that express the neuropeptide PDF promote wakefulness and are targets for the GABA inhibition of wakefulness (Parisky et al. 2008; Sheeba et al. 2008; Chung et al. 2009). How these components interact to control all aspects of sleep and wakefulness is still unknown, but we can expect further work to "join the dots" of the circuitry in the coming years, and to develop a circuit model of how sleep can be controlled.

Finally, analysis of cellular phenotypes related to sleep in *Drosophila* has shown a striking reduction in synaptic markers in several regions of the *Drosophila* brain, which is prevented by sleep deprivation (Gilestro et al. 2009). This is consistent with sleep being a period in which unwanted synaptic connections are broken down or weakened. The molecular and genetic tools that allow this process to be monitored and manipulated promise a much better understanding of how central this process is to the nature and purpose of sleep.

In summary, *Drosophila* is emerging as a powerful model for the cellular and circuit mechanisms of sleep, which will certainly provide many testable models for the nature of human sleep, and thus provide a route to eventually unraveling the complex interrelationships between sleep disorders and a wide range of neuropsychiatric conditions.

3.4 Addiction

Addiction to psychoactive substances is a complex range of disorders that have a great human cost, and we are still far from understanding the cellular mechanisms involved. Typically, an addictive substance evokes positive reinforcement or rewarding experience, thought to be, at least in part mediated by elevating the levels or effects of extracellular dopamine in the mesolimbic dopamine system (Kauer and Malenka 2007; Heidbreder 2011 for detailed discussion). In addition, repeated exposures may result in either sensitization or tolerance, depending on the strength of exposure and intervals between exposures. Cessation of repeated exposures can impair normal physiological functions, resulting in withdrawal symptoms.

No substance has yet been demonstrated to evoke a full gamut of addiction in flies, but this could potentially improve as behavioral testing of flies becomes more sophisticated. Flies certainly show some of the elements of addictive behavior, and a number of psychoactive substances, including cocaine, nicotine and ethanol show strong behavioral effects in flies (McClung and Hirsh 1998; Bainton et al. 2000). Cocaine induces a dose-dependent range of responses, varying from increased grooming and decreased locomotion at lower concentrations, through abnormal locomotor patterns, loss of taxis behaviors, hyperkinetic activity and tremors, to total akinesia or death at higher concentrations (McClung and Hirsh 1998; Bainton et al. 2000; reviewed by Heberlein et al. 2009). Furthermore, repeated exposure to cocaine shows sensitization, with the maximum increase in response occurring when the second exposure is several hours after the first (McClung and Hirsh 1998). The behavioral response of flies to cocaine reflects the presence of its main pharmacological targets, the plasma membrane monoamine transporters including those for dopamine (DAT) and serotonin (SERT). As in mammals, dopamine plays a major role in the effects of cocaine in *Drosophila*. Pharmacological inhibition of dopamine synthesis reduces the behavioral effects of cocaine (Bainton et al. 2000) and, in an apparent contradiction, chronic blockade of both dopaminergic and serotonergic neurotransmission increases them (Li et al. 2000). The reason for the contradiction

is not clear, but may reflect the acute or chronic nature of the treatments; nevertheless, both results still implicate dopamine in the main cocaine response in flies.

The existence of such responses allows dissection of their control mechanisms in flies, for example by using powerful mutant screens, together with the powerful tools for targeted expression and circuit dissection. First, a functional circadian clock is required for cocaine sensitization in flies (Andretic et al. 1999), and this appears to reflect a target in dopaminergic neurons that innervate the PDF-expressing LNs, in which the circadian clock mechanism in turn regulates the ability of these neurons to control general locomotor activity. Ablation or impairment of LNs reduces but does not eliminate the overt behavioral effects of cocaine (Tsai et al. 2004). Remarkably, this situation is similar to that in mammals, where there is also strong interaction between cocaine and clock function (reviewed by Manev and Uz 2006); cocaine alters clock gene expression, and mutations in circadian clock gene expression significantly alter mouse responses to cocaine. Whether this similarity reflects a deep underlying conservation of the neuronal circuitry that regulates rhythmic behavior is still unclear, but it is sufficient to justify further study in flies of how circadian mechanisms contribute to the effects of cocaine and of other psychoactive drugs that target the dopaminergic system.

Genetic screens in flies have also implicated the BBB in responses to cocaine. This is surprising, given the known or likely permeability of this barrier to cocaine in both mammals and flies (reviewed by Daneman and Barres 2005). Partial loss-of-function of a G-protein-coupled receptor ("Moody"), which is necessary for integrity of the BBB that is formed by junctions between glial cells, increases both cocaine and nicotine sensitivity (Bainton et al. 2000). A direct effect on drug permeability seems unlikely. The BBB appears largely intact in the mutants and the physiological basis is therefore unclear. Further investigation of this, and continuing screens for new mutants affecting fly responses to cocaine, promise further insights into the mechanisms by which it acts. In particular, this work has only scratched the surface regarding the mechanisms of cocaine sensitization, and there is no doubt room for further progress.

Probably the most widely used psychoactive substance is ethanol – but unlike cocaine, the molecular and neuronal targets for its psychoactive effects are not clear. Flies therefore offer a useful system for dissecting these effects and identifying their molecular mechanisms. Indeed, ethanol evokes a set of responses in flies that shows many similarities to its effects in humans. After initial exposure to ethanol vapor, flies first become hyperactive, but then gradually uncoordinated and sedated (Moore et al. 1998); a further effect is apparently disinhibited courtship of other males (Lee et al. 2008). On repeated exposures, these responses can show either tolerance (Scholz et al. 2000) or sensitization (Scholz et al. 2005; Lee et al. 2008), depending on the response assayed and the kinetics of exposure.

Mutant screens for flies with altered sensitivity to ethanol have revealed some of the complexity of the mechanisms, and suggest a role of a number of forms of synaptic plasticity, in defined neurons. Inhibition of cAMP (cyclic AMP)-dependent protein kinase (PKA) causes increased sensitivity to ethanol

(Moore et al. 1998). The precise cells responsible have not yet been identified, although a relatively small set of cells has been identified in which PKA inhibition by contrast decreases ethanol sensitivity (Rodan et al. 2002), indicating a variety of targets for the different components of the behavioral response to ethanol. cAMP also has an important role in mammalian responses to ethanol; for example, knockout of the AC1 calcium-sensitive adenylyl cyclase increases sensitivity of mice to ethanol-induced sedation (Maas et al. 2005), similar to the effects of loss of the equivalent enzyme in *Drosophila rutabaga* mutants (Moore et al. 1998). A further similarity to mammals is that neurons expressing or responding to the NPY homolog, NPF, also contribute to sensitivity to ethanol, without affecting responses to other sedative agents (Wen et al. 2005; Chen et al. 2008).

What of the mechanisms of tolerance, one of the processes that contributes to addiction? Genetic and pharmacological approaches have identified both a rapid tolerance induced by a single large dose of ethanol, and a longer term tolerance to prolonged exposure to a low concentration, which is dependent on protein synthesis (Scholz et al. 2000; Berger et al. 2004). Genetic screens have identified mutations that have helped to dissect the rapid tolerance phase into a component that is dependent on octopamine signaling (regarded as the fly equivalent of norepinehrine) and another component regulated by the putative transcription factor Hangover, which is also required for tolerance to other stress treatment (Scholz et al. 2005). Both of these mechanisms must regulate proteins that mediate tolerance and a good candidate for such a protein is the BK-type calcium-activated K^+ channel Slowpoke, which contributes positively to tolerance (Cowmeadow et al. 2005, 2006; Wang et al. 2009a, b). In human HEK cells BK channel properties depend on ethanol concentration (Yuan et al. 2008) and also develop tolerance to acute ethanol exposure.

In summary, analysis of *Drosophila* mutants has started both to reveal the complexity of the psychoactive effects of ethanol, and to distil some of the mechanisms out of this complexity. The recent and continuing availability of more mutants and ethanol-responsive genes (Scholz et al. 2005; Morozova et al. 2006, 2007, 2009; Bhandar et al. 2009) should help to define the cellular and circuit mechanisms further. A surprising theme emerging from some of these screens is that mutations affecting a number of cytoskeletal or cellular trafficking proteins also affect either sensitivity or tolerance to ethanol in flies, for example a RhoGAP, Homer, the endoplasmic reticulum protein Arl6IP and the cell adhesion molecule integrin (Rothenfluh et al. 2006; Urizar et al. 2007; Li et al. 2008; Bhandar et al. 2009). Since some of these are known to affect synaptic function and plasticity, this is suggestive also that structural changes in synapses may play a role in the effects of ethanol.

3.5 Social Behaviors

Many neuropsychiatric diseases have profound effects on human social behavior. Compared to humans, or even to the social insects such as hymenopterans, *Drosophila* is less obviously a social animal. Nevertheless, flies do show a variety of social

behaviors from as apparently simple ones like aggregation (Lefranc et al. 2001; Tinette et al. 2004), to complex ones like courtship and aggression. Some unexpected depths in their social behavior have emerged in recent years. The power of *Drosophila* genetics and circuit analysis tools is allowing dissection of the mechanisms of these behaviors, and while we still have much to learn, there are a few cases of striking parallels with human behavior.

Courtship. Flies have sophisticated courtship behaviors based on visual, auditory and pheromonal cues, all involving high level neuronal control (recently reviewed by Dickson 2008). These behaviors ensure that males will court only females of the right species, that only unmated females are receptive to males and that males learn to avoid repeating unproductive courtship encounters, e.g., with mated females. In addition to these obvious behaviors, more subtle social behaviors related to courtship are being uncovered. For example production of courtship pheromones and the behaviors that they control are sensitive to social context (Kent et al. 2008; Krupp et al. 2008); and it has recently been discovered that *Drosophila* females can copy the mate choice preferences of other females (Mery et al. 2009), an ability that would evoke amazement even when found in vertebrate species.

The highly species-specific nature of courtship obscures any mechanistic similarities between fly and human sexual behavior. However, genetic manipulations of the sexual identity of neurons in flies can have profound effects on sexual behavior (e.g., Clyne and Miesenböck 2008; Rideout et al. 2010), illustrating the extent to which such behaviors are under genetic control – notwithstanding the complexity of nature versus nurture arguments. The need for sexually dimorphic behavior must be as evolutionary ancient as sexually dimorphic multicellular animals and the genetic and circuit analysis tools of *Drosophila* are arguably turning it into the animal in which we can best understand the neural control of sexual behavior. Flies therefore offer at least a perspective that will be informative for neuronal control of human sexual behavior, even if direct mechanistic similarities are as yet not obvious.

Learning. While initial analysis of associative learning failed to find any group influences on individual learning, more sophisticated analyses have found a strong influence of social context on some forms of learning and memory in *Drosophila* (Chabaud et al. 2009). A form of long lasting memory, anesthesia-resistant memory (ARM), can be induced by massed training in an aversive learning olfactory choice paradigm. Remarkably, retrieval performance of individuals in this paradigm is enhanced by testing in the presence of other trained individuals; whether the basis for this is purely behavioral or is pheromonal is unknown.

Aggression. *Drosophila* is not normally thought of as an aggressive animal, but the potential of arthropods to show aggressive behavior is obvious in insects such as ants or wasps and in crustacea such as crabs and lobsters. To apply the genetic and circuit tools of *Drosophila* to the study of aggression, attempts have been made to design scenarios in which aggression can reproducibly be generated in *Drosophila*. For example, *Drosophila* males will fight each other in the presence of an immobilized (headless but living) female (Chen et al. 2002), and females will fight in the presence of a limited food source (Nilsen et al. 2004). Aggression can be modified

by experience and hierarchical relationships among flies develop on repeated encounters (Yurkovic et al. 2006).

Targeted expression, circuit analysis, mutant and pharmacological approaches have identified some surprising similarities between the neurobiology of fly and human aggression. Male aggressive behavior is almost completely abolished in the absence of the neurotransmitter octopamine, considered to be the invertebrate equivalent of norepinephrine (Hoyer et al. 2008) and a small set of octopaminergic neurons has been identified that mediates aggression (Zhou et al. 2008). Moreover, serotonin promotes aggression in both flies and mammals, and NPF (or NPY in mammals) inhibits aggression. All these similarities suggest that aggressive behavior is an evolutionarily conserved behavioral repertoire with a conserved neural basis. It still remains to be seen whether the major regulatory neurons are also evolutionarily conserved and homologous in both mammals and flies. However, the similarities that are already established provide a good basis for further work on flies, that can be used to generate models of the molecular, cellular and circuit mechanisms that control aggression in mammals including humans.

4 Human Neuropsychiatric Disorders in *Drosophila*?

It is clear that *Drosophila* has a wide and sophisticated behavioral repertoire that has enough in common with other animals including humans, to be a powerful model system for the molecular and circuit mechanisms of these behaviors, and for impairments in them. However, the most common human neuropsychiatric disorders have very complex effects on a range of higher human behaviors. Can these be modeled in flies?

4.1 Schizophrenia

Schizophrenia is a complex and heterogeneous disorder, the best known symptoms of which include hallucinations and delusions, and which may overlap with other disorders including bipolar disorder. Since it is currently impossible to conceive how to identify these behavioral phenomena in flies, modeling these aspects of the disease in flies is not feasible. However, these higher level behavioral phenomena must to some extent reflect underlying neurobiological processes. Consistent with this, schizophrenia has a strong genetic component and some convincing susceptibility loci have started to emerge, thus providing an opportunity to use flies to understand the functions of the affected genes (see also Gondo et al. 2011; O'Tuathaigh et al. 2011). Causative neurobiological processes are still only vaguely defined, although emerging themes include impairment of connectivity between the prefrontal cortex and temporal lobe, and subtle dysfunction of glutamatergic and dopaminergic synapses (see also O'Tuathaigh et al. 2011). The complexity of the

disease mechanisms is reflected in the length of time taken to map even a few loci that are associated with the condition (Stefansson et al. 2008; The International Schizophrenia Consortium 2008; Walsh et al. 2008), and in the recent identification of large number of loci that make small contributions to disease susceptibility (Stefansson et al. 2009; The International Schizophrenia Consortium 2009). However, now that risk genes are being identified, *Drosophila* has considerable potential to illuminate how these genes might underlie the cellular and neurobiological basis of the condition.

DISC1 and PDE4B. Disappointingly, one of the strongest schizophrenia candidate genes, *DISC1* (Chubb et al. 2008), has no obvious ortholog in flies. DISC1 is a large predicted coiled-coil protein that diverges relatively rapidly in evolution, with likely orthologs in lower chordates showing only barely detectable homology to human DISC1 (data not shown). Therefore, either DISC1 is specific to the deuterostome lineage of animals (including humans), or protostomes including *Drosophila* might have a DISC1 ortholog, but one that is too divergent to be identified by sequence homology searches. However, flies do have well studied homologs of some known DISC1 binding partners, including the cAMP phosphodiesterase PDE4B, itself also implicated in psychiatric illness (Millar et al. 2005). The *Drosophila PDE4B* ortholog, *dunce*, is a learning and memory mutant (reviewed by McGuire et al. 2005), and the molecular and circuit mechanisms of how *dunce* and related genes contribute to neuronal and behavioral plasticity are the subject of intense study (see Sect. 3 above on learning and memory). If a *Drosophila* DISC1 ortholog emerges in future, then flies will offer a wealth of neurobiological context in which to study its function.

Dysbindin (*DTNBP1*). *Drosophila* has been highly informative on functions of dysbindin that may be relevant to a causative role in schizophrenia. Dysbindin, encoded by the *DTNBP1* locus, is a binding partner of dystrobrevin and a component of the BLOC-1 complex that has an emerging role in trafficking in the endosomal–lysosomal pathway (reviewed by Ryder and Faundez 2009). It has been implicated in schizophrenia in a number of linkage and association studies and the condition is associated with lowered levels of dysbindin expression (Straub et al. 2002; Ross et al. 2006). Strikingly, loss-of-function mutations in *Drosophila* dysbindin were recovered in a screen for mutations affecting presynaptic homeostatic upregulation of glutamatergic release at the NMJ, in response to acute blockade of postsynaptic receptors (Dickman and Davis 2009). *dysbindin* mutations showed only a modest effect on baseline neurotransmission, but severe impairment of this homeostatic regulation, which is likely to be a key process in regulating the strength of synaptic transmission to an appropriate level. This finding provides a plausible model of how dysbindin mutations might cause neurobiological defects that can lead to the behavioral symptoms of schizophrenia and suggests that defects in homeostatic regulation of glutamatergic transmission rather than in basal transmission mechanisms are a causative factor (among others) in the condition. The fly NMJ is a highly amenable system to study synaptic regulation, including processes known to involve the endosomal–lysosomal system (e.g., Sweeney and Davis 2002; Wucherpfennig et al. 2003; Wang et al. 2007) and offers many possibilities to test

whether other schizophrenia susceptibility genes affect synaptic homeostasis, or other aspects of synaptic regulation.

22q11 deletion. Deletions in the 22q11 region are associated with increased susceptibility to schizophrenia (Karayiorgou et al. 1995). The relative contributions of genes in this region remain to be determined, but strong candidates include *COMT1*, which encodes the dopamine-metabolizing enzyme catechol-*O*-methyltransferase (Gothelf et al. 2005) and proline dehydrogenase, which encodes a mitochondrial enzyme that mediates one potential pathway for glutamate biosynthesis (Gogos et al. 1999). Despite having a functional dopaminergic system, *Drosophila* has no ortholog of COMT1, since sequence homology searches using human COMT1 detect more similar homologs in organisms as distant as bacteria and fungi than in flies (data not shown). The *Drosophila* homolog of proline dehydrogenase is *sluggish-A*; mutations in this gene do not affect basal glutamatergic synaptic transmission, although they cause adult locomotor defects and mild neurodegeneration (Shayan et al. 2000; Fergestad et al. 2008), which are suggestive of some subtle neuronal dysfunction. *Sluggish-A* mutants may therefore deserve testing for synaptic phenotypes similar to those of *dysbindin* mutants.

Neuregulin (*NRG1*). Among the other major susceptibility loci (Ross et al. 2006), neuregulin (encoded by *NRG1*) also has a *Drosophila* homolog, the EGF-like protein known as Vein. This has been implicated in multiple developmental processes (www.flybase.org) but so far has not been linked to synaptic phenotypes, although this deserves to be investigated further in light of the above findings on *Drosophila* dysbindin function.

DAOA encodes a short 153 amino-acid residue protein that is evolving rapidly, with no orthologs yet detected outside primates (Chumakov et al. 2002).

Other regions are associated recurrently as copy number variants with schizophrenia (Stefansson et al. 2008; The International Schizophrenia Consortium 2008; Walsh et al. 2008). One of the smallest of these, 15q11.2, contains only four candidate genes, three of which have *Drosophila* orthologs that are required for normal synaptic development and function. First, mutations in the *Drosophila* ortholog of *CYFIP1* result in aberrant axon pathfinding and synaptogenesis, apparently by affecting its ability to antagonize the action of actin cytoskeletal regulator Rac1 and the Fragile X protein ortholog FMR1 (Schenk et al. 2003). Second, the single *Drosophila* ortholog of *NIPA1* (causative for a dominant form of hereditary spastic paraplegia) and *NIPA2* regulates synaptic organization by inhibiting BMP receptor endocytic trafficking and signaling (Wang et al. 2007); interestingly, schizophrenic symptoms are observed in some subgroups of hereditary spastic paraplegia patients, but this has not yet been noted for patients carrying *NIPA1* mutations (McMonagle et al. 2006).

In conclusion, the usefulness of *Drosophila* as a model to gain mechanistic insights into schizophrenia is likely to grow. Expecting flies to be a simple model for schizophrenia is too naïve. Nevertheless, as more susceptibility genes are identified there is a growing need to understand their neurobiological role and *Drosophila* offers a wealth of possibilities for this. As the neurobiological basis underlying schizophrenia and related conditions emerges, *Drosophila* offers the

opportunity to study the relevant neurobiological phenomena in a model with powerful genetic and cell biological tools.

4.2 Other Developmental Disorders: Bipolar Disorder, Autism

As in the case of schizophrenia, to expect mutant or transgenic flies that can be considered as simple models of these genetically and phenotypically complex conditions is overly naïve. Nevertheless, there is sometimes a wealth of understanding from flies on the neurobiological roles of the emerging collection of risk alleles.

Bipolar disorder shows an overlapping spectrum of phenotypes with schizophrenia, and although it has a strong genetic component, the individual loci are as yet even less well defined than for schizophrenia. However, the two conditions share some susceptibility genes (The International Schizophrenia Consortium 2009; Craddock and Sklar 2009), meaning that flies should yield comparable insights into the mechanisms of bipolar disorder as for schizophrenia.

Autism and autism spectrum disorders also present similar difficulties in diagnosis and mechanistic study to schizophrenia and bipolar disorder, presenting with a range of features including impaired social awareness and communication. Again there is a strong genetic component, but no compelling single mechanism (Abrahams and Geschwind 2008). A small proportion of autism cases can be ascribed to a heterogeneous collection of specific loci, and genome-wide screens are now identifying additional susceptibility regions as single-nucleotide polymorphisms and copy-number variants (Glessner et al. 2009; Wang et al. 2009a; Weiss et al. 2009).

Mechanisms for the human disorders are still elusive, but since many of the susceptibility loci encode synaptic proteins, this points at as yet undefined defects in synaptogenesis or synaptic organization (Abrahams and Geschwind 2008; Glessner et al. 2009; Wang et al. 2009a; Weiss et al. 2009). Some loci encode synaptic cell surface molecules such as cadherins, neurexins and neuroligins, the L-type calcium channel CACNA1C, the neurexin-like protein Caspr2 (encoded by *CNTNAP2*) known for its role in organizing protein localization at nodes of Ranvier, and semaphorin 5A (*SEMA5A*), best known for its role in axonal guidance, the postsynaptic density protein Shank3, and the Fragile X protein FMR1. Ube3A protein was recently shown to regulate postsynaptic receptor trafficking (Greer et al. 2010), and Tsc1 and Tsc2 regulate dendritic spine size and synapse function (Tavaziole et al. 2005). *MECP2*, responsible for a syndromic form of autism, Rett Syndrome, encodes a methylcytosine-binding protein with a predicted role in regulation of chromatin conformation and gene expression. Interestingly, *MECP2* expression in glia influences the morphology and function of adjacent neuronal dendrites (Ballas et al. 2009).

Despite the growing characterization of *Drosophila* social behavior it would be highly speculative to model the behavioral aspects of autism spectrum disorders in flies. However, the main value of flies lies in their usefulness as a tool to understand

the functions of susceptibility genes and studies with fly mutants point to functions for many of these genes in synapse development and differentiation:

- Neurexin-1 is required to establish normal synaptic architecture, including apposition of the presynaptic active zone with the postsynaptic receptor field; loss-of-function mutants show defects in this architecture, and moderate reductions in synaptic strength (Li et al. 2008).
- Neurexin-IV (Caspr2) has a role in neuron–glia interactions, e.g., in glial migration, ensheathment, and subdivision of groups of commisural axons (Stork et al. 2009; Wheeler et al. 2009).
- Much of what we know about the role of FMR1 in regulating the synaptic cytoskeleton and in translational control comes from flies (Zarnescu et al. 2005). Work in *Drosophila* continues to reveal new roles of FMR1, e.g., in regulation of sleep and rhythms (Bushey et al. 2009; Gatto and Broadie 2009). The availability of whole-organism phenotypes for *FMR1* mutants have also recently allowed a screen for small molecules that rescue *FMR1* loss-of-function phenotypes (Chang et al. 2008) – an approach with future potential for other genes implicated in neuropsychiatric disorders.
- Levels of semaphorins specify positional targeting of axons during development (e.g., Zlatic et al. 2009).

4.3 Mood, Stress, and Attention

Mood disorders such as depression and anxiety also have complex etiologies, with both environmental causes such as stress, and genetic contributions (see Krishnan and Nestler 2011). They involve systems including the serotonergic system and a number of neuropeptides (reviewed by Leonardo and Hen 2006). Comparable behavioral conditions are hard to identify in *Drosophila*, although there may be some parallels. For example, NPY helps to counteract the behavioral responses of stress in humans (Eaton et al. 2007); recently it has been shown that the homologous NPF system in *Drosophila* inhibits avoidance responses to external noxious stimuli, at least in part by negatively regulating neuronal excitation that results from activation of TRP channels by such stimuli (Xu et al. 2010). As discussed above (Sect. 2.2) *Drosophila* also has a serotonergic system, although the functions of most of its serotonergic neurons are unknown, and at this point in time it can only be speculated on whether they mediate any higher level mood states. Given the emerging realization of the sophistication of higher order fly behaviors, it would not be surprising if such states existed, although detecting them will require equally sophisticated behavioral experimental approaches. Regardless of progress on this front, association studies will doubtless identify novel genetic influences on human mood disorders (e.g., McMahon et al. 2010) and flies will be invaluable in analyzing their underlying neurobiological function, as they have for other disorders.

5 Conclusions

In spite of their small size and apparent simplicity, *Drosophila* show a range of sophisticated behaviors that have much in common with what are regarded as "simple" human behaviors that can be impaired in neuropsychiatric disorders, including behaviors as fundamental as Pavlovian learning and sleep. Here, the powerful genetic and circuit analysis tools of *Drosophila* allow investigation of the mechanisms of these behaviors and of how they can be impaired. Higher order human behaviors, that are impaired in some of the most devastating neuropsychiatric disorders, cannot be modeled so simplistically in flies, but even here there is potentially much to learn from them. Flies may display some of the underlying simpler component behaviors (such as ethanol tolerance), or may provide mutant phenotypes in homologs of susceptibility genes (such as dysbindin) that can provide important clues on underlying neurobiological mechanisms of the human condition. Future use of *Drosophila* in the field is likely to increase, first from increasing understanding of higher order fly behaviors, and secondly from the use of flies to understand the functions of the growing number of susceptibility genes emerging from genome-wide association studies.

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Transgenic Animal Models of Huntington's Disease

Shang-Hsun Yang and Anthony W. S. Chan

Contents

Abstract Huntington's disease (HD) is a devastating neurodegenerative disorder that currently has no cure. In order to develop effective treatment, an understanding of HD pathogenesis and the evaluation of therapeutic efficacy of novel medications with the aid of animal models are critical steps. Transgenic animals sharing similar genetic defects that lead to HD have provided important discoveries in HD mechanisms that cell models are not able to replicate, which include psychiatric impairment, cognitive behavioral impact, and motor functions. Although transgenic HD rodent models have been widely used in HD research, it is clear that an animal model with comparable physiology to man, similar genetic defects that lead to HD, and the ability to develop similar cognitive and behavioral impairments is critical for explaining HD pathogenesis and the development of cures. Compared to HD rodents, HD transgenic nonhuman primates have not only developed comparable

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neuropathology but also present HD clinical features such as rigidity, seizure, dystonia, bradykinesia, and chorea that no other animal model has been able to replicate. Distinctive degenerating neurons and the accumulation of neuropil aggregates observed in HD monkey brain strongly support the hypothesis that the unique neuropathogenic events seen in HD monkey brain recapitulate HD in man. The latest development of transgenic HD primates has opened a new era of animal modeling that better represents human genetic disorders such as HD, which will accelerate the development of diagnostic tools and identifying novel biomarkers through longitudinal studies including gene expression and metabolite profiling, and noninvasive imaging. Furthermore, novel treatments with predictable efficacy in human patients can be developed using HD monkeys because of comparable neuropathology and clinical features.

Keywords Animal models · Huntington's disease · Nonhuman primates · Transgenesis

1 Introduction to Huntington's Disease

Huntington's disease (HD) is an inherited autosomal dominant disease which causes neurodegeneration in humans and was first described by the American physician, George Huntington in 1872. HD affects the central nervous system (CNS), resulting in distinct clinical symptoms related to neuronal and brain dysfunction (Li and Li 2006). The prevalence of HD in the Caucasian population is approximately 7–10 patients per 100,000 population; whereas prevalence decreases in other ethnic groups, such as the African and Asian population (Walker 2007). The progression of HD is evident by the degeneration of gray and white matter in the brain, resulting in the loss of neurons (DiFiglia et al. 1997). Classical HD symptoms include personality changes, emotional disturbances, mental deterioration, involuntary movements, progressive cognitive dysfunction, chorea, dystonia, weight loss, and motor deficits (Li and Li 2006; Walker 2007). The average onset for HD is 37–38 years and median life span is 16.2 years after disease onset. In the absence of a cure for HD, patient care places a major burden on the sufferer, their family, and medical services.

The *Huntingtin* (*HTT*) gene was first cloned in 1993 and is located at the 4p16.3 region of the short arm of chromosome 4 (The Huntington's Disease Collaborative Research Group 1993; Gusella et al. 1983). *HTT* is expressed ubiquitously in the body with the highest levels in the brain and testis (DiFiglia et al. 1995; Li and Li 2004, 2006; Sharp et al. 1995; Trottier et al. 1995). The human *HTT* gene (also called *IT15*) has 67 exons spanning more than 200 kb. Wild-type HTT protein is approximately 350-kDa with a polymorphic stretch of 6–35 glutamine residues in the N-terminal domain of exon 1 (The Huntington's Disease Collaborative Research Group 1993). HD is the result of an expansion of CAG trinucleotide repeats (polyglutamine; polyQ; >37 residues) in the *HTT* gene (Li and Li 2006; Walker 2007). The number of CAG repeats is highly correlated with the severity of

the disease. HD patients with > 60 CAG repeats develop symptoms at ~15–20 years of age. Such patients are categorized as juvenile HD patients. Individuals with CAG repeats in the range of 37–60 develop HD later in life, and are categorized as adult HD patients (Estrada Sanchez et al. 2008).

Mutant *HTT* gene results in a toxic gain-of-function by eliciting cytotoxicity in affected cells, primarily in the brain. Mutant HTT causes neurodegeneration, particularly in the striatal region and deeper layers of the cortex (Li and Li 2006; Walker 2007). The key feature of the early stages of HD is the degeneration of striatal medium spiny neurons (MSNs); however, cortical degeneration may also be observed prior to the development of clinical features (Rosas et al. 2005; Vonsattel and DiFiglia 1998). Other brain regions, such as the hippocampus, the hypothalamus, and the cerebellum are also affected in HD patients (Vonsattel et al. 1985).

Another key determining factor of HD is the size of the HTT fragment, which is highly correlated with the severity and pathogenesis of HD in patients and animal models. Smaller HTT fragments result in more severe phenotypes and earlier onset of HD in transgenic HD mouse models (DiFiglia et al. 1997; Graham et al. 2006; Gutekunst et al. 1999; Li and Li 2006; Schilling et al. 1999). In HD patients and transgenic mouse studies, the N-terminal HTT fragments were misfolded, forming aggregates and inclusions in the brain (DiFiglia et al. 1997; Gutekunst et al. 1999). These studies suggest the proteolysis of full-length HTT to smaller fragments is important in HD pathogenesis. The *HTT* gene contains numerous unique protease cleavage sites within the first 550 amino acids (Graham et al. 2006; Li and Li 2006; Wellington et al. 2002). These cleavage sites are specific for capsase-3, caspase-6, calpain, and several unknown aspartic proteases (Gafni and Ellerby 2002; Kim et al. 2001, 2006; Li and Li 2006; Lunkes et al. 2002; Wellington et al. 2002). When the caspase-6 site was blocked in HD transgenic mice, neuronal function was protected and striatal neurodegeneration was ameliorated (Graham et al. 2006). This suggests proteolysis of full-length HTT contributes to neurodegeneration and HD pathogenesis. Lysosomal proteases, such as cathepsins D, B, and L, also regulate the processing of mutant HTT and the levels of cleavage products (Kim et al. 2006), suggesting their roles in the generation of N-terminal HTT fragments and the clearance of mutant HTT.

2 Cellular and Neuropathology of HD

HTT plays an important role in intracellular trafficking and interacts with proteins that are related to transcription, intracellular signaling, trafficking, endocytosis, and metabolism (Cowan and Raymond 2006; Li and Li 2004). Based on previous studies, wild-type HTT participates in protein trafficking between the Golgi and the extracellular space (Strehlow et al. 2007). In addition, mutant HTT can sequester dynactin p150 and kinesin to influence axonal transport (Gunawardena et al. 2003; Szebenyi et al. 2003) and affects vesicle transport, such as the transport of brain-derived neurotrophic factor (BDNF) along microtubules (Gauthier et al. 2004). The impairment of cellular transport caused by mutant HTT leads to

neuronal toxicity and the loss of neurotrophic support, which results in neuronal cell death (Gauthier et al. 2004). Therefore, mutant HTT may lead to HD through impairment of cellular transport.

Other important factors affecting the progression of HD at the cellular level are the proteins that interact with mutant HTT. In the past 15 years, a number of cytoplasmic proteins, such as HTT-associated protein 1 (HAP1) and HTT-interacting protein 1 (HIP1), have been identified that interact with HTT based on a yeast two-hybrid screen and *in vitro* binding assays (Borrell-Pages et al. 2006; Li and Li 2006; Li et al. 1995). These proteins have been shown to be involved in the HD pathogenesis. For example, the binding of HAP1 with HTT is enhanced by an expansion of polyQ repeats, which regulates the intracellular trafficking of mutant HTT (Li et al. 1995; Rong et al. 2006, 2007). HIP1, p150, and 14-3-3 are all involved in the cascade of protein trafficking (Gauthier et al. 2004; Rong et al. 2007), similar to HTT itself. Additionally, HIP1 is involved in the functions of neuronal cytoskeleton and endocytosis, and also regulates the normal function of HTT with weak binding affinity (Kalchman et al. 1997; Li and Li 2006). Other cytoplasmic proteins, such as Huntington associated protein of 40 kDa (HAP40) and Rab protein 5 (Rab5), are involved in modulating the aggregation and toxicity of mutant HTT through macroautophagy (Pal et al. 2006, 2008; Ravikumar et al. 2008). Furthermore, nuclear factors, such as the coactivators, cAMP response element-binding protein (CREB)-binding protein (CREBBP) and specificity protein 1 (Sp1), and transcription factors, such as TATA-binding protein (TBP) and TBP-associated factors 130 (TAF 130), also play a critical role in HD (Li and Li 2006; Mantamadiotis et al. 2002; Sugars and Rubinsztein 2003). These studies suggest various cytoplasmic and nuclear proteins play a regulatory role in HD pathogenesis.

The hallmark neuropathological feature of HD is neuronal loss in the caudate and putamen, especially targeting MSNs (Vonsattel et al. 1985). In the pathological diagnosis of HD, two signature characteristics are neuropil aggregates and intranuclear inclusions in neurons (Li and Li 2006; Maat-Schieman et al. 2007; Walker 2007). In the postmortem brains of HD patients and transgenic mice, HTT aggregates are primarily observed in the nucleus, and mutant HTT accumulates in striatal neurons as HD progresses (Li and Li 2006; Lin et al. 2001). Based on a structural biology study, mutant HTT is involved in the aggregation process, forming dimers, trimers, and oligomers inside the cells (Walker 2007). In addition, HTT inclusions are the result of protein misfolding which is caused by the mutant polyQ expansion (Li and Li 2006). The expression pattern of HTT also relates to the progression of HD, especially in neurons. In healthy individuals, HTT protein is primarily located in the cytoplasm; whereas mutant HTT protein forms aggregates in the nucleus (Li and Li 2006). In early-stage HD, patients' brains have more neuropil aggregates than nuclear inclusions (DiFiglia et al. 1997; Gutekunst et al. 1999); in late-stage HD, more nuclear inclusions develop. This transition of HTT expression and aggregation may be related to the development of HD and neural degeneration in the brain. Furthermore, the truncated N-terminal proteins also misfold, aggregate and form inclusions in HD patients' brains (DiFiglia et al. 1997; Gutekunst et al. 1999), which results in the accumulation of toxic mutant HTT leading to neurodegeneration and cell death.

3 Aberrant Gene Regulation in HD

Although the relationship between polyQ repeats, N-terminal size, and the severity of HD has been determined in different modeling systems, the effect of mutant HTT on gene regulation remains unclear. Normally, the HTT complex is involved in cellular gene regulation; therefore, mutant HTT is expected to influence gene expression. Based on previous reports, mutant HTT interacts with transcription factors and coactivators at different binding sites, and several transcription factors also contain the polyQ-rich domains, which may interact with other polyQ protein residues (Li and Li 2006; Ryu et al. 2006). For example, mutant HTT represses the transcription of the p53-regulated promoters, p21 (WAF1/CIP1) and MDR-1 (Bae et al. 2005; Steffan et al. 2000) and also interacts with transcription factors carrying polyQ or proline-rich domains, such as CREBBP, TBP, and TAF130 (Li and Li 2006; Perez et al. 1998; Shimohata et al. 2000; Steffan et al. 2001). The CAG repeats in HTT also change the normal nuclear location of CREBBP to the nuclear aggregates of HD (Nucifora et al. 2001; Steffan et al. 2000). Transcription activator, Sp1, and coactivator, TAFII130, are also suppressed by mutant HTT, resulting in transcriptional inhibition of the dopamine D2 receptor gene, which causes cellular toxicity (Chen-Plotkin et al. 2006; Dunah et al. 2002). By sequestering transcription factors, such as Sp1, mutant HTT reduces the opportunities for binding to the promoter region, thus down-regulating the expression of target genes (Chen-Plotkin et al. 2006). Furthermore, TBP was found in the HTT aggregates and interacted with polyQ-containing proteins. With the abnormal interaction of these proteins that are essential for the survival of target neurons, mutant HTT aggregates lead to neuronal death in HD (Huang et al. 1998). Since the aberrant expression of mutant HTT protein was also detected ubiquitously and affected gene expression patterns in peripheral tissues, such as the muscle and blood (Borovecki et al. 2005; Luthi-Carter et al. 2002), the gene regulation pathway may also cause dysfunction in peripheral tissues. For example, HTT aggregates affected liver function and resulted in the dysregulation of the ubiquitin–proteasome system (Chiang et al. 2009), CCAAT/enhancer binding protein (C/EBPα) and peroxisome proliferator-activated receptor-γ (PPARγ), which led to the impairment of urea cycle (Chiang et al. 2007).

HTT interacts with several essential transcription repressors to regulate gene expression. REST (Repressor element 1 silencing transcription factor, also known as NRSF, neuron-restrictive silencing factor) is normally expressed in the cytoplasm of neurons; however, in HD, REST is sequestered by mutant HTT and accumulates in the nucleus of neurons. These changes repress the expression BDNF, a REST target gene and decrease the survival of striatal neurons by altering the cooperation of chromatin-modifying proteins (Ooi and Wood 2007; Zuccato et al. 2003). REST potentially interacts with more than 1,000 sites in the human or mouse genome, and some sites are putative targets related to neuronal function and differentiation (Bruce et al. 2004; Johnson et al. 2006; Zuccato et al. 2007), thus the regulation of REST may contribute to the development of HD. These studies

suggest that mutant HTT may have a compound effect on regulating gene expression via transcription factors and cofactors.

Recently, posttranscriptional regulation of noncoding RNAs in metazoan, mice, and humans, especially in the area of microRNA (miRNA), has been found. Several miRNAs have been identified which are involved in neuronal functions and development (Lim et al. 2005; Vo et al. 2005; Yu et al. 2008). In several neuronal diseases, such as Fragile X syndrome (Jin et al. 2004; Li et al. 2008; Qurashi et al. 2007), Parkinson's and Alzheimer's disease (Kim et al. 2007; Lukiw 2007), miRNAs play a critical role in pathogenesis. Furthermore, mutant HTT has been proven to alter the association status with argonaute 2 protein and P bodies (Savas et al. 2008), suggesting mutant HTT's role in posttranscriptional processes. Therefore, it is tempting to speculate that miRNA also plays a role in neuropathogenesis of HD.

Several miRNAs are expressed during brain development; for example, miR-124 is preferentially expressed in the brain (Lim et al. 2005; Yu et al. 2008) and miR-132 is enriched in neurons (Vo et al. 2005). These miRNAs are involved in the control of the neuronal transcriptome and regulation of neuronal morphogenesis and dendrite development (Lim et al. 2005; Vo et al. 2005; Yu et al. 2008), suggesting a regulatory role of miRNAs in the brain. Another polyglutamine disease, spinocerebellar ataxia type 3 (SCA3) (Gondo et al. 2011) is also modulated by the miRNA, bantam, which acts downstream of SCA3 to prevent degeneration (Black et al. 2002). Mutations of Dicer in SCA3 enhance polyQ toxicity in both Drosophila and HeLa cells (Black et al. 2002). In a similar study, the depletion of Dicer led to dysfunction in miRNA processing and resulted in the death of Purkinje cells (Schaefer et al. 2007). This evidence implies a neuroprotective role of miRNAs in neural diseases (Schaefer et al. 2007) and gives insight into the miRNA pathway in HD.

Due to the interaction between HTT and the transcriptional repressor, REST, HD may be regulated by miRNAs while REST regulates the expression of several neuronal miRNAs (Conaco et al. 2006). REST suppresses the expression of miR-124a in nonneuronal cells and neural progenitors, leading to the existence of nonneuronal transcripts (Conaco et al. 2006). In the brain tissue of HD mice and human patients, upregulation of REST enhanced the repression of neuronal-specific miRNA, mir-132, leading to higher levels of p250GAP mRNA (Johnson et al. 2008). It is possible that other miRNAs may also be involved in the pathogenesis of HD at the posttranscriptional level.

Epigenetic modification also contributes to HD pathogenesis by altering nucleosome dynamics, chromatin remodeling and subsequent transcriptional dysregulation. Histone modification is one epigenetic change that is important in HD. Several studies have shown aberrant histone modification patterns in HD patients and animal models. For example, the extent of acetylation and deacetylation of histones differs between HD and wild-type in cell, fly, and mouse models based on the expression level of histone acetyltransferase (Giorgini et al. 2008; Sadri-Vakili et al. 2007; Steffan et al. 2001). In addition, altered histone methylation and histone methyltransferase are important for transcription-induced neuronal death in HD (Ryu et al. 2006). Recently, treatments targeting HD epigenetic modification have

been developed, including histone H3 (K9) methyltransferase and histone deacetylase inhibitors (Dompierre et al. 2007; Ryu et al. 2006). These studies support a potential role of mutant HTT in global gene dysregulation at an epigenetic level (McCampbell et al. 2001; Ryu et al. 2006; Steffan et al. 2001).

The development of HD may be dependent on the effect of mutant HTT on gene regulation; therefore, studies of gene regulation are important in fully understanding HD. The genome-wide DNA microarray analysis of HD is a powerful tool to monitor thousands of gene expression profiles in relation to their pathological status. As a result, several genomic profiling studies using microarray technologies have demonstrated the effect of mutant HTT overexpression on the gene expression profile in different HD mouse models and patients (Borovecki et al. 2005; Chan et al. 2002; Crocker et al. 2006; Luthi-Carter et al. 2002). There are two categories of expression changes in HD microarray studies: direct responses to mutant HTT and indirect/secondary compensatory effects to mutant HTT. Therefore, it is a challenge to identify the most important transcripts directly related to HD. Because cellular and molecular alterations in neurons or other somatic cells must precede clinical symptoms such as motor dysfunction, identifying a set of genes that are specifically altered at different stages of HD could be useful in early diagnosis and determining the extent of HD progression. Furthermore, clarifying the transcriptional changes throughout the course of HD will provide important information for understanding the mechanism of neurodegeneration and the development of HD.

4 Rodent Models of HD and Limitations

Transgenic technology has led biomedical research into a new era for animal modeling (Gondo et al. 2011), which has accelerated the understanding of gene function and disease development. In the HD field, several transgenic organisms have been used as HD models for investigating mechanisms of disease progression. These include yeasts (Giorgini et al. 2008), flies (Marsh et al. 2003; Williams et al. 2008), zebrafish (Diekmann et al. 2009; Williams et al. 2008), mice (Schilling et al. 1999; Wang et al. 2008), rats (Bugos et al. 2009; von Horsten et al. 2003), and primates (Brouillet et al. 1995; Palfi et al. 1996, 2007; Yang et al. 2008). Yeasts, flies, and zebrafish are important model systems that allow easy and simple genomic manipulation. Additional advantages include their smaller size and easy maintenance. While novel discoveries of HD mechanism have been achieved in these model systems, genome composition and physiological functions remain the limiting factors that distinguish them from higher mammals.

Transgenic mice expressing mutant *HTT* controlled by different promoters, including a *HTT* promoter and a neuron-specific promoter (e.g., prion), show similar neuropathology and motor impairment compared to HD patients. One of the most commonly used transgenic mice, R6/2, expresses exon 1 of *HTT* with 115–156 CAG repeats under the control of human *HTT* promoter (Davies et al. 1997; Mangiarini et al. 1996). These mice develop a progressive neurological

phenotype including motor and cognitive deficits (Carter et al. 1999). A dramatic loss of MSN in the striatum and the formation of intranuclear inclusions in neurons are similar to those observed in HD patients (Carter et al. 1999; Li et al. 2003; Li and Li 2006; Lione et al. 1999). R6/2 mice also lose weight at approximately 9 weeks old, suggesting metabolic dysfunction and abnormal regulation of weight-regulating factors (van der Burg et al. 2008). Furthermore, pre- and post-symptomatic R6/2 mice reveal alterations in corticostriatal synaptic transmission (Levine et al. 2004) and abnormalities in dopaminergic cell function, which lead to progressive motor and cognitive symptoms (Cha et al. 1998; Johnson et al. 2006; Kung et al. 2007). Due to the similarity of R6/2 mice and HD patients in neuropathological alterations and clinical characteristics, R6/2 mice are broadly used in the investigation of HD pathogenesis, which include study of the ubiquitin/proteasome system (Bennett et al. 2007; Bett et al. 2009; Chiang et al. 2009; Maynard et al. 2009), pathological deficits (Phan et al. 2009; Rossi et al. 2006; Sawiak et al. 2009), mitochondria functions (Acevedo-Torres et al. 2009; Bogdanov et al. 2001; Ferrante et al. 2004; Yang et al. 2009), and the development of potential biomarkers (Chopra et al. 2007; Strand et al. 2005; Tsang et al. 2006). Furthermore, the R6/2 mice are one of the most broadly used HD mouse models for the development of novel treatments, such as sodium butyrate chemotherapy1, mithramycin2, V(L)12.3, Happ13, histone deacetylase inhibitors4, and Coenzyme Q(10)5.

Another widely used transgenic HD mouse, N171-82Q, expresses the first 171 amino acids of HTT with 82 CAG repeats under the control of neuronal specific *prion* promoter (Schilling et al. 1999). N171-82Q mice display similar neurological symptoms to R6/2 mice in a similar time frame. The HTT N-terminal fragments are prone to form intranuclear inclusions and neuropil aggregates (Schilling et al. 1999; Wang et al. 2008). These mice also develop behavioral abnormalities, including loss of coordination, tremors, hypokinesia, and abnormal gait (Schilling et al. 1999). Studies on N171-82Q mice further support the neurotoxicity of N-terminal fragments of HTT with expanded CAG repeat. Due to the well-defined characteristics of N171-82Q mice, they have been broadly used in the investigation of neuroprotective effects (Gardian et al. 2005; Vamos et al. 2009), chaperone function (Orr et al. 2008), epigenetic effect (Zadori et al. 2009) and to identify therapeutic targets and biomarkers of HD (Ramaswamy et al. 2009; Zadori et al. 2009). However, due to the aggressive development of HD at young age and their short life span (<6 months), R6/2 and N171-82Q, are difficult for maintain for long-term study.

To prevent the impact of position effect and retain endogenous regulation elements, yeast artificial chromosome (YAC) HD mice were generated. YAC HD mice carry full-length *HTT* with 46 or 72 CAG repeats under the regulation of the human *HTT* promoter. Studies using YAC HD mice revealed the cleavage of HTT leading to neuronal cytoplasmic toxicity followed by nuclear translocation of the N-terminal fragments of HTT (Hodgson et al. 1999; Van Raamsdonk et al. 2007a, b). Mice with expanded CAG repeats show motor and cognitive impairment, striatal degeneration (Hodgson et al. 1999; Van Raamsdonk et al. 2005, 2007a) and exhibit higher sensitivity to NMDA-induced apoptosis in MSNs cultured from

postnatal pups (Fernandes et al. 2007; Hodgson et al. 1999; Shehadeh et al. 2006; Slow et al. 2003). Studies on YAC HD mice also suggested that HTT aggregates are not necessary for neurodegeneration, while proteolytic cleavage of HTT remains important in HD pathogenesis (Hodgson et al. 1999; Li and Li 2006).

In addition to overexpression of mutant HTT, gene targeted HD mice were also generated. HTT is an essential protein responsible for intracellular trafficking and transcription. Knock-out mice were embryonically lethal, suggesting the vital role of HTT in early embryo development (Dragatsis et al. 1998). While knock-out mice are lethal, *HTT* knock-in mice represent a precise genetic replica with mutant *HTT* under the control of normal regulatory elements. *HTT* knock-in mice display behavioral phenotypes at approximately 70 weeks, including motor deficit and gait abnormality (Heng et al. 2007; Lin et al. 2001). Neuropathological characteristics of a *HTT* knock-in mouse include the development of reactive gliosis and the formation of neuronal intranuclear inclusions, predominantly in the striatum (Heng et al. 2007; Lin et al. 2001; Woodman et al. 2007). Widespread aggregates in the brain, dysregulated gene expression in the striatum and cerebellum and decreased expression level of specific chaperones are similar to R6/2 mice (Woodman et al. 2007). The overall neuropathology found in *HTT* knock-in mice is similar to early stage in HD patients (Lin et al. 2001).

In addition to transgenic HD mice, transgenic HD rats have also been developed because their larger brain size is better suited to noninvasive imaging and more sophisticated and versatile behavioral tests are available. Larger brains are also advantageous for cell transplantation, cerebrospinal fluid collection, intravenous cannulation and microsurgery that are relatively limited in mice. Additionally, rat, not mouse, displays unique genomic characteristics with specific genes related to human pheromones, immunity, chemosensation, detoxification, and proteolysis (Gibbs et al. 2004), which may have profound impact on HD pathogenesis. Most importantly, most of the human disease related genes have orthologs in the rat.

A rat model of HD was first described by Schwarcz et al. (1977). Kainic acid was stereotaxically injected into rat striatum and resulted in neuronal degeneration similar to HD (Schwarcz et al. 1977). In fact, potential therapeutic agents for HD were first developed in the late 1970s using the kainic acid-induced model (Beaumont et al. 1979; Borison and Diamond 1979; Coyle 1979). Additionally, a similar focal injection approach using quinolinic acid instead of kainic acid has also resulted in motor deficit and pyruvate-mediated neuroprotection in rats (Block et al. 1993; McBride et al. 2003; Ryu et al. 2004). Other chemicals, such as malonate, or viruses expressing mutant *HTT* have also been introduced into the striatum of rats to induce lesion that led to HD-like phenotypes (McBride et al. 2003; Meldrum et al. 2000). In addition to the focal injection rat model, the first transgenic HD rat to carry a truncated *HTT* cDNA fragment with 51 CAG repeats under control of the native rat *HTT* promoter was reported in 2003 (von Horsten et al. 2003). Transgenic HD rats exhibited similar neurological phenotypes including reduced anxiety, cognitive impairments, and motor dysfunction. Histopathological analysis revealed neuronal nuclear inclusions and neuropil aggregates in different regions of the brain, which was similar to HD patients and transgenic HD mice. Because of their larger

body size, it is possible to perform magnetic resonance imaging (MRI) and positron emission tomography (PET) in rats. MRI studies revealed enlarged lateral ventricles and focal lesions in the striatum, while PET scans suggested metabolic abnormalities in transgenic HD rat (von Horsten et al. 2003).

Besides neuropathology and noninvasive imaging studies in transgenic HD rat, mitochondria dysfunction was shown to play a critical role in HD pathogenesis. Ca^{2+}-dependent impairments of mitochondrial oxidative phosphorylation was revealed as one of the primary causes of HD in transgenic HD rat (Gellerich et al. 2008). Potential therapy was also developed using the transgenic rat model. Bilateral globus pallidus deep brain stimulation in HD rat resulted in improvement in cognitive and motor symptoms, which was considered as a possible treatment for HD patients (Temel et al. 2006).

Although HD rodent models have had a significant impact in breakthrough HD research, they have limitations. For example, neural anatomy and gene functions in rodents are distinct from higher primates. In fact, the extent of overall neurodegeneration in transgenic HD rodents HD patients even when similar neurological symptoms were developed (Davies et al. 1997; Li and Li 2006; Schilling et al. 1999). Possible explanations for these limitations are (1) the extent of neurodegeneration is limited by the short life span of rodents (Li and Li 2006), (2) gene function may vary between rodents and humans, and (3) there are fundamental differences, especially in the brain, between rodents and humans. While transgenic HD rodent models will continue to play a critical role in HD research, it is important to develop an animal model with closer proximity to humans (Chan 2004; Yang et al. 2008) not only physiologically but also genetically, to better capture the clinical features of HD and further accelerate our understanding of HD pathogenesis and the development of effective therapeutic strategies.

5 Development of HD Nonhuman Primate Models

Nonhuman primates are ideal for modeling human neurodegenerative diseases for a number of reasons. First, brain functions and anatomy of nonhuman primates are highly similar to those of humans (Presty et al. 1987; Small et al. 2004; Walker et al. 1988). Second, the complex behavior of nonhuman primates enables advanced studies of cognition, social and motor development that are progressively impaired in neurodegenerative disorders (Peters et al. 1996). Third, the large brain size and comparable anatomical structure of nonhuman primates are suitable for high resolution in vivo imaging such as MRI and PET, which are powerful diagnostic tools in humans. Fourth, nonhuman primates also share high similarity in genome constitution and physiologic functions with humans, thus the likelihood of identifying human ortholog genes in nonhuman primates such as rhesus macaques is higher than for other species (Chan 2004). Finally, longitudinal studies are possible in nonhuman primates because of their longer life span. By integrating findings in *in vivo* imaging, cognitive and behavioral studies, metabolite profiling and gene

expression profiling in peripheral blood as disease progresses, HD nonhuman primates provide a unique model for developing early diagnostic tools, identification of biomarkers and therapeutic targets that will accelerate the development of effective treatments (Chan 2004).

Nonhuman primate models of HD were first reported by chemical induction and subsequent development of focal lesion in the brain by stereotaxic delivery of lentiviruses expressed mutant *HTT* (Burns et al. 1995; Isacson et al. 1990; Lee and Chang 2004; Palfi et al. 1996). Apomorphine was first used to induce excitotoxic caudate-putamen lesions that resulted in HD-like phenotypes, such as dyskinesias (Isacson et al. 1990). Similar to rat lesion models, focal injection of quinolinic acid in the caudate and putamen of rhesus monkeys caused spontaneous HD-like chorea and has led to a new model system for movement disorders (Burns et al. 1995). Furthermore, focal injection of 3-nitropropionic acid, a mitochondrial inhibitor, has led to the development of pathophysiological HD phenotypes which include movement disorder, progressive striatal degeneration, and frontostriatal syndrome of cognitive impairment. These unique behavioral phenotypes and motor deficits strongly suggested the high similarity between nonhuman primates and humans, which are superior to the other model systems especially in replicating cognitive behavioral and motor impact in HD.

Stereotaxic bilateral injection of lentiviruses carrying mutant *HTT* into dorsolateral sensorimotor putamen has led to the classic symptoms of HD in nonhuman primates for up to 30 weeks (Palfi et al. 2007). These monkeys displayed progressive chorea, dystonia, spontaneous dyskinesia, and ipsilateral turning behavior. Neuropathological studies revealed neuritic and nuclear HTT aggregates, reactive astrocytes, and the loss of the neurons. This further demonstrated that the expression of mutant *HTT* in a specific brain region of nonhuman primates is capable of replicating HD phenotypes comparable to human patients.

Although the creation of focal lesions in monkey brain by lentiviruses has successfully induced clinical features of HD and has proven to be a very useful model for HD research the approach has limitations. Focal lesion primate models develop neuropathological and behavioral changes similar to those observed in HD patients but the systemic effect of mutant HTT and its impact on specific neuronal targets cannot be investigated due to the limited number of target cells that will be randomly infected by lentiviruses at the injection site. Efficient diffusion of viruses to surrounding tissues has also limited successful gene delivery into the target cells. Thus, variations in mutant HTT expression and cellular responses to mutant HTT are expected. This may lead to misinterpretation due to suboptimal gene transfer in the target cell population or simply because of variations among different individuals. Similar limitations apply with focal chemical induction models. Thus, focal chemical induction and the focal transgenic model might not be sufficient to elicit a degeneration process and consistent clinical responses between individuals. In addition to its potential impact on the outcome of the study, focal transgenic model characteristics cannot be inherited by the next generation and do not allow the study of systemic effects.

6 Germline Nonhuman Primate Model of HD

The three basic criteria for a successful transgenic animal are (1) stable integration of the gene of interest (transgene) into the host cell genome, (2) expression of the transgene and bioactivity, and (3) transmission of the transgene to the next generation (germline transmission). The first two points (1 and 2) determine the genotype and phenotype of a transgenic animal, whereas germline transmission is important for preserving the unique genotype and phenotype and is critical for extending availability of the transgenic model to broader applications. When a transgenic animal is identified as a useful model for human conditions, it is important to preserve its unique genotype and phenotype. If germline transmission is achieved, these animals can be reproduced by traditional breeding processes, and the unique genotype and phenotype will be preserved throughout generations. However, since rhesus monkeys have a long gestation period (5.5 months) and limited availability, the generation of transgenic nonhuman primates such as rhesus monkeys is a challenging task. With the aid of assisted reproductive techniques (ART), larger numbers of mature oocytes can be recovered from females stimulated by hormonal regimens, similar to the approach used in human fertility clinics. Together with *in vitro* fertilization and culture techniques, high quality embryos can be generated for gene transfer followed by transplantation into surrogate females for the generation of transgenic monkeys. Additionally, cryopreservation of gametes derived from transgenic animals will further ensure the preservation of unique genetic configurations. Due to the advancement in ART, cryopreserved gametes can also be used to reproduce offspring at any time.

One of the prerequisite techniques in developing transgenic nonhuman primate models is an efficient gene delivery method requiring a relatively small number of animals in the development process. Therefore, despite the fact that nonhuman primates are one of the best animal models for human disease, the development of a transgenic nonhuman primate model has not been considered feasible until such fundamental techniques as efficient gene transfer methods were established. Lentiviruses has been successfully used for the generation of different transgenic species including mice, rats, prairie voles, pigs, cattle, and monkeys (Butland et al. 2007; Chapman et al. 2005; Donaldson et al. 2009; Hofmann et al. 2004; Lois et al. 2002; Park 2007; Sasaki et al. 2009; Whitelaw et al. 2004; Yang et al. 2008) with successful germline transmission and have been shown to be one of the most important breakthrough technologies in transgenic research.

Five transgenic HD monkeys generated by lentiviral gene transfer in early embryos were first reported in 2008, which has led to a new era of animal modeling of human inherited genetic disorder (Yang et al. 2008). Transgenic HD monkeys carried the exon 1 of human *HTT* gene with expanded polyQ tract under the regulation of human ubiquitin promoter. The fact that CAG repeats are highly unstable, CAG repeat numbers ranging from 29 to 88 was revealed in HD monkeys. Among the five HD monkeys, four were euthanized at 1 day to 1 year of age due to the severity of their HD symptoms, including dystonia (Fig. 1a), chorea, seizure,

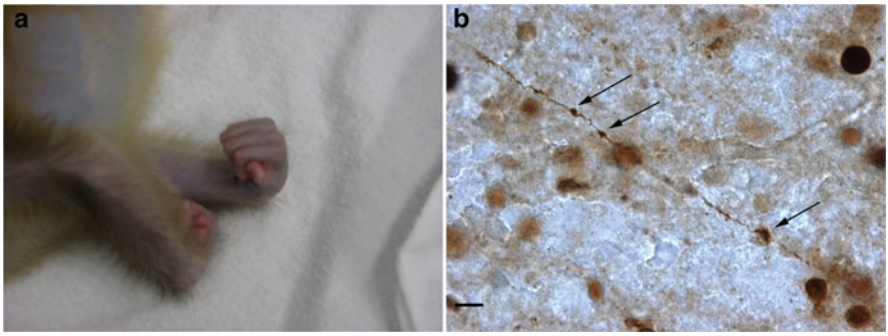

Fig. 1 Clinical features and neuropathology in transgenic HD monkeys. (**a**) Transgenic HD monkey at 1 month of age showing involuntary dystonic posture of the arms. (**b**) EM48 immunostaining of the striatum of HD monkeys that expressed exon-1 HTT with 147Q. Mutant HTT is abundant in the nucleus and forms aggregates in neuronal processes. *Arrow* indicates aggregates in the neuronal processes with disrupted appearance. *Scale bar* = 5 μm

and weight loss which were comparable to human HD patients. The remaining HD monkey carries a single copy of mutant *HTT* with 29 CAG repeats. Although the number of CAG repeats is below the threshold for HD in human patients, rhesus monkeys have only 10–11 CAG repeats flanking the *HTT* gene. Thus, if ever developed, later onset of HD is expected in the living HD monkey. While expression of mutant HTT was determined in placental tissues and peripheral blood, longitudinal studies include noninvasive imaging, genomic and metabolite profiling, and cognitive behavioral evaluation are ongoing. Due to the extreme toxicity of the mutant *HTT* construct (consisting of the Exon 1 and long CAG repeats) as shown in the HD monkeys, the next generation of HD monkeys with less aggressive phenotypes that develop a later onset of HD should be considered. To achieve this purpose, weaker promoters, such as *HTT* promoter, or shorter CAG repeats with larger HTT fragments will be considered.

HD monkeys revealed the selective expression of mutant HTT in different regions of the brain and peripheral tissues, which was similar to the expression pattern in HD patients. Mutant HTT was expressed predominantly in the cortex and striatum with the formation of intranuclear aggregates (inclusion bodies) and neuropil aggregates in HD monkeys, which are signature neuropathological features in HD patients (Yang et al. 2008) and are comparable to prior studies in HD transgenic rodents (Davies et al. 1997; Schilling et al. 2007; Vonsattel et al. 1985; Wang et al. 2008). One distinctive observation was the formation of neuropil aggregates along the swollen and disrupted neuronal processes, which captured the process of neuronal degeneration (Fig. 1b) (Wang et al. 2008). In addition, the expression of mutant HTT was also observed in glial-like cells, which suggested mutant HTT may affect both neuronal and nonneuronal cell types in the brain (Wang et al. 2008). Based on prior study, mutant HTT has been reported to accumulate in glial nuclei in HD brains (Shin et al. 2005; Bradford et al. 2009, 2010a, 2010b), which suggest the impact of nonneuronal cells in HD pathogenesis.

Besides neuropathology, neuroimaging and cognitive behavioral testing, lymphoblast cell lines have been widely used in studies involved in pharmacogenetics (Hashem et al. 2004), neurodegeneration (Acehan et al. 2007; Panov et al. 2002; Toneff et al. 2002), transcriptional profiling (Abuhatzira et al. 2005), and the development of new medications (Hashem et al. 2004). The advantages of using lymphoblast cell lines are the easy access of peripheral blood and a well-established protocol for the establishment of lymphoblast cell lines. One may have concerns about using lymphoblasts to represent neuronal cell types, the primary site of neurodegeneration, and although there are fundamental differences between lymphoblasts and neurons, recent studies have demonstrated that gene expression profiling in peripheral blood is highly correlated with HD progression and response to experimental treatment (Borovecki et al. 2005; Sharp et al. 2006). Recent studies have found abnormalities in lymphocytes, which include elevated levels of oxidative DNA damage (Morocz et al. 2002) and an increased number of apoptotic monocytes (Bergman et al. 2002). Thus, the alteration of gene expression patterns that are specifically observed in lymphocytes may be used as diagnostic information complementary to clinical evaluation and as a biomarker indicating the progression of the disease. Furthermore, genomic profiling of neurological diseases using blood has been suggested as a monitoring scheme of the progression of disease and response to treatment (Sharp et al. 2006; Tabchy and Housman 2006). Lymphoblast cell lines from HD patients have also been used to investigate mitochondrion function and comparative study of htt proteolytic fragments with human brains (Toneff et al. 2002). In addition to the versatile applications of lymphoblast cell lines, genomic profiles and cellular responses correlated with disease progression could be closely monitored due to the fact that peripheral blood could be collected at different stages of HD. Furthermore, lymphoblast cell lines established at different stages of HD could be a powerful tool for evaluating the efficacy of novel therapies and chemicals that were designed to target specific stages of HD, thus stage specific response to novel treatment could also be determined. Therefore, lymphoblast cell lines in HD monkeys are unique tools for monitoring the alteration of genomic and cellular function in HD, which could be correlated with neuroimaging, cognitive and behavioral changes in HD monkeys longitudinally.

7 Limitations of Nonhuman Primate Models and Future Directions

Although the advancement of ART in nonhuman primates and the latest development of lentiviral gene transfer technology have overcome some of the major barriers in developing transgenic nonhuman primate models of human inherited genetic diseases (Chan 2004; Sasaki et al. 2009; Yang et al. 2008), the physiological constraints of nonhuman primates such as age of puberty (34 years old), long

gestation time (150–160 days), and singleton pregnancies have become the limiting factors in transgenic primate modeling. Thus, one of the major challenges in the field is to generate sufficient numbers of HD monkeys and to make them available for the biomedical community. Although ART could overcome most of the limitations in traditional breeding schemes, a strategic breeding approach needs to be designed specifically for each primate model. For transgenic HD monkey models, thorough characterization of HD monkeys should be accomplished prior to the derivation of offspring from a HD monkey founder with a unique and known phenotype, which should be confirmed and evaluated before puberty. When HD monkeys reach puberty, depending on their gender, different strategies could be in place for the establishment of a cohort of HD monkeys with the aid of ART. Female monkeys can either be enrolled in traditional breeding schemes (the most time consuming approach) or used for hormone stimulation, followed by *in vitro* fertilization and culture, with a transfer of embryos into surrogate females. This approach could generate 8–10 babies in 1–2 years based on current success rate in rhesus macaques. On the other hand, male HD monkeys have an advantage over females when breeding of a selected HD monkey founder is needed. Female monkeys could be inseminated using HD monkey semen or a surrogate female could receive embryos derived from HD monkey sperm. Thus, HD monkey offspring could be generated in 5–6 months (gestation time of monkey) while the limiting factor is still the availability of adult females for insemination or as surrogates.

In addition to physiological limitations, a transgenic monkey model is limited to a dominant genetic disorder by overexpression of the mutant gene. The generation of a gene targeted monkey by creating a chimeric monkey may not be a realistic approach because of the long gestation time, singleton pregnancy, and limited availability of monkeys. Even if chimeric monkeys are generated, subsequent breeding processes may reduce the practicality of the approach. One possible method to overcome the hurdle of chimerism is by cloning or nuclear transplantation. However, there have to date been no successful attempts to generate a viable cloned monkey. Thus, until the perfection of gene targeting or nuclear transfer technology has been achieved, transgenic primate models of human inherited genetic diseases is expected to be primarily focused on dominant genetic defects or the overexpression of regulatory RNA such as small hairpin RNA to knock down the expression level of the target genes.

Although recent advancement in modeling human genetic diseases such as HD has further suggested that higher primates hold great promise in mirroring human conditions, the high cost of primate research often governs our interest in applying primate models in critical studies. A transgenic primate animal model could potentially help us explain the pathogenesis of Alzheimer's, Parkinson's, and Huntington's diseases because other animal models cannot replicate key aspects of disease pathology, simply due to their physiological differences from humans (Yang et al. 2008). However, due to the limited availability of primates and primate facilities, and relatively high costs, one should only consider working with diseases that could clearly benefit from a transgenic primate.

8 Conclusion

Transgenic animal modeling of human diseases has accelerated the progression of biomedical research in the past decades and has proven to be one of the most valuable resources in explaining human disease mechanisms and pathogenesis. It is an exciting time in animal modeling as significant advances in transgenic primate research in recent years has opened the door for researchers to consider primates as a model system with the potential of replicating unique human conditions that may not be possible to achieve with other species. However, one should keep in mind that since there is no perfect animal model for humans, it is important to embrace the value of comparative medicine in bringing us a step closer to understand human diseases, and thus develop effective cures and preventative strategies.

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Molecular Genetic Models Related to Schizophrenia and Psychotic Illness: Heuristics and Challenges

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Contents

Abstract Schizophrenia is a heritable disorder that may involve several common genes of small effect and/or rare copy number variation, with phenotypic heterogeneity

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across patients. Furthermore, any boundaries vis-à-vis other psychotic disorders are far from clear. Consequently, identification of informative animal models for this disorder, which typically relate to pharmacological and putative pathophysiological processes of uncertain validity, faces considerable challenges. In juxtaposition, the majority of mutant models for schizophrenia relate to the functional roles of a diverse set of genes associated with risk for the disorder or with such putative pathophysiological processes. This chapter seeks to outline the evidence from phenotypic studies in mutant models related to schizophrenia. These have commonly assessed the degree to which mutation of a schizophrenia-related gene is associated with the expression of several aspects of the schizophrenia phenotype or more circumscribed, schizophrenia-related endophenotypes; typically, they place specific emphasis on positive and negative symptoms and cognitive deficits, and extend to structural and other pathological features. We first consider the primary technological approaches to the generation of such mutants, to include their relative merits and demerits, and then highlight the diverse phenotypic approaches that have been developed for their assessment. The chapter then considers the application of mutant phenotypes to study pathobiological and pharmacological mechanisms thought to be relevant for schizophrenia, particularly in terms of dopaminergic and glutamatergic dysfunction, and to an increasing range of candidate susceptibility genes and copy number variants. Finally, we discuss several pertinent issues and challenges within the field which relate to both phenotypic evaluation and a growing appreciation of the functional genomics of schizophrenia and the involvement of gene × environment interactions.

Keywords Gene × environment interaction · Mutant model · Phenotype · Psychotic illness · Schizophrenia · Susceptibility gene

1 Introduction

Schizophrenia is an enigmatic and debilitating psychotic illness that is associated with profound human, societal and economic costs on a world-wide basis; having a lifetime prevalence of 0.3–0.6% and an incidence of 10–20 per 100,000 person-years, patients with schizophrenia show not only substantive functional impairments but also increased mortality that is due primarily to higher rates of suicide, risk factors for medical morbidity and accidents/misadventures (McGrath et al. 2008; Tandon et al. 2008; Van Os and Kapur 2009). This disorder is characterised by a multifactorial aetiology that involves genetic liability interacting with epigenetic and environmental factors to increase risk for developing the disorder (Waddington et al. 2007; Gill et al. 2009; Kirby et al. 2010; O'Tuathaigh and Waddington 2010). Furthermore, any boundaries vis-à-vis other psychotic disorders are far from clear.

Over the past several years, molecular genetics has identified a number of candidate risk genes, as documented in recent systematic reviews and meta-analyses (Allen et al. 2008; Gogos 2007; Shi et al. 2008), with the most recent studies indicating a substantive role for rare structural variants (copy number

variations (CNVs); International Schizophrenia Consortium 2008, 2009). Some consensus is emerging to suggest that schizophrenia likely involves the action of both common alleles of small effect *and* individually rare but highly penetrant CNVs (Waddington et al. 2007; Williams et al. 2009). In relation to these common alleles, the risk variant has yet to be agreed upon and it is at present unclear whether they each contribute individually in an incremental fashion, act in a more complex manner, or exert background effects to modify the effect of rare variants (Gill et al. 2009). Such candidate genes may not confer risk for schizophrenia per se; rather, there may be: (a) specific relationships between individual risk alleles and different domains of psychopathology and underlying pathobiology; (b) specific relationships between individual risk alleles and different *endophenotypes* within the overall schizophrenia syndrome; (c) more general relationships between risk alleles and aspects of psychotic illness that transcend conventional diagnostic boundaries, for example in relation to bipolar disorder as well as schizophrenia; and (d) interactions between individual susceptibility genes (epistasis) and/or interaction between susceptibility gene(s) and exposure to one or more environmental adversities.

A role for several of these genes is supported by biological plausibility, such as their involvement in important neurodevelopmental processes and, thus, putative disease mechanisms. It should be noted, however, that evidence for biological plausibility should not be viewed in an exclusionary manner, so as to reduce risk for discounting or ignoring novel targets for therapeutic interventions. Preliminary evidence from genome-wide association studies, which should have greater power to detect the role of common alleles of small effect, have indicated associations between risk for schizophrenia and several neurodevelopmental proteins previously implicated in schizophrenia pathobiology, including reelin (Shifman et al. 2008) and colony-stimulating factor-2 receptor alpha (CSF2RA; Lencz et al. 2007), as well as as-yet uncharacterised genes such as zinc finger protein 804A (ZNF804A; Moskvina et al. 2009). However, the absence of validating disease biomarkers necessarily places a handicap on any efforts to identify common risk alleles, as does the increasing demand for ever larger sample sizes to endow sufficient power to yield statistically significant findings.

Most recently, there has been intense interest in multiple, rare CNVs, each of which may confer risk for schizophrenia in a very small number of cases. These variations usually consist of DNA sequences that vary between individuals as a result of duplication or deletion of chromosomal material. Early evidence for the significance of CNVs came from patients with velocardiofacial syndrome, which is caused by a chromosome 22q11 microdeletion and is associated not only with cardiac anomalies, craniofacial dysmorphology and learning disabilities but also with a substantive increase in risk for psychosis (Murphy et al. 1999; Murphy and Owen 2001; Karayiorgou and Gogos 2006). Subsequent evidence indicates that there appears to be a greater number of low-frequency CNVs, at numerous loci, in individuals with schizophrenia (International Schizophrenia Consortium 2008; Walsh et al. 2008; Kirov et al. 2009a). Several studies have identified common CNV loci, which map to chromosomes 1q21.1 and 15q13.2 (International Schizophrenia Consortium

2008; Stefansson et al. 2002). The latter region (15q13.2) is known to contain the α7 nicotinic cholinergic receptor gene (CHRNA7). As a prelude to detailed consideration below, mice containing heterozygous deletion of the CHRNA7 gene display cognitive defects (Young et al. 2007), while agonists at the α7 nicotinic cholinergic receptor may have some efficacy against the negative symptoms and perhaps also the cognitive deficits of schizophrenia (Freedman et al. 2008).

Interestingly, several studies have provided strong evidence for an association between schizophrenia and microdeletions affecting the gene neurexin 1 (NRXN1; Kirov et al. 2008, 2009b; Ikeda et al. 2010; Rujescu et al. 2009). Neurexins are presynaptic proteins that function as synaptic recognition molecules and mediate various aspects of synaptic function via binding to neuroligins (Ichtchenko et al. 1995). Mice containing deletion of NRXN1α display behavioural and electrophysiological deficits relevant to schizophrenia, including disruption to synaptic transmission and prepulse inhibition (PPI) (Etherton et al. 2009). In summary, while the involvement of CNVs is undeniable, the size of their contribution as risk factors and their relationship with specific symptom sub-types is unclear (Gill et al. 2009; Kirby et al. 2010).

2 Mutant Approaches

Technological developments in high throughput trangenic techniques (see Gondo et al. 2011 for discussion), alongside improved understanding of the complexity of the genetic basis of the disorder, have provided an important stimulus to genetic modelling of schizophrenia and related psychiatric disorders. Fundamentally, two complementary approaches, i.e. gene-driven (reverse genetics – gene to phenotype) or phenotype-drive (forward genetics – phenotype to gene), have been used to create mice with modification of disease-relevant genes. Recombinant DNA and evolving techniques now constitute a primary strategy for clarifying the functional role(s) of putative risk genes and of biological entities for which investigative pharmacological tools are not yet available, and for identifying novel drug candidates. Phenotype-driven approaches, most notably chemical mutagenesis, have been used as a means of revealing novel risk pathways involved in related pathobiological phenotypes (see Gondo et al. 2011).

2.1 Constitutive Mutants

Deletion of a particular gene ('knockout' [KO]), insertion of additional gene copies ('knockin') or insertion of human disease mutations ('transgenic') in mice has led to increased understanding of the role of candidate disease genes in pathogenesis (Chen et al. 2006; O'Sullivan et al. 2006; O'Tuathaigh et al. 2007a). Mutant models have also shown considerable heuristic value in the evaluation of pharmacological

interventions, as well as identification of genes that modify phenotypic severity. Mice with altered expression of a neurochemical pathway component can be used as animal models for basic and therapeutically targeted research. This is particularly important in the absence of appropriately selective pharmacological agents; for example, mutant models can provide a unique opportunity to determine the subunit composition of native receptors with putative involvement in therapeutically relevant central nervous system (CNS) actions. Similarly, when the targeted gene is expressed in the brain, the phenotype of mutant mice may reveal genetic mechanisms underlying normal behaviours and may increase our knowledge of genetic factors in neuropsychiatric disorders (Waddington et al. 2005, 2007).

Although constitutive mutants continue to provide invaluable information regarding molecular genetic, pathophysiological and pharmacological processes related to schizophrenia, such models are not without their limitations. The excised or otherwise mutated gene is often critical for the viability of the organism, such that knockout of that gene may be associated with gross deficits or even embryonic lethality that preclude further phenotypic evaluation; compensatory mechanisms, biological redundancy and genetic background can each confound phenotypic assessment; pleiotropy can result in phenotypes that involve diverse brain regions and functions, thereby complicating analysis of the resultant phenotype. In addition, it has been increasingly shown that neuropsychiatric phenotypes in candidate gene mutants can be modulated by gene × gene and gene × environment interactions (see Lesch 2011 for discussion) and by epigenetic factors (see Bountra et al. 2011); further study of mechanisms underlying the action of such phenotypic modifiers has the potential to provide greater insight into variable penetrance of disease genes across individuals and populations (O'Sullivan et al. 2006).

2.2 Conditional Mutants

Generation of conditional mutants, allowing spatially and/or temporally controlled gene mutation, minimises some of the limitations associated with constitutive mutants. The Cre/*lox*P system has been widely used as a tool for tissue-specific mutation and, in association with the use of tetracycline responsive promoters, also for time-specific gene mutation. Generation of conditional mutants (Sauer 1993; O'Sullivan et al. 2006) requires two elements: (a) a conventional transgenic mouse line with Cre targeted to a specific tissue or cell type, and (b) a mouse strain that embodies a target gene (endogenous gene or transgene) flanked by two *lox*P sites in a direct orientation ("floxed gene"). Recombination (e.g. excision and consequent inactivation of the target gene) occurs only in those cells expressing Cre recombinase, hence the target gene remains active in all cells and tissues which do not express Cre. These techniques can also be used for the removal of a transgene, which has been overexpressed in a specific tissue at a certain time point, so as to study the reciprocal effect of downregulation of that transgene.

The ability to manipulate both spatial and temporal gene expression in a single mouse model affords the investigator greater control over gene activity and makes it possible to address more focused questions relating to the pathobiology of schizophrenia. These techniques have been particularly utilised in relation to the in vivo characterisation of the *disrupted-in-schizophrenia 1* (*DISC1*) gene (see Sect. 4.3.1), where regionally selective, inducible mutants have illuminated DISC1 involvement in the development of schizophrenia endophenotypes (Hikida et al. 2007; Li et al. 2007; Pletnikov et al. 2008; Shen et al. 2008).

2.3 Chemical Mutagenesis

Chemical mutagenesis using *N*-ethyl-*N*-nitrosourea (ENU) provides an example of a phenotype-driven approach for modelling genetic disorders (O'Sullivan et al. 2006 and Gondo et al. 2011). Treatment with the mutagen ENU, usually administered via series of systemic injections in adult male mice, has been shown to induce point mutations in a genome-wide fashion, enabling identification of previously unknown genes implicated in disease-relevant phenotypes and resulting in the development of large-scale mutagenesis projects (Nolan et al. 2000). A notable obstacle is that for any given phenotype identified, the positional cloning techniques necessary to identify the causative gene are laborious and time consuming (O'Sullivan et al. 2006; Gondo 2008; Gondo et al. 2011). While ENU mutagenesis has been used predominantly to investigate dominant mutations (Gondo 2008; see also Gondo et al. 2011), phenotypic effects of recessive mutations can also be examined, although they require extensive breeding strategies and considerable experimental numbers (O'Sullivan et al. 2006).

The primary advantage of ENU relative to conventional transgenic techniques is the ability to generate a diverse range of mutations, from loss- or gain-of-function, to hyper- or hypomorphs. In a complementary manner, researchers have also focused on a subset of known candidate genes and screened ENU mutagenesis libraries for mutations in the target loci. Consequently, this allows determination of an allele series for each gene by phenotyping different mice harbouring specific missense or nonsense mutations at a particular locus (Cordes 2005; O'Sullivan et al. 2006). It should be noted, however, that different mouse strains exhibit differential sensitivity to ENU dosage and that background strain may also predispose to select phenotypes (Acevedo-Arozena et al. 2008).

2.4 RNAi Gene Silencing

RNA interference (RNAi) strategies consist of targeted in vivo post-transcriptional gene silencing initiated by double-stranded RNA (dsRNA) homologous to the target gene (Mahairaki et al. 2009). Insertion of an RNA molecule complementary to the target mRNA enables endogenous RNA processes to suppress translation and

thereby downregulate the expression of the target gene (Zamore 2001; Nielsen et al. 2009).

The use of RNAi has spurred the development of efficient delivery methods capable of producing long-term knockdown of gene function in mice, either globally or regionally specific (Singer and Verma 2008; Nielsen et al. 2009). The majority of vectors use widely expressed polymerase III (pol III) or polymerase II promoters to promote the expression of short hairpin RNAs (shRNA) that are processed into siRNAs (Brummelkamp et al. 2002). More recently, shRNAs have been engineered to utilise the endogenous micro RNA (miRNA) machinery to drive the expression of RNAi; studies have reported a favourable efficiency and toxicity profile relative to shRNA-based vectors (Nielsen et al. 2009).

Over recent years, lentiviral vectors have received considerable attention as gene delivery vehicles, with the capability of infecting a variety of dividing and non-dividing cells, integrating stably into the host genome and resulting in long-term expression of the transgene. Through stereotaxic injection, lentiviral particles can be injected into specific brain areas, enabling the expression of shRNAs that target disease-associated genes. Given that lentiviral vectors integrate into the host genome, the offspring of a transgenic animal generated using a lentiviral vector is likely to inherit the provirus and express the transgene or silencing cassette. Other viral vectors, including adenoviruses and adeno-associated viruses (AAVs), remain as episomal DNA in transduced cells and do not produce germ-line transmission of viral DNA. Recent applications included a study showing that delivery of lentiviruses expressing shRNAs and targeting the CD81 gene into the nucleus accumbens resulted in abolition of cocaine-induced behaviour (Bahi et al. 2005). In a mouse study, lentiviral silencing of brain-derived neurotrophic factor (BDNF) in the dentate gyrus but not the CA3 regions of the hippocampus was associated with a pronounced depression-related phenotype (Taliaz et al. 2010), illustrating the power of this approach to refine knowledge of gene–endophenotype relationships in relation to neuropsychiatric disorders.

With traditional methods of generating transgenic animals being both time consuming and laborious, researchers have begun to capitalise on the capacity of lentiviral vectors to generate transgenic animals, using either zona pellucida removal or sub-zonal injection to deliver lentiviral particles to the embryo (Tiscornia et al. 2003). One notable advantage of this technique has been the ability to generate efficient transgenesis in species that are incompatible with conventional knockout technology (Singer and Verma 2008; see Chan et al. 2011).

3 Modelling the Psychopathology of Schizophrenia in Mutants

The psychopathology of psychotic illness is characterised by positive symptoms (such as hallucinations and delusions), negative symptoms (such as social withdrawal, anhedonia and flattened affect) and cognitive deficits (such as deficits in working memory and executive function). Such complexities of psychopathology, together

with diagnostic uncertainty associated with limited understanding of etiopathology, have long confounded efforts to 'capture' schizophrenia in valid animal models (Arguello and Gogos 2006, 2010; Desbonnet et al. 2009a, b; Kirby et al. 2010). Given increasing understanding of the genetics of schizophrenia and of mutant technologies, contemporary modelling strategies have de-emphasised any unitary, quasi-holistic approach in seeking to delineate specific gene–endophenotype relationships; researchers have chosen to focus on a series of specific behavioural abnormalities, each of which may be more readily modelled in rodents and could be related to a specific disease endophenotype (Powell and Miyakawa 2006; Low and Hardy 2007; Desbonnet et al. 2009a; Jaaro-Peled et al. 2010; O'Tuathaigh and Waddington 2010).

3.1 Positive Symptoms

Given the well-characterised role for dopaminergic (DAergic) hyperfunction and antagonism in relation to psychotomimetic and antipsychotic activity, respectively (Guillin et al. 2007), it is unsurprising that validity for positive symptomatology is commonly assessed using DA-linked behaviours. In this context, phenotypic modelling of positive symptoms in mutants has focused on 'proxy' indices of baseline- and psychotomimetic drug-induced locomotor hyperactivity and disruption of PPI. The latter phenomenon, whereby presentation of a weak prestimulus inhibits the reaction of an organism to a subsequent startle stimulus, is disrupted in schizophrenia and by amphetamine in animals, and can be ameliorated by antipsychotics; this phenomenon, involving sensory gating mechanisms at the interface of psychotic and cognitive processes, can be viewed as a model related, in part, to positive symptoms (Powell and Geyer 2007; Van den Buuse 2010). Latent inhibition (LI), where the association between paired stimuli is attenuated by non-reinforced pre-exposure to the conditioned stimulus, is also disrupted in schizophrenia and by amphetamine in animals, and is reversible by antipsychotics. These processes, involving selective attention to relevant over irrelevant stimuli and salience attribution, are also at the interface of psychotic and cognitive processes (Moser et al. 2000; Bay-Richter et al. 2009; Weiner and Arad 2009).

3.2 Negative Symptoms

Modelling negative symptoms in animals is yet more problematic, being confounded by ongoing clinical debates such as the distinction made between 'primary' vs. 'secondary' negative symptoms; the former reflect persistent negative symptoms that are a component of the illness and the latter relate to state phenomena such as depression and/or extrapyramidal side effects (Blanchard and Cohen 2006; Winograd-Gurvich et al. 2006). In addition, certain negative

symptoms, including poverty of speech, are especially difficult to measure in animals and may comprise abilities unique to humans. However, symptoms such as social withdrawal, anhedonia and motivational deficits represent constructs that are conserved across species and expressed across a diversity of species-specific behaviours (Panksepp 2006; O'Tuathaigh et al. 2010a).

In particular, disturbances in social behaviour represent a core negative symptom in schizophrenia that is amenable to modelling in animals. Social approach-avoidance behaviours, including species-typical affiliative (e.g. investigative sniffing) or agonistic (e.g. biting, pinning) behaviours can be assessed dyadically in a novel environment (File 1980; O'Tuathaigh et al. 2010a). Alternatively, choice paradigms for affiliative behaviours, where mice are given the choice to spend time in a chamber containing an unfamiliar mouse vs. an empty chamber are now commonly used to test interest to engage in social interaction in mouse mutant models related to schizophrenia and other psychiatric disorders characterised by asociality (Brodkin et al. 2004; Moy et al. 2006; Babovic et al. 2008).

Mouse models for anhedonia have employed reduction in sucrose consumption as evidence of decreased reward function in rodents (Sammut et al. 2001; O'Tuathaigh et al. 2010a). However, using sucrose volume intake as an index of anhedonia may be complicated by extraneous factors such as conditioned taste aversion, presence of competing behaviours such as locomotion or stereotypies, or visceral malaise (Kirby et al. 2010; O'Tuathaigh et al. 2010a). Furthermore, it should also be noted that voluntary sucrose consumption has been used to model anhedonia in relation to depression. This highlights the lack of sufficient specificity of behavioural measures in small rodents to effect the necessary distinctions between features related to clinically similar symptoms in schizophrenia and depression.

3.3 Cognitive Deficits

Although the nature of cognitive impairment in schizophrenia is the subject of ongoing debate, specifically whether it constitutes a generalised deficit or one involving specialised domains of cognitive dysfunction, there is some agreement that impairments in working memory and executive function are core deficits; these may extend to attentional processing, verbal and recognition memory (Arguello and Gogos 2006, 2010). Given the paucity of pharmacotherapeutic strategies available for treating cognitive impairment in schizophrenia, researchers have emphasised the importance of developing novel, targeted treatments for ameliorating the cognitive deficits of psychotic illness and model systems are essential tools to this end (Arguello and Gogos 2010). Aside from verbal memory, rodent analogues of such cognitive processes are readily available, where a particular emphasis has been placed on spatial working memory and executive function, as reviewed in detail elsewhere (Kellendonk et al. 2009; Arguello and Gogos 2010).

3.4 Structural and Pathological Features

While the lack of a prominent neuropathological signature has long been considered an obstacle to the development of accurate preclinical models of schizophrenia, growing evidence not just for structural but also for more subtle neuropathological changes in the brain of patients with schizophrenia provide a basis for validation of putative mouse models; the most widely supported of these structural and neuropathological endophenotypes include enlarged ventricles, as reported in brain imaging studies, dendritic changes in pyramidal neurons and modification of GABAergic interneurons in the prefrontal cortex (Jaaro-Peled et al. 2010; O'Tuathaigh and Waddington 2010). The growing body of evidence implicating risk genes in the development of such structural and neuropathological markers encourages further investigation in mutants of mechanisms underlying the cellular/molecular characteristics of schizophrenia (Jaaro-Peled et al. 2010).

4 Genetic Models Related to Schizophrenia

4.1 Genetic Models Relating to Putative Dopaminergic Pathophysiology

Over the last 40 years, the prevailing DAergic hyperfunction hypothesis of schizophrenia has been the subject of iterative advances: while positive symptoms appear to be related to increased release of DA onto subcortical D2 receptors, negative symptoms may reflect associated reduction in cortical release of DA, particularly involving D1 receptors in prefrontal cortex (Guillin et al. 2007; Howes and Kapur 2009).

Constitutive KO of individual DA receptor subtypes has facilitated elucidation of their role in mediating behaviours that show analogous disruption in schizophrenia patients. This approach has also been important in understanding which DA receptor subtypes underpin the behavioural effects of psychotomimetic and antipsychotic drugs. The indirect DA agonist amphetamine has been shown to disrupt sensorimotor gating in the PPI paradigm in D1, D3 and D4 KO but not D2 KO mice, suggesting the D2 receptor is required for amphetamine-induced disruption of PPI. Interestingly, the disruptive effect of direct DA agonists on PPI differs, as apomorphine disrupts PPI in D2 but not D1 KO mice (Ralph-Williams et al. 2002; Ralph et al. 1999). Recently, dissociable roles for D1 and D2 receptors in habituation and sensitization to acoustic startle, a component of PPI, have been identified using a KO approach (Halberstadt and Geyer 2009); this may be of relevance to subgroups of patients that display habituation abnormalities.

Antipsychotic drugs enhance low levels of LI, an effect that is reproduced by D2 and D1 KO in females but only by D2 KO in males (Bay-Richter et al. 2009); this

suggests that there may be sex differences in the relative contribution of D1 and D2 receptors to LI itself and potentially to the manner in which antipsychotic drugs exert their behavioural effects. In relation to the D1-like receptor family, divining unique roles for D1 vs. D5 receptors across behavioural models related to schizophrenia has so far proven difficult, due to a lack of pharmacological tools of sufficient selectivity. To date, studies employing KOs have not yet clarified any distinct contribution from D1 vs. D5 receptors in these forms of behaviour.

The D2 receptor has two isoforms, deriving from alternative splicing: long (D2L) and short (D2S). Studies in isoform-specific KOs have indicated that it is the D2L isoform that may be important for antipsychotic drug action (Xu et al. 2002) and of greater importance for extrapyramidal side effects (Wang et al. 2000). However, effects in behavioural paradigms related more specifically to schizophrenia are yet to be investigated fully using this approach.

Given the importance of the prefrontal cortex in mediating cognitive functions known to be disrupted in schizophrenia, such as working memory and executive function, together with the rich density of D1 receptors in this region, it is not surprising that D1 KOs show deficits in spatial working memory and reversal learning (El-Ghundi et al. 2007; Holmes et al. 2004). The D2 receptor may also be important in set shifting and reversal learning, as D2 KOs show impairment in adjusting responding to previously reinforced stimuli when unexpected outcomes are encountered, and both D2 KOs and antipsychotic drugs have been shown recently to produce similar reversal learning deficits in a set shifting paradigm (Kruzich and Grandy 2004; DeSteno and Schmauss 2009). D2 and D3 KOs also show deficits in spatial working memory (Glickstein et al. 2002; Karasinska et al. 2005).

Mutants with selective over-expression of subcortical D2 receptors exhibit deficits that include reduced incentive motivation, as measured by reduced lever pressing for food reward in both an operant timing task and under a progressive ratio schedule of reinforcement (Drew et al. 2009); however, the extent to which this might represent an avolition phenotype remains to be clarified. While distinct behavioural profiles have been documented for D3, D4 and D5 KOs (Waddington et al. 2005), no substantive evidence for a role in negative symptom-related processes has been found (O'Tuathaigh et al. 2010a). Clinical studies have indicated that variation in the DA transporter (DAT) gene may be associated with negative symptoms (Fanous et al. 2004); in agreement, DAT KOs show impairment in social interaction (Rodriguiz et al. 2004; Gainetdinov 2008); stereotyped patterns of social behaviour have also been observed in DAT KOs (Tillerson et al. 2006). However, at variance with a negative symptom profile, DAT KOs develop a more positive bias towards a hedonically positive tastant (Costa et al. 2007), while mutants with DAT knockdown, to 10% of the complement in WT, show no change in responsivity for sucrose reward in a sucrose consumption task (Cagniard et al. 2006).

Additional DA-related genes, such as AKT1, GSK3β and Nurr1, have been subjected to varying breadth and depth of interrogation and are considered elsewhere (Desbonnet et al. 2009a; Kirby et al. 2010).

4.2 *Genetic Models Relating to Putative Glutamatergic Pathophysiology*

There is a substantive body of evidence for hypoglutamatergic function in schizophrenia: *N*-methyl D-aspartate (NMDA) receptor (NMDAR) antagonists have been shown to possess psychotomimetic properties in healthy subjects (Adler et al. 1999; Kegeles et al. 2000; Coyle 2006) and to exacerbate psychotic symptoms in patients with schizophrenia (Malhotra et al. 1997). NMDAR deficits have been reported in schizophrenia, both in postmortem brain (Stone et al. 2007) and in living patients using SPECT imaging (Pilowsky et al. 2007).

Hypomorphic mutants with 90% reduction in the NMDAR NR1 subunit display abnormalities across several schizophrenia-related phenotypes (Mohn et al. 1999; Duncan et al. 2004): decreased responsivity to the NMDAR antagonists PCP and MK-801, hyperactivity in a novel environment and deficits in PPI and social interactions that were reversible by antipsychotics, with second-generation agents (clozapine, quetiapine) appearing more effective than their first-generation counterpart (haloperidol; Mohn et al. 1999; Duncan et al. 2006). Another NMDAR mutant line, involving deletion of the NR1-associated NR2A (GluR-1) subunit, exhibited hyperactivity that was ameliorated by haloperidol and risperidone, together with deficits in spatial and latent learning and augmented DA metabolism in striatum and frontal cortex (Miyamoto et al. 2001).

Glycine acts at an accessory site necessary for NMDAR function and thus facilitates NMDA-mediated transmission; glycine agonists promote NMDA-mediated transmission and may evidence some efficacy against the negative symptoms of schizophrenia (Patil et al. 2007). Two mutant lines carrying point mutations in the NMDAR glycine binding site, Grin1 (D481N) and Grin1 (K483Q), have been described; they exhibit 5- and 86-fold reductions in receptor glycine affinity, respectively (Ballard et al. 2002). Grin1 (D481N) mice display hyperactivity in a novel environment that is not reversible by antipsychotics (Ballard et al. 2002). These mutants also show reduced sociability; this abnormality was reversed by administration of D-serine and, to a lesser extent, clozapine (Labrie et al. 2008). Grin1 (D481N) mice also show cognitive deficits, including abnormalities in spatial learning and memory as well as spatial recognition (Labrie et al. 2008).

Abnormalities in various components of the NMDAR signalling complex have been implicated in schizophrenia; these include the glial glutamate and aspartate transporter (GLAST), which has been shown to be expressed differentially in the dorsolateral prefrontal cortex, anterior cingulate cortex and thalamus in post-mortem brain of patients with schizophrenia (Smith et al. 2001; Bauer et al. 2008). GLAST KOs display haloperidol-sensitive hyperactivity in a novel environment, increased hyperlocomotor responsivity to MK-801 and impaired sociability (Karlsson et al. 2008, 2009). Knockout of the NMDAR signalling molecule SynGAP is associated with hyperactivity in the open field and reduced sensitivity to the motor stimulatory effects of MK-801 (Guo et al. 2009). SynGAP mutants evidence intact sociability but

impairment in social novelty preference, disrupted PPI, enhanced startle reactivity and deficits in spatial working memory (Guo et al. 2009).

Vesicular glutamate transporters 1 and 2 (VGluT1 and VGluT2) are recognised markers of glutamatergic neurons that are responsible for the vesicular packaging of glutamate in the presynaptic axon terminal (Fremeau et al. 2001). Altered VGluT1 expression has been documented in the striatum and hippocampus of patients with schizophrenia (Oni-Orisan et al. 2008). Heterozygous deletion of VGluT1 was associated with greater attenuation of sucrose consumption following exposure to a chronic mild stressor (Garcia-Garcia et al. 2009). Mutants with conditional, heterozygous deletion of VGluT2 in the cortex, hippocampus and amygdala during the third postnatal week exhibited reduced social dominance in the tube test and increased sociability (Wallén-Mackenzie et al. 2009).

Additional glutamate-related genes, such as ionotropic and metabotropic receptor subtypes/subunits, αCamKII, calcineurin, DAO, DAOA (G72/G30), D-serine and SynGAP, have been subjected to varying breadth and depth of interrogation and are considered elsewhere (Desbonnet et al. 2009a; Kirby et al. 2010).

4.3 Mutant Models Relating to Candidate Risk Genes

In this section, we outline work relating to the three most extensively studied candidate genes (DISC1, DTNBP1, NRG1) and to widely considered 22q11-associated genes. Additional putative risk genes, such as FEZ1, PPP3CC, reelin and RGS4, have been subjected to varying breadth and depth of interrogation and are considered elsewhere (Desbonnet et al. 2009a; Kirby et al. 2010).

4.3.1 Disrupted-in-Schizophrenia-1

A study in a Scottish pedigree demonstrated that a familial mutation in the disrupted-in-schizophrenia-1 (DISC1) gene, due to a balanced chromosomal translocation at 1q42.1–1q42.3, segregated with several psychiatric disorders, including schizophrenia; this association between DISC1 and schizophrenia has been replicated across diverse populations (Chubb et al. 2008; Hennah et al. 2009; Schumacher et al. 2009). During embryonic development, DISC1 appears to play an important role in neurodevelopment and neuronal plasticity via interaction with several proteins, including phosphodiesterase-4B, Fez1, NudEL and LIS1; these functions likely alternate, depending upon the stage of development (Chubb et al. 2008).

The assumption underlying the majority of mutant DISC1 models to date has been that the chromosomal translocation is associated with the production of a truncated DISC1 protein that is functionally disruptive. In a line generated using ENU-induced mutagenesis in exon 2 of DISC1 (Clapcote et al. 2007; Gondo et al. 2011), mutants were characterised by hyperactivity, antipsychotic-sensitive disruption of PPI and LI

and impaired working memory but intact spatial learning. In a transgenic line with inducible and reversible expression of a DISC1 C-terminal fragment under the αCAMKII promoter, positive symptom indices have yet to be reported; mutants evidenced reduced sociability with impaired spatial working memory (Li et al. 2007). In a transgenic line with expression of a dominant-negative truncated form of DISC1 under the αCaMKII promoter, mutants exhibited hyperactivity but no effects on PPI, social behaviour or cognition (Hikida et al. 2007). In a double transgenic line expressing human DISC1 under the CMV promoter, with tetracycline under the αCaMKII promoter, mutants showed a hyperactive phenotype, some evidence for impaired social behaviour and spatial memory but no effect on PPI (Pletnikov et al. 2008). In a transgenic line with expression of truncated DISC1, mutants displayed disruption to LI (Shen et al. 2008). Mice of the commonly employed 129S6 Sv/Ev strain have been shown to carry a 25 bp deletion in exon 6 of the DISC1 gene which abolishes expression of the DISC1 protein. While there is no evidence for positive- or negative symptom-related endophenotypes in this line, they show impaired spatial working memory without impairment in recognition, reference and short-term memory, spatial learning or associative learning (Koike et al. 2006; Kvajo et al. 2008).

The cognitive phenotypes observed in some DISC1 mutant models may be consistent with clinical evidence for association between DISC1 risk variants and cognitive deficits in patients with schizophrenia, including P300 response (a measure of early attentional processing; Blackwood et al. 2000) and various measures of memory (Cannon et al. 2005; Burdick et al. 2007). In addition, these studies indicate that genetic variation in DISC1 may affect risk for schizophrenia by modifying the morphology and function of hippocampal and prefrontal grey matter (Callicott et al. 2005; Cannon et al. 2005; Di Giorgio et al. 2008). In one transgenic line expressing truncated DISC1, reduction of hippocampal synaptic transmission as a consequence of decreased hippocampal dendritic complexity has been observed (Li et al. 2007; Kvajo et al. 2008). Other structural and pathological markers relevant to schizophrenia have been reported in DISC1 mutants, including reduced immunoreactivity of parvalbumin in the hippocampus and prefrontal cortex (Hikida et al. 2007; Shen et al. 2008) and enlargement of the lateral ventricles (Hikida et al. 2007).

4.3.2 Dysbindin

Association between risk for schizophrenia and the gene encoding dystrobrevin binding protein 1 (DTNBP1; dysbindin) has been replicated across diverse samples (Allen et al. 2008; Gill et al. 2009); however, studies have differed in the risk haplotypes reported. A series of studies have reported dysbindin risk variants to be associated with impaired cognitive function: decreased performance in tests of higher-order cognitive domains (Corvin et al. 2008), enhanced neuronal activity in prefrontal networks associated with encoding and retrieval of episodic information (Thimm et al. 2010) and increased activation of the bilateral middle frontal gyrus, a component of dorsolateral prefrontal cortex, during a working memory task

(Markov et al. 2009); general and domain-specific cognitive deficits (Fallgatter et al. 2006; Donohoe et al. 2007) and disruption to early information processing, as measured by the P1 response (Donohoe et al. 2008) in schizophrenia. Dysbindin has been implicated in reduced exocytosis of glutamate-containing synaptic vesicles (Weickert et al. 2004; Bray et al. 2005) and binds synaptic and microtubule-interacting proteins (Talbot et al. 2006; Camargo et al. 2007); a number of these proteins interact with DISC1 (see Sect. 4.3.1), indicating a putative link between DISC1 and DTNBP1 risk pathways in mediating aspects of neurodevelopment that are disrupted in schizophrenia, including neuronal migration and synaptic plasticity.

The *sdy* mouse arose as a spontaneous mutation in the DBA/2J strain due to a large in-frame deletion of two exons of the mouse DTNBP1 gene; *sdy* mice express no dysbindin protein. *Sdy* mice have been shown to manifest several schizophrenia-like phenotypes, although the evidence to date has been inconsistent. This mutant has been proposed as a murine model of Hermansky–Pudlak syndrome (Li et al. 2003), a disorder characterised by albinism, bleeding tendency and lung disease, which are not a feature of schizophrenia. Assessment of *sdy* mutants has produced inconsistent findings of unaltered (Feng et al. 2008; Bhardwaj et al. 2009), decreased (Takao et al. 2007; Hattori et al. 2008) or increased (Cox et al. 2009) spontaneous exploratory activity. Increased DA turnover has also been reported in *sdy* mutants (Hattori et al. 2008). While the DBA/2J strain does not show robust PPI, precluding assessment thereof, backcrossing of the *sdy* mutation onto a C57BL6 line revealed a disruptive effect on PPI (Halene et al. 2009).

Sdy mutants evidence: reduction in social contacts and social contact time in a dyadic paradigm (Feng et al. 2008; Hattori et al. 2008); impaired long-term memory retention in the Barnes maze, as well as a working memory deficit in the T-maze forced alternation task (Takao et al. 2008); impaired encoding or maintaining an item in spatial memory, as assessed in a delayed non-match-to-position test (Jentsch et al. 2009); impaired spatial reference memory and object recognition memory (Feng et al. 2008). *Sdy* mutants also show morphological changes in excitatory asymmetrical synapses on hippocampal CA1 dendritic spines: presynaptically fewer but larger glutamatergic vesicles; narrower synaptic cleft; and broader postsynaptic density (Chen et al. 2008).

4.3.3 Neuregulin-1

Stefansson et al. (2002) first reported a link between a risk haplotype located at the 5′ end of neuregulin-1 (NRG1) and schizophrenia; evidence from diverse populations has sustained an association between variation at the NRG1 locus and schizophrenia (Bertram 2008; Gong et al. 2009). Post-mortem brain studies have shown altered mRNA and protein levels of NRG1 isoforms in the dorsolateral prefrontal cortex and hippocampus (Law et al. 2007); NRG1 has been found to produce at least 31 different isoforms by consequence of alternative splicing

(Mei and Xiong 2008; O'Tuathaigh et al. 2009a). NRG1 is a growth factor containing an epidermal growth factor (EGF)-like domain and synthesised as a membrane-bound form; after undergoing cleavage by the proteases at a juxtamembrane site, soluble NRG1 is released into synaptic cleft. In response to NRG1 stimulation, ErbB tyrosine kinase transmembrane receptors become dimerised to form homo- or hetero-dimers and play a significant role in neuronal development, neuronal migration, axon guidance and synaptic plasticity (Harrison and Law 2006; Mei and Xiong 2008; O'Tuathaigh et al. 2009a).

Targeted mutations of various NRG1 isoforms (I–III) have facilitated delineation of some of their specific functions. As a consequence of the vital role played by NRG1 in cardiac and lung development, homozygous deletion of NRG1 in mice is associated with death in mid-embryogenesis; hence, the majority of studies on constitutive mutants are conducted using heterozygotes. While survival is essential for behavioural studies, heterozygous gene deletion may be less or sometimes more likely to reveal subtle phenotypes, depending on extent of recruitment of compensatory processes (Gogos et al. 1998; Babovic et al. 2008). Most NRG1 proteins are synthesised with a transmembrane (TM)-domain. Mutants with heterozygous deletion of TM-NRG1 exhibit hyperactivity in exploration- and anxiety-related tasks (Stefansson et al. 2002; O'Tuathaigh et al. 2006, 2007b; Karl et al. 2007; Van den Buuse 2010), an effect that is partially reversed by treatment with the antipsychotic clozapine (Stefansson et al. 2002); ethologically based assessment of exploratory activity in TM-NRG1 mutants reveals sex-specific increases in both initial exploratory behaviour and subsequent habituation to the environment (O'Tuathaigh et al. 2006). Deficits in PPI have also been reported (Stefansson et al. 2002), although the size of this effect is likely to be modest (Van den Buuse 2010). TM-NRG1 mutants also show abnormalities of social behaviour, including a reduction in social novelty preference (O'Tuathaigh et al. 2007b) and increased aggression during dyadic social encounter (O'Tuathaigh et al. 2008); no changes across cognitive measures, including spatial learning and working memory, have been noted (O'Tuathaigh et al. 2007b).

TM-NRG1 mutants demonstrate differential sensitivity to the locomotor- stimulating and disruptive social effects of subchronic NMDAR antagonist administration (O'Tuathaigh et al. 2010b); they also evidence enhanced sensitivity to the effects of Δ^9 tetrahydrocannabinol (THC; the primary psychoactive constituent of cannabis) on locomotor activity, PPI and the development of specific anxiety-related behaviours (Boucher et al. 2007a). Investigations into potential underpinnings of behavioural abnormalities in TM-NRG1 mutants have indicated deficits in NMDA receptor expression (Stefansson et al. 2002; however, for contrary findings see Dean et al. 2008), elevated levels of 5-HT_{2A} receptors and the 5-HT transporter (Dean et al. 2008) and an increase in c-Fos activation, a marker of neuronal activation, following exposure to the stress of behavioural testing (Boucher et al. 2007b). Constitutive loss of TM-NRG1 has also been associated with structural brain changes on MRI, notably an increase in cerebellar but reduction in olfactory bulb and ventricular volume (O'Tuathaigh et al. 2010b).

Heterozygous deletion of TM-domain NRG1 disrupts the expression of several NRG1 isoforms (I–III). Findings in mutants with more isoform-specific NRG1 deletions indicate that type III NRG1 mutants show greater deficits in PPI relative to TM-NRG1 mutants (Chen et al. 2007). Type III NRG1 mutants also evidence impairment in working memory that is not evident in TM-NRG1 mutants; these effects may be related to reduced dendritic spine density, enlarged lateral ventricles and hypofunctionality of PFC and CA1 region of the hippocampus also reported in this mutant line (Chen et al. 2007). However, neither type III NRG1 mutants nor targeted disruption of type I/type II NRG1 (NRG1 isoforms containing an Immunoglobulin (Ig)-like domain) share the hyperexploratory profile evident in TM-NRG1 mutants; rather, Ig-NRG1 mutants display impairment in a modified LI paradigm with intact PPI and unaltered activity (Rimer et al. 2005). Mutants with heterozygous deletion in the EGF-like domain (a pan-isoform mutation) exhibit an initial increase in exploratory activity but subsequently show more rapid habituation; while baseline PPI was unaltered, PPI was disrupted by MK-801 but not by amphetamine (Duffy et al. 2008). In contrast with the TM-NRG1 phenotype, recent findings in EGF-NRG1 mutants indicates reduction in social approach behaviours as well as deficits in mismatch negativity, a measure of early information processing, which is disrupted in patients with schizophrenia (Ehrlichman et al. 2009).

Mutants with heterozygous deletion of the ErbB4 receptor, but not ErbB2 or ErbB3, show hyperactivity in the open field environment (Gerlai et al. 2000). A hypoactive phenotype has been described in mutants with loss of ErbB signalling in oligodendrocytes (Roy et al. 2007). B-site APP-cleaving enzyme 1 (BACE-1) plays a significant role in NRG1 signalling via proteolytic processing of NRG1. Mutants with knockout of BACE1 KO demonstrate hyperactivity with disruption to PPI and heightened responsivity to MK-801 (Savonenko et al. 2008).

4.3.4 22q11-Associated Genes

Velocardiofacial syndrome (VCFS) is a 22q11.2 deletion syndrome characterised by a 25-fold increase in risk for psychosis (Murphy et al. 1999; Murphy and Owen 2001; Prasad et al. 2008). The phenotype of mutants carrying a multigene deletion across the 22q11 region includes impaired PPI, neuronal migratory defects and disruption of cortical neurogenesis, with haploinsufficiency of genes located in this region such as Tbx1, Gnb1l and Dgcr8 implicated in phenotypic deficits (Meechan et al. 2007; Stark et al. 2008). Among the genes located on this chromosomal region that have been associated with the expression of the 22q11-associated psychiatric phenotype, the three most well supported are proline dehydrogenase (PRODH), Zinc finger DHHC domain containing 8 (ZDHHC8) and catechol-*O*-methyltransferase (COMT).

The PRODH gene codes for proline dehydrogenase and has been modestly implicated in risk for schizophrenia (Liu et al. 2002; Li and He 2006). Mutant mice with knockdown of PRODH demonstrate a reduction of exploratory activity in

a novel environment, enhanced behavioural responsivity to amphetamine, reduced locomotor responsivity to the NMDA antagonist MK-801 and impaired PPI and associative learning (Gogos et al. 1999; Paterlini et al. 2005). These phenotypic effects occurred alongside changes in hippocampal glutamatergic and cortical dopaminergic transmission and upregulation of COMT mRNA in the frontal cortex. In addition, administration of the COMT inhibitor tolcapone to PRODH mutants resulted in increased disruption of PPI and impairment of working memory; thus, an epistatic interaction between PRODH and COMT might contribute to aspects of schizophrenia and the psychotic phenotype associated with 22q11.2 deletion (Paterlini et al. 2005).

The enzyme COMT is involved in the catabolism of DA, with functional polymorphisms in the COMT gene indicated to exert differential regulation of DA metabolism in prefrontal cortex (Seamans and Yang 2004; Tunbridge et al. 2006). Although studied extensively, evidence linking COMT genotype with risk for schizophrenia (Allen et al. 2008), or an executive cognition/working memory endophenotype in schizophrenia (Tunbridge et al. 2006; Barnett et al. 2008), remains controversial.

While no gross changes in locomotor activity have been observed in COMT KOs (Gogos et al. 1998), ethologically based assessment of exploratory activity revealed a subtle and distinct exploratory profile (Babovic et al. 2007). Although sociability and social novelty preference are unaltered in both heterozygous and homozygous COMT KO (Babovic et al. 2008), heterozygous COMT mutants evidence increased aggression in a resident intruder assay (Gogos et al. 1998). In agreement with clinical studies suggesting a role for COMT in cognition, COMT KOs exhibit sex-specific enhancement in spatial working memory (Babovic et al. 2008; Papaleo et al. 2008), while COMT transgenics with overexpression of a human COMT-Val polymorphism exhibit deficits in attentional behaviour, working memory and recognition memory; amphetamine disrupted recognition memory in wildtypes but ameliorated recognition memory in COMT-Val transgenics (Papaleo et al. 2008), providing support for an inverted-U relationship between extent of PFC-mediated DAergic transmission and cognitive function (Seamans and Yang 2004; Tunbridge et al. 2006).

The ZDHHC8 gene regulates a brain-expressed putative palmitoyltransferase and has been implicated in schizophrenia not only because of its chromosomal location and involvement in synaptic transmission but also, more variably, through association studies (Liu et al. 2007; Allen et al. 2008). ZDHHC8 KO mice evidence a sex-specific PPI deficit in females, decreased exploratory activity and reduced sensitivity to the locomotor stimulatory effects of MK-801 (Mukai et al. 2004). In addition, decreased density of dendritic spines and glutamatergic synapses in mutants containing a 1.3-mb deletion in the 22q11.2 chromosomal region are reversed by reintroduction of enzymatically active ZDHHC8 protein; furthermore, ZDHHC8 can palmitoylate post-synaptic density-95, an adaptor molecule implicated in dendritic formation (Mukai et al. 2008). Thus, the evidence seems to indicate that ZDHHC8 may contribute to aspects of schizophrenia and the psychiatric phenotype associated with 22q11.2 deletion.

5 Mutant Models: Conceptual Issues and Methodological Challenges

5.1 Divining Phenotypes: Genetic Background, Sex and Phenotypic Battery

When analysing the phenotype of mice with mutation of risk genes, one must take into account two extra-phenotypic sources of variation that may be operating: (a) the genetic technology itself and (b) the manner in which the associated phenotype is assessed. The former refers to the influence of background strain as well as potential compensatory processes, while the latter factors include the influence of sex, the selected behavioural phenotyping screen and the interplay of gene and environment (Waddington et al. 2005; Desbonnet et al. 2009a; Kirby et al. 2010). The dangers potentially associated with these factors have been highlighted in a recent editorial which suggested certain standards necessary for mutant studies; these include appreciation of the importance of background strain and flanking genes, use of appropriate breeding strategies for experimental animals (e.g. the use of heterozygous breeding pairs), and consideration of sex-specific phenotypic effects (Crusio et al. 2009).

5.1.1 Influence of Background Strain

Reviews have repeatedly emphasised the need for extensive knowledge of existing inbred mouse strains to minimise their potentially confounding effect on the interpretation of mutant phenotype (Waddington et al. 2005). Constitutive mutant strains are usually constructed by injecting a genetically modified embryonic stem (ES) cell, derived commonly from a 129/Sv inbred strain, into a C57BL/6 blastocyst; resultant male chimaeras are then mated with C57BL/6 females and heterozygous offspring subsequently mated to generate homozygous ‘knockouts’. In light of well-characterised phenotypic differences between and within mouse strains (Waddington et al. 2005), these genetic and phenotypic variations between the inbred mouse strains used to construct genetic models may confound the interpretation of phenotype derived from a mutant on a mixed genetic background (e.g. 129Sv/C57BL6), irrespective of the entity targeted by the mutation (Gerlai and Clayton 1999; Crusio 2004; Crusio et al. 2009).

One approach to resolving this issue involves back crossing such animals into one strain (e.g. C57BL6) to obtain (by, arbitrarily, up to five back-crosses) incipient or (by, arbitrarily, greater than five back-crosses) more complete congenicity; a minimum of ten backcrosses has been advocated (Crusio et al. 2009). Research from our laboratory using D1 KOs has illustrated the importance of establishing congenicity in a given knockout line: systematic comparison of the behavioural phenotype of the same D1 receptor mutation on mixed (C57BL6/129Sv) vs. congenic (C57BL6) backgrounds

revealed differences in both exploratory behaviour profiles and behavioural responsivity to selective D1-like agonists (Clifford et al. 2001; McNamara et al. 2002), emphasising the necessity for well-defined genetic backgrounds in establishing phenotypic profile.

5.1.2 Influence of Sex

For pragmatic reasons, the majority of mutant studies have been carried out either in males, in mice of 'either gender' or, sometimes, in mice of unspecified gender. In many cases, only small numbers of newly generated mutants are available and it is common to pool data from males and females to obtain larger sample sizes (Crusio et al. 2009). However, only systematic comparisons between the sexes will allow clarification of any phenotypic interaction between sex and genotype. Data on NRG1 and COMT mutants from our laboratory indicate sex-specific alterations across various behavioural and cognitive domains (O'Tuathaigh et al. 2006; Babovic et al. 2007, 2008). Such sex-specific expression of mutant phenotypes has been documented numerous times, for example mutants lacking the genes encoding phosphodiesterase 1B, the D_3 dopamine receptor and DARPP-32 (Waddington et al. 2005). This is of particular relevance in the case of genes associated with risk for psychiatric disorders characterised by sexual dimorphism; for example, epidemiological studies in schizophrenia have repeatedly shown gender differences in the development and outcome of the disease (Tandon et al. 2008).

5.1.3 Choice of Phenotyping Battery

The validity of any mutant model related to schizophrenia is predicated on the validity and reliability of the phenotypic and statistical tools used to evaluate the endophenotype at issue (Powell and Miyakawa 2006; O'Tuathaigh and Waddington 2010). One substantive issue is that of fundamental structural and functional differences due to divergent evolutionary processes between rodents and humans. However, the evidence to date, particularly as it relates to measures of early information processing and working memory, provides some basis for a positive outlook. It is clear that, given ethologically appropriate indices of emotional, social and cognitive behaviour, development of animal analogues of human behaviours which share an overlapping neural basis is an achievable goal; however, higher brain functions, such as language and executive function, some of which are disturbed in schizophrenia and appear uniquely human, may not be conserved in rodents to allow such modelling (Jaaro-Peled et al. 2010; O'Tuathaigh et al. 2010a).

Refining the schizophrenia-relevant behavioural repertoire into more accessible constructs, with incorporation of murine behavioural assessments that can be more easily translated to human symptoms, will enhance understanding of pathological mechanisms and help bridge the translational gap between preclinical and clinical studies (Desbonnet et al. 2009a; Kirby et al. 2010). In a complementary manner,

integration of molecular, cellular and morphological approaches into phenotypic analysis will also strengthen the validity of mutant models for schizophrenia; molecular changes induced by genetic manipulation occur upstream from overt behavioural alterations and may therefore constitute more direct measures of the functional role(s) of schizophrenia-related genes. Therefore, multiple levels of analysis are necessary to reveal the relationship of a specific gene to pathobiology.

5.2 *Advances in Understanding the Functional Genomics of Schizophrenia*

Emerging evidence from the field of molecular epigenomics suggests a putative deficit in microRNA processing in schizophrenia. MicroRNAs are small, noncoding RNAs that are believed to target at least a third of protein coding genes (Bartel 2004; Filipowicz et al. 2008) and have been functionally linked with regulation of mRNA levels for numerous genes in the human cerebral cortex (Zhang and Su 2008).

One possible explanation for lack of consistency regarding risk alleles for schizophrenia may be dysregulation by miRNAs or other noncoding RNAs of rate of transcription/translation, leading to abnormal schizophrenia risk gene expression of phenotypic relevance (Perkins et al. 2007). Several studies have now indicated altered levels of brain-expressed miRNAs in schizophrenia (Dinan 2009).

A recent study has indicated pharmacological (MK-801-induced) or genetic (NR1 hypomorphic) disruption of NMDA receptor function to be associated with decreased levels of a brain-specific miRNA, miR-219 that has been shown to target CaMKII; pretreatment with either antipsychotic drugs or miR-219-specific anti-miR attenuated the hyperlocomotor effects of MK-801 (Kocerha et al. 2009). Increased understanding of the role of miRNAs in regulating phenotypic expression has the potential to provide further insight into the mechanisms by which genes may contribute to specific aspects of the schizophrenia phenotype.

5.3 *Modelling Gene × Environment Interactions in Mutant Models of Schizophrenia*

It is posited schizophrenia is a brain disorder of developmental origin in which perturbations in neurodevelopmental processes, associated with a combination of genetic and environmental factors, long precede the onset of diagnostic psychopathology in adulthood. Hence, assessing putative interactions between genetic and environmental manipulations in terms of brain function may inform on how candidate risk schizophrenia genes might modify response to environmental insults (see also Lesch 2011).

Twin studies conducted in schizophrenia generate a heritability estimate of 70–80%. A variety of environmental factors are also implicated, including prenatal infection, obstetric complications, urbanicity, psychosocial stress and substance abuse (Welham et al. 2009); the severity and timing of these environmental events is also important (Caspi et al. 2005).

5.3.1 Perinatal Environment

Animal models of maternal infection and immune activation represent one of the more commonly used paradigms for experimental investigation of the human association between early environmental adversity and the development of neuropsychiatric disorder in offspring.

Analyses of adult phenotype in the offspring of maternally infected mice reveal persistent behavioural changes, including deficits in sensorimotor gating, spatial memory and sociability, with increase in anxiety and enhanced sensitivity to NMDA receptor antagonists (Asp et al. 2009; Meyer et al. 2008, 2009; Ibi et al. 2010). Investigations into the long-term effects of perinatal immune activation in DISC1 mutants on adult phenotype revealed gene × environment interactions, whereby the combination of both genetic abnormality and maternal infection exacerbated the behavioural and physiological deficits produced by either DISC1 mutation or maternal infection alone (Ibi et al. 2010).

Perinatal immune activation has also been associated with altered expression of NRG1 (Asp et al. 2009), suggesting that the signalling pathway regulated by NRG1 plays a role in the pathological mechanisms resulting therefrom. The involvement of NRG1 in the regulation of immune-related neuropathology is further indicated by a positive dose–response relationship between NRG1 expression and exposure to the endotoxin LPS in human umbilical endothelial cells (Hoffmann et al. 2010); the presence of a polymorphism in the NRG1 promoter region correlated positively with NRG1 expression and was associated with a reduced risk for adverse neurologic outcome in newborn infants, pointing towards a possible neuroprotective role for NRG1 in early life. In the same vein, prenatal stress may have a greater impact on the behavioural profile of synaptosomal-associated protein of 25 kDa (Snap-25) mutants, particularly sensorimotor gating and sociability (Oliver and Davies 2009). Additionally, maternal separation stress in mutants with KO of complexin II, another putative schizophrenia risk gene involved in synaptic plasticity and connectivity, disrupts long-term potentiation and spatial learning to greater extent than in WT counterparts (Yamauchi et al. 2005).

5.3.2 Adolescent Environment

Onset of the schizophrenia occurs typically in late adolescence or early adulthood. Psychosocial factors, such as those associated with social defeat (Selten and

Cantor-Graae 2007) and/or social fragmentation (Kirkbride et al. 2008), together with the varying psychosocial and/or biological adversities associated with living in cities (Kelly et al. 2010), can contribute to risk for schizophrenia. Exposure to early post-natal isolation in combination with heterozygous gene deletion of Nurr1, a mutant characterised by DAergic dysfunction and behavioural abnormalities related to schizophrenia, has been shown to produce disruption to PPI that was not evident in Nurr1 mutants without such exposure (Eells et al. 2006).

There is also evidence to suggest that drug abuse during adolescence, particularly in those with underlying genetic abnormalities, is associated with increased levels of psychotic symptoms (Henquet et al. 2006). Caspi et al. (2005) found that development of psychotic features in early adulthood was predicted by an interaction between Val/Met polymorphism in the COMT gene and adolescent-onset cannabis use. Recent work in our laboratory has provided further evidence for this relationship, with the cognitive and social effects of adolescent exposure to chronic delta-9 tetrahydrocannabinol (the psychoactive constituent of cannabis) differentially expressed in mutants with KO of the COMT gene (O'Tuathaigh et al. 2009b). Additionally, subchronic treatment with phencyclidine (PCP) in NRG1 mutants, followed by washout, resulted in heightened sensitization of the behavioural response to acute rechallenge with PCP (O'Tuathaigh et al. 2010b).

6 Conclusions

Although there have been rapid advances over the past several years, it is clear that we continue to face substantive conceptual, technical, methodological, analytical and interpretive challenges in applying mutant models to better understand the pathobiology of schizophrenia. Among these is increasing evidence that genes and pathologies implicated in schizophrenia overlap with other psychotic disorders, particularly bipolar disorder. Yet, the breadth and depth of ongoing studies in mutants holds the prospect of addressing these shortcomings. Overall, there is a new body of literature supporting a role for both genetic and environmental factors in the etiopathology of schizophrenia. A contemporary formulation revisits the 'two-hit hypothesis', whereby inherent biological vulnerability resulting from genetic variation interacts with an adverse environmental exposure to trigger the development of the disorder (Bayer et al. 1999; Le Strat et al. 2009). This is receiving increasing attention and is leading to the emergence of new, improved and influential mutant models of gene × environment interaction.

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Animal Models of Depression: Molecular Perspectives

Vaishnav Krishnan and Eric J. Nestler

Contents

Abstract Much of the current understanding about the pathogenesis of altered mood, impaired concentration and neurovegetative symptoms in major depression has come from animal models. However, because of the unique and complex features of human depression, the generation of valid and insightful depression models has been less straightforward than modeling other disabling diseases like cancer or autoimmune conditions. Today's popular depression models creatively merge ethologically valid behavioral assays with the latest technological advances in molecular biology and automated video-tracking. This chapter reviews depression assays involving acute stress (e.g., forced swim test), models consisting of prolonged physical or social stress (e.g., social defeat), models of secondary depression, genetic models, and experiments designed to elucidate the mechanisms of antidepressant action. These paradigms are critically evaluated in relation to their ease, validity and replicability, the molecular insights that they have provided, and their capacity to offer the next generation of therapeutics for depression.

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Abbreviations

5HT	5-Hydroxytryptamine or serotonin
BDNF	Brain-derived neurotrophic factor
CRF	Corticotropin-releasing factor
CUS	Chronic unpredictable stress
DBS	Deep brain stimulation
DNA	Deoxyribonucleic acid
ECT	Electroconvulsive therapy
FST	Forced swim test
GR	Glucocorticoid receptor
HPA	Hypothalamic–pituitary–adrenal
KO	Knockout
LH	Learned helplessness
SERT	Serotonin transporter
TST	Tail suspension test

1 Introduction

Major depressive disorder (MDD) or *depression* is a heritable neuropsychiatric syndrome characterized by relatively subtle cellular and molecular alterations distributed across a circuit of neural substrates (Krishnan and Nestler 2008). This disease claims a malignant toll on health: a 2007 World Health Organization study of over 200,000 adults across the world showed that depression produces the greatest decrement in health when compared with chronic diseases like diabetes and arthritis (Moussavi et al. 2007). In spite of a large variety of available antidepressant medications and alternative therapeutic modalities including several forms of psychotherapy (e.g., cognitive behavioral therapy) and several other approaches such as yoga, exercise, and sleep deprivation, depression suffers a huge treatment gap worldwide, whereby large numbers of individuals who require care do not receive treatment (Kohn et al. 2004). Depressive disorders cause morbidity across the entire age spectrum (Kessler et al. 2005): they can be difficult to diagnose and treat in the pediatric and adolescent period (Prager 2009), complicate the course of patients with chronic illness (Evans et al. 2005), and increase overall medical burden in the elderly (Lyness et al. 2006).

Over and above this alarming public health problem, shortfalls in treatment pose a grave concern. Even if major depression is accurately diagnosed and treated in all

individuals with perfect treatment compliance, the best remission rates with standard antidepressants are only *30–40%* (Rapaport et al. 2003; Trivedi et al. 2006). This is in stark contrast with other chronic disorders such as diabetes mellitus (Krishnan and Nestler 2008), where the correct combination of medications ultimately *can* ensure normoglycemia and prevent diabetic complications in a large majority of patients. Several explanations have been put forth for this discrepancy between the treatment of depression and other chronic disabling conditions. First, the diagnosis of depressive episodes is made when patients display a certain number of vaguely defined clinical symptoms (e.g., depressed mood, anhedonia, sleep changes, appetite changes, guilt, etc.) for a 2-week period. In the absence of more objective diagnostics such as neuroimaging, genetic variations, biomarkers, or biopsies, this rudimentary "symptom-counting" approach creates obvious limitations for the development of animal models, clinical trials, and neuropathological investigations (Krishnan and Nestler 2008). While the symptomatic heterogeneity of depression (*atypical* vs. *melancholic* vs. *psychotic*, etc.) is well recognized (Rush 2007), little insight has been gained into the etiological and pathophysiological distinctions between these subtypes. Drug efficacy trials are seldom conducted on subtype-segregated groups, thereby increasing the chance of abandoning therapies that may be subtype-specific. Since all available pharmacological treatments for depression work through altering monoaminergic transmission (Berton and Nestler 2006), it is possible that only one type of depression is being treated ("*monoamine-responsive*"). Due to high placebo response rates (Brunoni et al. 2009) and side effect concerns, monoamine-based agents still constitute a significant proportion of "new" antidepressants being tested in clinical trials (Mathew et al. 2008). And finally, given that genetic, neuroimaging, postmortem analyses and laboratory investigations (e.g., markers in serum or cerebrospinal fluid) have yielded limited insight into the neurobiology underlying depression (Krishnan and Nestler 2008), most current theories of depression are based largely on animal models of the disease, which are also inherently limited.

2 Can Depression Be Modeled in Laboratory Animals?

If the full psychiatric syndrome of depression cannot be recapitulated in rodents or nonhuman primates, then is it worthwhile to infer anything at all from animal models of depression? While symptoms such as guilt, suicidality and sad mood are likely to be purely human features, other aspects of the depressive syndrome have been replicated in laboratory animals, and in several instances ameliorated with antidepressant treatment. These include measures of helplessness, anhedonia, behavioral despair and other neurovegetative changes such as alterations in sleep and appetite patterns. From an evolutionary perspective, depression has been proposed to be an analog of the *involuntary defeat strategy* (IDS), which is triggered when an animal perceives defeat in a hierarchical struggle for resources (Sloman 2008). Features of psychomotor retardation, hyperarousal, anhedonia and

sleep disturbances in the setting of losing such a struggle are postulated to have an adaptive advantage in that they serve to protect losers from further attack and focus cognitive assets on planning ways out of complex social problems (Nesse 2000; Watson and Andrews 2002). Most, if not all, animal models of depression aim to quantitatively assay some form of experimentally induced *defeat* or *despair*, even though this aspect of mammalian behavior is likely *physiological* (i.e., adaptive) rather than *pathological*. In addition, while despair behavior is often extrapolated as being *depression-like*, the application of stress to rodents also produces *anxiety-like* changes that are manifestations of the *fight or flight response* (reduced exploration, freezing, stress-induced hyperthermia, etc.). Just as anxiety and depression often overlap clinically, the distinction between stress-induced *depression-like* and *anxiety-like* behaviors is difficult to ascertain, particularly since both types of behaviors respond to antidepressants. Thus, an important challenge of the field has been to produce a long-lasting state of depressive pathology in laboratory animals, which has seldom been achieved.

Today's depression models are often evaluated by fulfilling three main criteria (a) *face validity* (the requirement for a reasonable degree of symptomatic homology), (b) *construct* (or *etiological*) validity (the requirement for similar causative factors), and (c) *pharmacological validity* (which requires the reversal of depressive symptoms by available antidepressants). These criteria serve as guides to compare models against each other, but each criterion suffers basic flaws (Nestler and Hyman 2010). For instance, in the olfactory bulbectomy model of depression, surgically bulbectomized adult rats display increased locomotor activity, increased aggression, and spatial memory impairments that are all reversed by the chronic administration of a diverse array of antidepressants (Song and Leonard 2005). While this model may appear to be weak in construct and face validity, its pharmacological validity is excellent: virtually all classes of available antidepressants reverse these behavioral changes with a therapeutic delay. Of course, people with depression do not have olfactory lesions. Nevertheless, our assessment of poor construct validity is of limited value, since the etiology of depression is incompletely understood. Strict applications of face validity pose the risk of excessive anthropomorphization, particularly when assessing rodents such as mice, rats or tree shrews, which each have their own distinct behavioral repertoires (Crawley 2000). Since candidate models of depression are often assessed for reversibility with known monoamine-based antidepressants, there exists the alarming possibility that the most popular models of depression may, by design, be insensitive to the antidepressant effects of nonmonoamine-based agents (Berton and Nestler 2006). A potential fourth criterion is *pathological* validity, whereby animal models are validated by their recapitulation of known postmortem pathological or serological changes found in human depressed patients. Given our current state of knowledge, this is a very difficult requirement, but with increasing efforts in this field stemming from more widespread access to human postmortem tissue, the elucidation of pathological validity criteria may potentially eliminate the circular arguments that lie at the core of modeling depression.

This chapter evaluates the current status of animal models in depression and highlights certain novel neurobiological insights which have been generated using these models. Given the emphasis on molecular perspectives, we focus on data from rodent studies. Preclinical studies in nonhuman primates have largely focused on the behavioral and endocrinological impacts of early life stress (Gilmer and McKinney 2003) and, while this is clearly a critical research avenue, this field has been limited by a variety of factors which restrict nonhuman primate research. Instead of attempting to be comprehensive, this review highlights key methodological strengths and limitations and provides recommendations for further experimentation. The reader is referred elsewhere for a recent systematic and concise description of the neurobiology of depression (Krishnan and Nestler 2010).

3 Animal Models of Depression and Molecular Insights

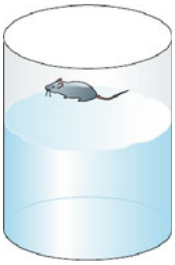
3.1 Models of Acute Stress

3.1.1 Forced Swim Test and Tail Suspension Test

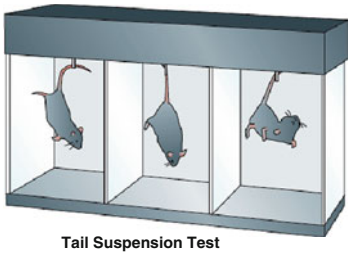
The forced swim test (FST) and tail suspension test (TST) are the most widely used tests of antidepressant action and are also used to infer "depression-like" behavior. In the *Porsolt* test (Porsolt et al. 1977), also known as the FST test, a mouse or rat is placed in an inescapable cylinder of water and, following an initial period of struggling, swimming and climbing, the animal eventually displays a floating or immobile posture. In the TST, immobility is scored while mice are suspended by their tails. Since water is not required, the TST is not confounded by challenges to thermoregulation (Cryan and Mombereau 2004). FST or TST immobility has been interpreted as an expression of *behavioral despair* or *entrapment* (Cryan et al. 2005; Lucki et al. 2001), and is reversed by the acute administration of almost all available antidepressants. This poses a problem for the model, since antidepressants restore mood in depressed humans only after many weeks of administration. Numerous agents that act independently of monoamine signaling have also been shown to reduce immobility time, such as recombinant ghrelin (Lutter et al. 2008), ketamine (Maeng et al. 2008), and estradiol (Dhir and Kulkarni 2008), to name a few. The foremost strength of these models is their ability to rapidly screen novel agents and phenotype genetically manipulated mice, and both paradigms have been successfully automated to reduce errors in subjective scoring. As shown in Fig. 1, a large number of mutant mice have been screened through the FST or TST. There appear to be a much larger number of "antidepressant-like" knockouts (KO), i.e., those mice that exhibit reduced immobility, compared with the number of KOs that exhibit increased immobility, but this may reflect a constraint of the model since it was originally designed to capture antidepressant effects. These studies illustrate

Increased Immobility
(PROdepressant)

α adrenergic receptor 2A KO (Schramm et al., 2001)
Adenosine receptor 3A KO (Fedorova et al., 2003)
Aromatase KO (Dalla et al., 2004)
#**BDNF forebrain KO** (Monteggia et al., 2006)
Conditional CREB overexpression (Newton et al., 2002)
CRF receptor 2 KO (Bale and Vale, 2003)
Delta opioid receptor KO (Filliol et al., 2000)
Desert hedgehog KO (Umehara et al., 2008)
Galanin receptor 2 KO (Lu et al., 2008)
Melatonin receptor 1 KO (Weil et al., 2006)
NCAM KO (Aonurm-Helm et al., 2008)
PACAP KO (Hashimoto et al., 2009)
#**Relaxin-3 KO** (Smith et al., 2009)
Sigma receptor 1 KO (Sabino et al., 2009)
SLITRK1 KO (Katayama et al., 2008)
*Thyroid hormone receptor KO** (Wilcoxon et al., 2007)
***TRH receptor 1 KO** (Zeng et al., 2007)
TRH receptor 2 KO (Sun et al., 2009)
Vesicular monoamine transporter 2 HET (Fukui et al., 2007)



Forced Swim Test



Tail Suspension Test

Decreased Immobility
(ANTIdepressant)

α adrenergic receptor 2C KO (Sallinen et al., 1999)
Adenosine receptor 2A KO (El Yacoubi et al., 2001)
Adenylyl cyclase 5 KO (Krishnan et al., 2008)
Acid sensing ion channel-1a KO (Coryell et al., 2009)
Angiotensinogen KO (Okuyama et al., 1999)
CaV 1.3 (L-type channel) KO (Busquet et al., 2009)
***CD26 KO** (El Yacoubi et al., 2006)
***Clock mutant** (Roybal et al., 2007)
Conditional beta-catenin KO (Gould et al., 2008)
CRF conditional overexpression (Lu et al., 2008)
D-aspartate oxidase KO (Weil et al., 2006)
Dopamine receptor 5 KO (Holmes et al., 2001)
***Dopamine transporter KO** (Perona et al., 2008)
EMX1 KO (Cao and Li, 2002)
Forkhead box O 3A KO (Polter et al., 2008)
GABA receptor A3 KO (Fiorelli et al., 2008)
GABA receptor B1 KO (Mombereau et al., 2004)
GABA receptor B2 KO (Mombereau et al., 2005)
***GABA transporter KO** (Liu et al., 2007)
Glutamic acid decarboxylase 65 KO (Stork et al., 2000)
Ikaros KO (Kiehl et al., 2008)
Inositol transporter KO (Bersudksy et al., 2008)
Interleukin 6 KO (Chourbaji et al., 2006)
K channel TREK1/KcnK2 KO (Heurteaux et al., 2006)
Leukemia Inhibitory factor KO (Pechnick et al., 2004)
***M1 muscarinic receptor KO** (Miyakawa et al., 2001)
Metabotropic glutamate receptor 5 KO (Li et al., 2006)
Metabotropic glutamate receptor 7 KO (Cryan et al., 2003)
MGAT5 KO (Soleimani et al., 2008)
Monoamine oxidase A KO (Cases et al., 1995)
Monoamine oxidase B KO (Grimsby et al., 1997)
Mu opioid receptor KO (Yoo et al., 2004, Ide et al., 2010)
Neurokinin receptor 1 KO (Rupniak et al., 2001)
Neuronal nitric oxide synthase [nNOS] KO (Salchner et al., 2004)
Neuropeptide Y receptor 2 KO (Tschenett et al., 2003)
Neuropeptide Y receptor 4 KO (Tasan et al., 2009)
NMDA receptor 2A subunit KO (Boyce-Rustay and Holmes, 2006)
Nociceptin/orphanin FQ receptor KO (Gavioli et al., 2003)
Norepinephrine transporter KO (Perona et al., 2008)
Phosphodiesterase 4B KO (Zhang et al., 2007)
Purinergic receptor P2X7 KO (Basso et al., 2009)
Protein kinase C interacting protein KO (Barbier and Wang, 2009)
Serotonin receptor 1A KO (Ramboz et al., 1998)
#**Serotonin receptor 1B KO** (Jones and Lucki, 2004)
Serotonin receptor 7 KO (Hedlund et al., 2005)
Synaptotagmin IV KO (Ferguson et al., 2004)
Tachykinin 1 KO (Bilkei-Gorzo et al., 2002)
TGFα overexpression (Hilakivi-Clarke, 1994)
Tumor necrosis factor receptor 1 or 2 KO (Simen et al., 2006)

Fig. 1 The forced swim test (FST) and tail suspension test (TST) have been utilized to phenotype a large number of genetically manipulated mice, illustrating the sheer diversity of genes and pathways potentially involved in depression-related behavior. Knockout mice ("KO"), transgenic overexpressors or other types of mutants have been segregated into those that display increased immobility in *either* the FST or TST ("pro-depressant"), or reduced immobility (antidepressant-like). *Hash* indicates gender differences in the phenotype; *asterisk* indicates that results may be confounded by locomotor behavior. Shown are examples of genetic mutant mice examined to date; studies utilizing virally mediated gene transfer are not included. Abbreviations: *HET* heterozygote; *BDNF* brain-derived neurotrophic factor; *TRH* thyrotropin-releasing hormone; *CREB* cyclic adenosine monophosphate response element binding protein; *CRF* corticotropin-releasing factor; *NCAM* neuronal cell adhesion molecule; *PACAP* pituitary adenylyl cyclase activating peptide; *SLITRK* slit and NTRK-like family member 1; *GABA* gamma aminobutyric acid; *NMDA N*-methyl D aspartate; *TGF* transforming growth factor; *EMX* empty spiracles homolog; *MGAT* mannosyl glycoprotein acetylglucosaminyl transferase. Images obtained from Cryan and Holmes (2005)

the number and diversity of genes that may play a role in regulating stress-induced immobility, including transcription factors, growth factors, endocrine hormones, immune signaling molecules, and numerous genes encoding proteins required for synaptic neurotransmission.

Since the majority of mutants phenotyped thus far are constitutive KOs, their phenotype could be confounded by developmental compensatory effects, e.g., biochemical and anatomical alterations which are secondary to the loss of the gene of interest [see also Gondo et al. (2011); O'Tuathaigh et al. (2011) for further discussion]. These compensatory effects may, nevertheless, be relevant to the study of depression. For example, the profound antidepressant-like phenotype of TREK1 (*Twik-related K Channel 1*) KO mice is associated with markedly altered $5HT_{1A}$-receptor-mediated excitation in the hippocampus (Heurteaux et al. 2006), a change that is also observed following chronic treatment with a variety of antidepressants (Haddjeri et al. 1998). Performance on the FST and TST is also dependent on the background strain of the animals used: systematic comparisons of inbred mice reveal greater than a tenfold range of immobility (Liu and Gershenfeld 2003; Lucki et al. 2001; see also Gondo et al. 2011; O'Tuathaigh et al. 2011 for further discussion). While the effects of background strain tend to complicate phenotypic analysis of mutant mice, such variation has been exploited for QTL (*quantitative trait loci*) analyses, which have implicated genes in certain broad chromosomal regions in this type of behavioral response (Jacobson and Cryan 2007; Tomida et al. 2009).

The complexities of "simple" immobility testing are exemplified by data from serotonin transporter (SERT) KO mice. Since SERT is inhibited by many available antidepressants, one might expect SERT KO mice to display a robust antidepressant-like phenotype. However, they display increased FST immobility and decreased TST immobility on a *129S6* or 129S6/SvEV mixed background, have increased TST immobility on a *CD1* background, and yet have no phenotype on a *C57BL/6J* background (Alexandre et al. 2006; Holmes et al. 2002; Lira et al. 2003). Subsequently, a SERT KO rat has been generated through random ENU (*N*-ethyl-*N*-nitrosurea) mutagenesis, which displays increased immobility on the FST (Olivier et al. 2008). Thus, while SERT inhibition is required for the antidepressant effects of SSRIs (selective serotonin-reuptake inhibitors) (Holmes et al. 2002), it appears that the developmental loss of SERT produces a complex phenotype that is clearly dependent on background strain. While the precise mechanistic details remain unclear, the observed pro-depressant-like phenotypes may be related to pathologically elevated synaptic serotonin levels during development causing a decrease in the number and firing rate of serotonergic neurons (Lira et al. 2003) as well as disorganized limbic cortical development (Olivier et al. 2008).

3.1.2 The Learned Helplessness Model

Following an *uncontrollable* and *inescapable* stress such as exposure to inescapable electric shocks, animals develop a state of "helplessness" such that when

re-exposed to the same shocks, now with an easy escape route, animals will either display increased escape latency or completely fail to escape (Seligman et al. 1975). Following one or more sessions of inescapable shock, rats have been shown to develop persistent changes including weight loss, alterations in sleep patterns and HPA axis activity and loss of spine synapses in hippocampal regions (Cryan and Mombereau 2004; Haddjeri et al. 1998; Nestler et al. 2002). In mice, the learned helplessness (LH) syndrome appears to be short-lived (2–3 days), and several mutant lines of mice have been phenotyped on the LH assay, with results largely compatible with their corresponding FST data. Like the FST or TST, both mice and rats display a considerable degree of interstrain variation, and escape deficits are reversed by a variety of antidepressants (Henn and Vollmayr 2005).

One distinctive feature of LH is the considerable degree of variability in the expression of helplessness: anywhere from 10 to 80% of animals simply fail to develop escape deficits. While this may be a disadvantage in certain scenarios, this variability has been exploited to devise selective inbreeding strategies to create of helpless and nonhelpless strains of rats which differ across a variety of other indices, including measures of anhedonia, activity and sleep behavior (Henn and Vollmayr 2005). DNA microarray analyses performed on hippocampal tissues reveal that nonhelpless rats activate a distinct pattern of gene expression compared with helpless or stress-naïve rats, suggesting that their passive responsiveness may be due to distinct neurobiological changes (Kohen et al. 2005). In mice, the development of helpless behavior is inversely related to the activation of the transcription factor ΔFosB (a stable splice variant of FosB) in the periaqueductal gray (PAG) of the midbrain. The virally mediated overexpression of ΔFosB in PAG neurons protects against developing an escape deficit partly through the transcriptional repression of *substance P*, a neuropeptide known to modulate the physiology of serotonergic and other neurons (Berton et al. 2007).

Today, these acute stress models make up the first line of behavioral tests utilized to phenotype transgenic mice and are also exploited as tools to rapidly screen putative antidepressant compounds. Even though direct links to human depression may be weak since they use acute stressors and test acute antidepressant responses, these tests have directed the field toward a number of previously unappreciated molecular players (Fig. 1). Of course, to truly implicate these targets in the pathophysiology of depression without false positives and to shed light on complex relationships such as those observed in the case of the SERT KOs, positive hits on these screens require much further validation through a more diverse set of molecular and behavioral assays, ideally in conjunction with postmortem validation (Covington et al. 2009; Hunsberger et al. 2007; Krishnan et al. 2008; Svenningsson et al. 2006). Furthermore, the FST, TST and LH are highly sensitive to manipulations which impair motor function, and the LH model is particularly sensitive to alterations in central and peripheral pain sensitivity (Cryan and Mombereau 2004). Therefore, these screening assays should be followed up with tests of motor function or pain sensitivity.

3.2 Models of Secondary or Iatrogenic Depression

3.2.1 Hormones of the HPA Axis

The hypothalamic–pituitary–adrenal (HPA) axis is activated by a wide variety of stressful stimuli, and resultant increases in serum glucocorticoids serve an immediate adaptive role through increases in gluconeogenesis and lipolysis. The "cortisol" hypothesis suggests that certain symptoms of depression may be mediated by a persistently overactive HPA axis, brought about through (1) increased production of hypothalamic corticotropin-releasing factor (CRF) and (2) reduced negative feedback at the level of centrally expressed glucocorticoid receptors (Holsboer and Ising 2009). Clinical studies have demonstrated HPA axis dysregulation in some depressed individuals, mainly those with severe depression and psychotic symptoms (Gold and Chrousos 2002), and these patients may uniquely benefit clinically from pharmacological antagonists of the glucocorticoid receptor (Krishnan and Nestler 2008). In contrast, atypical depression (associated with increased sleep and appetite), posttraumatic stress disorder, chronic fatigue syndrome, and fibromyalgia are associated with reduced circulating glucocorticoid concentrations and heightened negative feedback (Krishnan and Nestler 2008), demonstrating that alterations in HPA axis activity in either direction can result in depressive features. A significant amount of preclinical effort has been devoted to generating animal models of impaired glucocorticoid function. Perhaps the most syndromically accurate model of melancholic depression is the forebrain glucocorticoid receptor (GR) knockout mouse, derived through conditional deletion of the GR allele via *cre-recombinase loxP* technology: these mice display enhanced basal serum glucocorticoid levels, dexamethasone nonsuppression, increased FST and TST immobility, and these changes are all reversible with chronic antidepressants (Boyle et al. 2005). Interestingly, the forebrain *overexpression* of GR leads to an identical behavioral phenotype (Wei et al. 2007), which suggests that the mood altering properties of glucocorticoid signaling are more complex than simple increases or decreases in steroid or receptor levels.

Depression is also commonly observed as an *iatrogenic* side effect of chronic glucocorticoid administration and is a key psychiatric symptom of Cushing's syndrome which is characterized by hypercortisolemia secondary to adrenal or pituitary corticotrophic hyperplasia. Thus, the negative consequences of heightened HPA axis activity are at least partially related to the adverse effects of glucocorticoids themselves (McEwen 2007; Pittenger and Duman 2008). Consistent with this hypothesis, mice exposed to 20 days of corticosterone dissolved in their drinking water to develop decreased responding for food pellets in an operant conditioning task (an anhedonic phenotype) and increased TST immobility, both of which are reversible by chronic amitriptyline (a tricyclic antidepressant) (Gourley et al. 2008). Such corticosterone exposure decreases activation of ERK1/2 (extracellular signal regulated kinase 1/2) in the dentate gyrus, which is itself sufficient to increase FST immobility and antagonize the action of antidepressants (Duman et al. 2007).

Increases in circulating serum cortisol in depression may also be secondary to increased CRF synthesis and secretion (Nemeroff et al. 1984). Many of CRF's strong effects on behavior occur through centrally mediated processes independent of adrenal function, i.e., are not reversed by adrenalectomy (Muller and Holsboer 2006). To tease out the behavioral significance of brain CRF signaling, numerous transgenic and knockout lines have been generated. While the loss of brain CRF has negligible behavioral consequences, the transient overexpression of CRF during development leads to reduced exploratory behavior (increased anxiety) and FST/TST immobility during adulthood, and constitutive CRFR1KO mice display increased exploration (anxiolysis) (Kolber et al. 2010; Muller and Holsboer 2006). These data, combined with postmortem evidence of enhanced CRF levels in depression, have encouraged pharmaceutical companies to invest in the development of a safe and effective CRFR1 antagonist to be used in depression and anxiety disorders (Mathew et al. 2008). However, despite decades of study and numerous pharmacological prototypes, this hypothesis remains to be tested effectively in humans. An important challenge in this field has been to selectively antagonize brain CRF signaling without altering natural HPA axis responsiveness.

3.2.2 Retinoic Acid Derivatives

Isotretinoin (Accutane ©), a retinoic acid derivative used as a highly effective treatment of severe acne, has been associated with an increased risk for depression and suicide (Bremner and McCaffery 2008). Mice chronically treated with isotretinoin develop increases in FST and TST immobility which have thus far been correlated with decreased hippocampal metabolism and neuronal proliferation (Crandall et al. 2004; O'Reilly et al. 2006). Isotretinoin is known to bind and activate retinoic acid receptors (RARs) which are widely distributed in the adult brain (Bremner and McCaffery 2008). RARs belong to the nuclear hormone receptor family of transcription factors, and the transcriptional consequences of isotretinoin exposure within limbic brain regions remain unexplored.

3.2.3 Cytokines and Immune System Dysregulation

Proinflammatory cytokines such as interferon-α are used in humans to treat several disease states. Many of these recombinantly derived proteins produce clinically significant depression as a side effect (Loftis and Hauser 2004). A large body of preclinical evidence suggests a bidirectional association between immune activation and depressive symptoms: certain cytokines have been shown to induce depression-like behavior in rodents and primates (Dunn et al. 2005; Felger et al. 2007), and several models of chronic stress produce significant changes in immune function (Miller et al. 2009). One such example is IL-1β (interleukin-1β): increases in IL-1β signaling in the hippocampus play a role in mediating the anhedonic and antineurogenic effects of chronic stress through the actions of the transcription

factor NFκB (nuclear factor-κB) (Koo and Duman 2008; Koo et al. 2010). A key priority in this field will be to progress from focusing on the *sickness behavior* induced by strong immune stimuli such as LPS (lipopolysaccharide) (O'Connor et al. 2009) to the behavioral consequences of more elegant manipulations of specific cytokine signaling axes, as well as defining the therapeutic relevance of a whole host of antiinflammatory therapeutics popularly prescribed for autoimmune conditions. Clearly, the answer is not simply *decreasing* inflammation. The immunization of rats with an altered version of MBP (myelin basic protein) activates weakly self-reactive T-cells and has been shown to render rats immune to the anhedonic effects of chronic unpredictable stress (CUS) (Lewitus et al. 2009), suggesting that specific activators of immune function may in fact promote stress resilience. Understanding the *immunology of depression* is particularly applicable to autoimmune diseases such as multiple sclerosis (MS) where up to 50% of patients experience clinically significant depression. Murine MS models display depression-like changes such as weight loss, anorexia and reduced social exploration well before the onset of neurologic deficits (Ghaffar and Feinstein 2007; Gold and Irwin 2009), suggesting the presence of shared pathogenic mechanisms.

Depression which is secondary to medical conditions (e.g., stroke, pancreatic cancer, hypothyroidism, hypercortisolemia, etc.) is clinically indistinguishable from so-called *endogenous* or primary depression. Without clear knowledge of the etiology of endogenous depression, models that are designed based on the direct application of clinical observations are positioned to play a critical role due to their strong construct validity. A direct comparison of the molecular changes associated with corticosterone, cytokine and/or isotretinoin exposures versus stress models are likely to provide insight into shared and distinct pathophysiological mechanisms between stress-induced, endogenous, and iatrogenic forms of depression. One obvious path of investigation would be to employ genome-wide transcriptional profiling techniques to look for shared patterns of molecular plasticity in both animal models and patient samples. These "common denominator" patterns could identify potential targets for antidepressant drug discovery, and such agents would likely be active against all forms of depression.

3.3 Chronic Stress Models

While acute stress paradigms are used broadly for their ease, automation, and rapid phenotyping abilities, they offer singular readouts that often cannot be unambiguously interpreted. For instance, increased immobility in the FST is often anthropomorphized as an expression of despair. However, it can also be understood as a successful and adaptive behavioral response that functions to conserve energy. Today's chronic stress models are distinguished by their remarkable ability to simultaneously produce a set of behavioral alterations with strong face validity for depression. However, this enhanced face validity often comes at the cost of low throughput: the precise application of these chronic stress models requires more

space and time and greater sample sizes and are consequently significantly more expensive than other models. Thus, fewer laboratories have experienced consistent success. Furthermore, even with their known pharmacological validity, their low throughput makes them poorly suited for the pharmacological validation of novel compounds. In essence, these models are composed of repeated applications of an uncontrollable and unpredictable stress that is coupled with a quantifiable assay of depression-like behavior. They are based on clinical evidence that stressful life events that significantly increase the risk of depressive episodes are generally of a chronic nature (divorce, financial problems, and sexual abuse) (Krishnan and Nestler 2008). As is discussed below, their main strengths lie in their ability to characterize the neuroplasticity associated with chronic stress or antidepressant exposures.

3.3.1 Chronic Mild Stress

Chronic mild stress (CMS), better described as CUS, paradigms involve the application of varied intermittent physical stresses applied over a relatively prolonged time period (between 1 and 7 weeks, Fig. 2). Sucrose drinking is the most commonly utilized assay to assess the impact of CUS and CUS-exposed rats or mice show deficits in their motivation to consume a dilute (1–2%) solution of sucrose measured either as total sucrose intake or as a preference against water (Willner 2005). CUS has also been shown to result in a number of other "emotional" changes that are difficult to objectively quantify, such as grooming deficits and changes in aggressive and sexual behavior. Many of these phenotypes are reversed by chronic antidepressants applied either during the stress or as a poststress treatment (Strekalova et al. 2006). This model has been the subject of considerable controversy related to poor reproducibility (Argyropoulos and Nutt 1997; Broekkamp 1997; Willner 2005), and while some groups have had consistent success in repeatedly generating anhedonic mice/rats with a given paradigm, others have not experienced the same reliability. It would appear that this model is particularly sensitive to subtle variations in design (the various permutations of stressors) and numerous other sources of variability endemic to behavioral research (e.g., time of testing, vendor differences, etc.) and has accordingly faded in popularity. While it may not have the pharmacological screening capabilities of the FST, when performed reproducibly and reliably, it has clear potential to generate important molecular insights into depression.

Aside from being a tool to study the physiological consequences of chronic stress, CUS has been applied recently to phenotype mouse mutants, study gender differences in stress responses, and validate novel antidepressants (Kong et al. 2009; LaPlant et al. 2009; Vitale et al. 2009). Like LH, CUS studies have reported significant individual differences. In one mouse study, decreased sucrose preference (anhedonia) was only observed in 61% of mice and was uniquely associated with increased immobility in the FST. In contrast, all CUS-exposed mice developed changes in locomotor behavior and decreased exploration, suggesting that segregating a subgroup of anhedonic mice identifies a unique susceptible population that

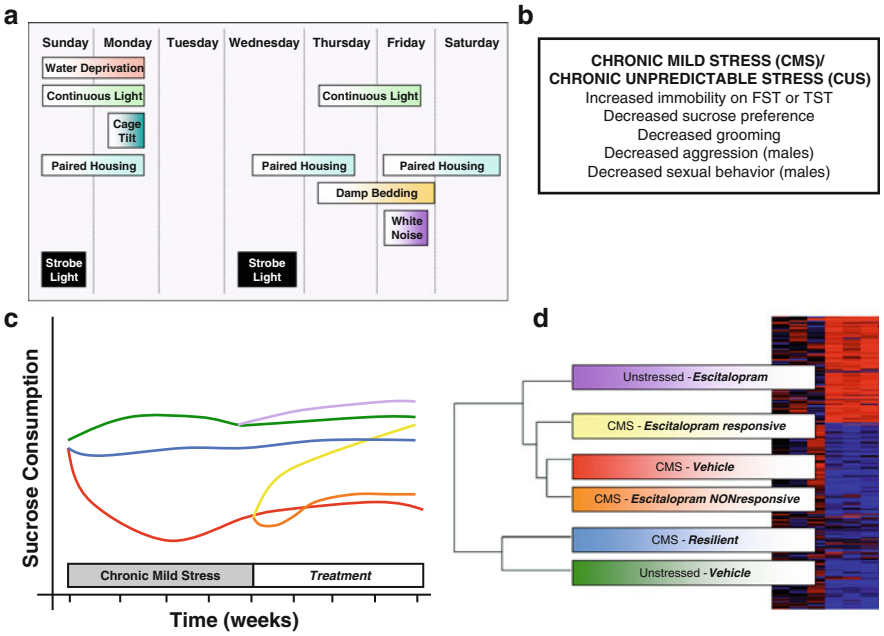


Fig. 2 The chronic mild stress (CMS)/chronic unpredictable stress (CUS) model of depression relies on a series of mostly physical stresses that are presented over 1–6 weeks (Willner 2005). (**a**) One example of a rat CUS protocol (Grippo 2009). (**b**) CUS paradigms in rats and mice produce a variety of behavioral changes. (**c**, **d**) The most popular assay for the effects of CUS is sucrose preference or sucrose intake whereby reductions in the consumption of a palatable sweet solution are interpreted as anhedonia. CUS has been applied to the study of stress resilience (CUS-resilient mice do not display a reduction in sucrose intake) and antidepressant resistance (escitalopram treated mice do not recover impairments in sucrose drinking). The key for the *colored lines* is provided in *Panel d*. DNA microarray technology combined with gene expression cluster analysis can aid in correlating behavioral groups with their gene expression patterns. For example, in this study examining total hippocampal tissue, genes modulated in CUS-resilient and vehicle-treated unstressed control rats were strongly overlapping, and these gene expression patterns were quite *distant* from unstressed rats treated with escitalopram (Bergstrom et al. 2007)

displays stress-induced depressive features (Strekalova et al. 2004). A similar degree of variability has been observed in rats and when CUS-sensitive (i.e., vulnerable) rats were treated with antidepressants two distinct populations emerged: antidepressant-sensitive and antidepressant-resistant (Jayatissa et al. 2006). This ability of CUS to model two poorly understood human phenomena, stress resilience and antidepressant resistance has inspired a series of microarray studies aimed at exploring the molecular signatures associated with these phenomena. Resistance to the antidepressant effects of escitalopram, an SSRI, is associated with the upregulation of proapoptotic genes including APP (amyloid precursor protein) and TNF (tumor necrosis factor) in hippocampus, while vulnerability to CUS-induced anhedonia is associated with reduced expression of genes required for cellular proliferation and differentiation (Bergstrom et al. 2007). Similar

experiments have been conducted in other brain regions including the amygdala and cingulate and frontal cortices (Orsetti et al. 2008; Sibille et al. 2009; Surget et al. 2009), each revealing unique region-specific molecular signatures associated with vulnerability to CUS.

At this stage, cellular heterogeneity represents a key limitation in the interpretation of these data: microarray studies performed on mixed samples of neuronal, glial, endothelial and immune cells are likely to result in poor reproducibility and low signal/noise ratio. Two important developments that are likely to address this problem are (1) laser capture microdissection techniques, which allow for precise isolation of limbic nuclei and subnuclei, and (2) mutant mice where subpopulations of neurons or other cell types are fluorescently labeled, allowing precise sorting of cells of interest through fluorescence-mediated techniques (Pollak et al. 2008; Sugino et al. 2006). As technological advances in DNA and protein array analysis allow for the rapid, reliable, and cost-effective genome-wide analysis of transcriptional regulation, these studies set the stage for an understanding of the complex gene network interactions involved in the pathophysiology of depression and antidepressant responsiveness.

3.3.2 Psychosocial Stress Models

One caveat with CUS is its questionable construct validity since certain routinely employed CUS stressors are physical (e.g., strobe lights, restraint or swim stress, or abrupt circadian disruptions) and are unlikely to be encountered by rats or mice in the wild. At least in this respect, models of psychosocial stress display their greatest strength since they entirely rely on innate social behavior. The central theme in these models (Fig. 3), whether they are conducted in rats, mice, or tree shrews, is to allow two or more subjects to socially and physically interact (an *agonistic* encounter) such that one achieves dominant status (*alpha*) and the others remain subordinate (*omega*). While some groups identify subordinates between age- and strain-matched pairs of mice or *dyads* (Avgustinovich et al. 2005; Malatynska and Knapp 2005), others employ a "forced subordination" strategy whereby reliably aggressive rodents (usually larger and/or of a more aggressive strain) are employed to consistently subordinate other subjects (Berton et al. 2006; Covington and Miczek 2005). In addition to the intense and unpredictable physical stress during social encounters, several laboratories add on the psychological stress of prolonged "sensory contact" through which subordinate mice are housed in the same cage as their dominant counterparts across a partition that prevents all but sensory interaction (Martinez et al. 1998). Following multiple defeat encounters, rodents display reduced social interaction, decreased exploration and locomotor behavior, anhedonia (e.g., decreased sucrose preference and sexual behavior), increased stress-induced immobility and alterations in HPA axis and autonomic function (Avgustinovich et al. 2005; Krishnan et al. 2007), many of which are reversed by chronic but not acute antidepressant administration (Becker et al. 2008; Rygula et al. 2008). Like CUS, the establishment and validation of such social stress

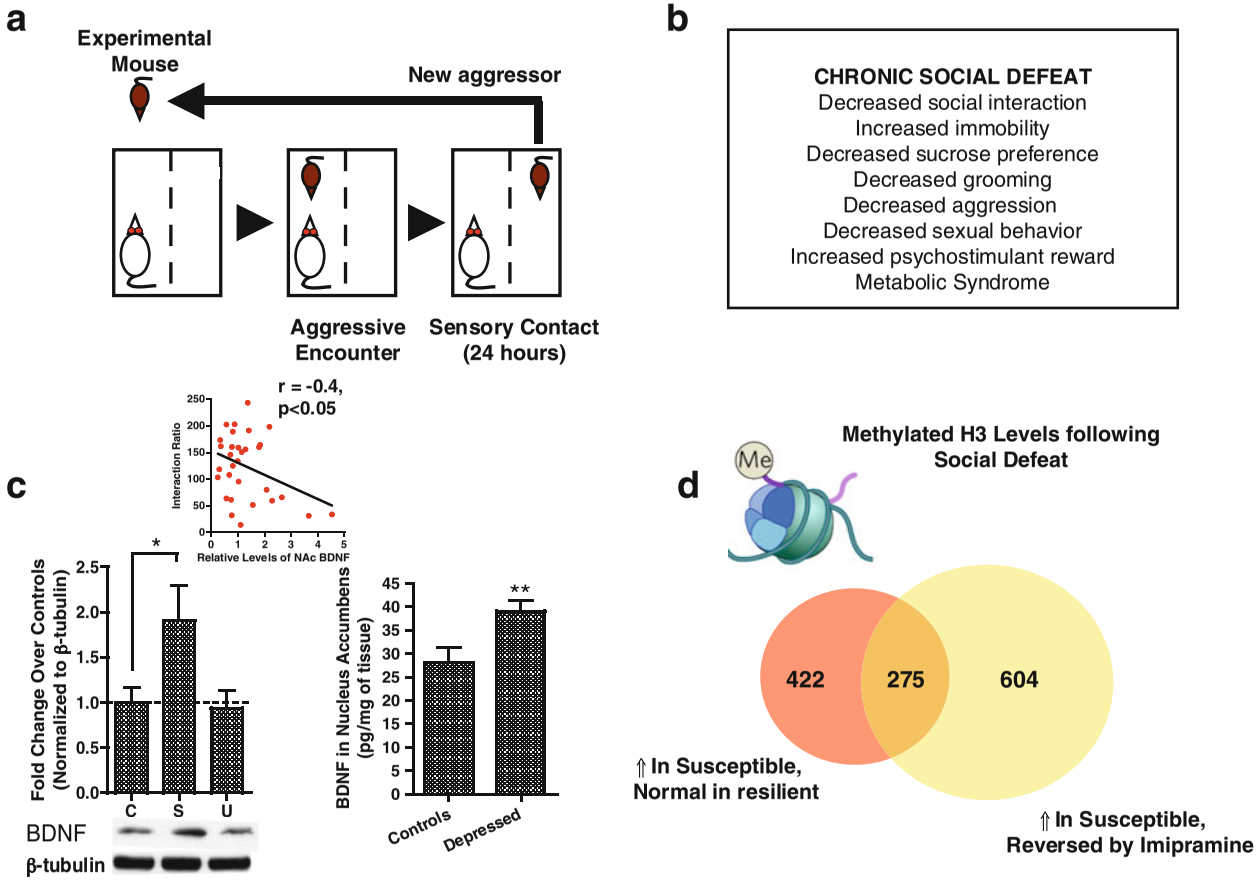


Fig. 3 Psychosocial stress models rely on innate social behavior among pairs or groups of male rodents allowing for the formation of stable dominant/subordinate relationships. (**a**) In the sensory contact adaptation of rodent social defeat, an intruder is periodically subordinated by a territorially aggressive

models can be cumbersome and expensive. Reliable expression of aggressive behavior can be easily disrupted by minor procedural variations such as changes in bedding or cage size. In addition, laboratory personnel performing social defeat experiments must attain a sense for the correct "quantity" of aggressive behavior: while excessively injurious physical interactions are both unethical and irrelevant to the study of depression, weakly aggressive encounters pose the risk of producing mild and short-lived phenotypes that may affect molecular analyses.

The decreased sociability following such defeats can be quantifiably assessed with automated tests of social interaction that permit an assessment of individual differences among defeated mice. By combining this type of highly quantitative behavioral analysis with standard molecular and cellular techniques, this model has shed light on a number of mechanistic hypotheses related to variability in stress responsiveness. These include the role of activity-dependent BDNF (brain-derived neurotrophic factor) signaling within the mesolimbic dopamine circuit (Feder et al. 2009; Krishnan and Nestler 2008), endogenous kappa-opioid signaling (McLaughlin et al. 2006), the contribution of adult hippocampal neurogenesis (Lagace et al. 2010) and the role of peripherally derived mediators of energy homeostasis (Chuang et al. 2010). Such significant variability even among age-matched members of an inbred strain suggests that this heterogeneity occurs independently of DNA sequence variations. One possibility is that *epigenetic* modifications of the genome, which occur stochastically during development, may contribute to this variability seen among inbred mice raised in near identical environmental conditions. These epigenetic mechanisms include covalent modifications to histones (e.g., histone acetylation, methylation or phosphorylation) or DNA (e.g., DNA methylation) (Krishnan and Nestler 2008; Bountra et al. 2011).

Social defeat itself has a powerful impact on the epigenome: defeated mice display increases in repressive histone methylation in the hippocampus and

Fig. 3 (continued) resident mouse and is forced to spend the remainder of the day across a partition that permits sensory contact without fighting. (**b**) The main behavioral consequences of repeated bouts of such social subordination. (**c**) Aside from face, construct, and pharmacological validity, one can further validate animal models by demonstrating the presence of identical molecular changes in human postmortem tissue. Here, 10 days of social defeat in C57Bl/6 mice increases BDNF protein levels (by immunoblot) in the nucleus accumbens such that vulnerable or susceptible mice (S) display the greatest increases in BDNF (*C* controls, *U* unsusceptible), with the inset demonstrating a significant inverse correlation between interaction scores and BDNF levels. This molecular change is also observed in postmortem accumbens samples from male depressed individuals (Krishnan et al. 2007). (**d**) ChIP–chip analyses (chromatin immunoprecipitation followed by DNA promoter arrays) examining genome-wide patterns of a repressive form of histone H3 methylation in the nucleus accumbens. The region of Venn overlap ("275") corresponds to 275 genes that are upregulated in susceptible animals and that are also reversed by imipramine and not seen in resilient animals (Wilkinson et al. 2009). Some examples of genes that fall within this overlap include CNK1D (casein kinase 1 delta), FGF1 (fibroblast growth factor 1) and HDAC4 (histone deacetylase 4). These results suggest that inhibiting the stress-induced histone methylation at these genes through inhibitors of histone methyltransferases constitutes a potential novel target for antidepressant development

nucleus accumbens (Tsankova et al. 2006) and increases in histone acetylation in the NAc (Covington et al. 2009). ChIP–chip techniques (chromatin immunoprecipitation combined with promoter array chips) have allowed for an appreciation of epigenetic profiles associated with the expression of susceptible or resilient behavior and antidepressant exposure (Wilkinson et al. 2009). This latter approach has illustrated a significant degree of overlap in patterns of epigenetic regulation between antidepressant-treated susceptible mice and vehicle-treated resilient mice, suggesting that certain individuals may avoid the deleterious effects of stress by naturally mounting an endogenous antidepressant-like response (Wilkinson et al. 2009). Furthermore, with the advent of pharmacological inhibitors of epigenetic enzymes such as histone deacetylase inhibitors (HDAC inhibitors; see Bountra et al. 2011), one can directly test epigenetic hypotheses in a more precise manner. For example, the antidepressant effects of systemically administered weakly selective HDAC inhibitors such as sodium butyrate and valproic acid (Gundersen and Blendy 2009; Schroeder et al. 2006; Tsankova et al. 2006) can be recapitulated by a localized infusion of more specific and selective drugs in the NAc (Covington et al. 2009). Similar strides have been made in understanding the behavioral impact of DNA methylation (LaPlant et al. 2010). Microarray analyses comparing the effects of systemic fluoxetine and localized HDAC inhibitor infusions reveal significant overlap in patterns of transcriptional activation and repression (Covington et al. 2009). On the other hand, genes influenced by HDAC inhibitors, and not by fluoxetine, may prove even more interesting in terms of identifying truly novel approaches for more effective antidepressant treatments.

Other forms of social stress are worth mentioning. Prolonged social isolation during adulthood results in reduced sucrose drinking and alterations in sexual reward behavior. While this model has received less recent attention, it displays excellent construct validity and requires minimal sophistication (Wallace et al. 2009; Wilkinson et al. 2009). Early life stress, typically applied in the form of maternal separation during early postnatal developmental periods, has been shown to result in cognitive and emotional changes that persist through adulthood. These phenotypes, such as altered HPA axis function, increased immobility, weakened prepulse inhibition, spatial learning deficits, etc., have been linked to a variety of neuropsychiatric syndromes with strong developmental hypotheses including schizophrenia (Fumagalli et al. 2007; Lupien et al. 2009). While studies in this field have traditionally almost exclusively emphasized the role of the HPA axis, more recent ventures have demonstrated how maternal separation paradigms are quite aptly designed to study epigenetic forms of neuroplasticity (Murgatroyd et al. 2009) as well as mechanisms by which early life stress can in fact promote resiliency during adulthood (Lyons et al. 2009). Since social defeat models rely on differences in intermale aggression, they cannot be directly applied to females. However, females do display depression-like features following other social stressors such as intermittent crowding or isolation (Herzog et al. 2009). Given the twofold preponderance of depression in females, further studies of pathophysiological mechanisms in female rodent models are a very high priority for the field and

these psychosocial stress models, in their ability to directly compare across sexes, are ideal candidates for such studies.

4 Insights from Models of Antidepressant Action

While the molecular targets of current antidepressant agents are known, there still remain large gaps in understanding their neuroanatomical sites of action and why these agents are associated with a significant therapeutic delay. Most of the current knowledge of these mechanisms has come from animal studies examining neurobiological changes following chronic antidepressant administration, voluntary exercise (through the exposure to a running wheel), or the application of ECT. More recent reports have exploited other strategies in rodents such as repetitive transcranial magnetic stimulation (rTMS) using a noninvasive cortical stimulating device (Vieyra-Reyes et al. 2008) as well as more creative cognitive paradigms such as *learned safety*, where a benign environmental stimulus that signals "safety" produces antidepressant-like effects (Pollak et al. 2008).

The most compelling and reproducible biological findings from these approaches are focused largely on the hippocampus, perhaps due to its well-understood anatomy. These studies have contributed to the development of neurotrophic model of depression, whereby stressful experiences through glucocorticoid signaling and other mechanisms reduce the level of neurotrophic factors such as BDNF in the hippocampus resulting in atrophic morphological changes. Antidepressants, by activating cellular signaling cascades that culminate in the activation of CREB (cyclic-AMP response element binding protein), function to enhance levels of BDNF and other growth factors like VEGF (vascular endothelial growth factor) and VGF (nonacronymic), which promote the proliferation and differentiation of hippocampal progenitors and alter monoaminergic synaptic transmission (Balu and Lucki 2009; Krishnan and Nestler 2008; Pittenger and Duman 2008). Other key molecular mediators have been identified, such as *p11*, a scaffolding protein induced by antidepressants that binds and enhances the surface expression and activity of the serotonin 1B (5-HT_{1B}) receptor, promoting an antidepressant-like response in laboratory assays (Svenningsson et al. 2006). p11 also enhances the activity of serotonin receptor type 4 (5-HT_4) (Warner-Schmidt et al. 2009), which is of particular significance since 5-HT_4 receptor agonists have rapid antidepressant-like activity. In the CUS model, while only 3–4 days of daily injections of RS67333 (a prototypical 5-HT_4 receptor agonist) alleviated the reduced sucrose intake in CUS-vulnerable rats: citalopram-treated controls required greater than 14 days of treatment to observe a significant improvement (Lucas et al. 2007). This study illustrates a key point related to pharmacological validity. Even though acute stress models are often criticized for their acute responses to antidepressants, efforts should nevertheless still be devoted to identifying novel agents that do not exhibit a therapeutic delay. The identification of such rapidly acting agents offers hope that antidepressants of the future will no longer be limited by their therapeutic delay.

A clinically validated example of one such a rapidly acting agent is ketamine (aan het Rot et al. 2010), and recent preclinical experiments reveal that ketamine's antidepressant effects may be mediated through rapid forms of glutamatergic synaptic plasticity (Li et al. 2010).

The neurotrophic hypothesis described above is consistent with the observation that certain subpopulations of depressed patients display small reductions in total hippocampal volume with consequent ventricular enlargement (Savitz and Drevets 2009). Aside from these correlative data, there is little direct clinical evidence that alterations in hippocampal activity alter mood per se. Functional neuroimaging studies designed specifically to reveal the neuroanatomical substrates of altered emotional processing in depression have indicated roles for the amygdala and frontal cortical regions such as the subgenual cingulate cortex (area cg25), where the application of deep brain stimulation (DBS) produces long-lasting antidepressant effects in treatment-resistant depression (Mayberg 2009). Such profound effects of DBS applied to Cg25 or the NAc (Bewernick et al. 2010) constitute not only an important therapeutic development, but also provide unequivocal evidence regarding neural substrates that participate in improving mood symptoms. Of interest, patients in these studies were noted to have an immediate and intolerable worsening of depressive symptoms when DBS stimulators were turned off (Bewernick et al. 2010), illustrating how the antidepressant effects of nucleus accumbens DBS are profound and yet short-lived. In the future, we can expect refinements in stimulation parameters and localization of DBS thanks to rodent studies which have begun to explore the effects of DBS and optogenetic stimulation (a spatiotemporally precise technique that relies on light-mediated activation of cation or anion channels) on circuit-level neurophysiology and molecular mediators (Gradinaru et al. 2009; McCracken and Grace 2009; Temel et al. 2007). While this approach is still in its infancy, it promises to improve understanding of the dispersed neurocircuitry involved in complex psychiatric symptoms such as anhedonia and may offer insight into how DBS may one day be combined with pharmacological interventions to enhance antidepressant efficacy.

5 Conclusions

Sadly, in spite of almost 40 years of research into depression's mechanisms, the newest agents released on to markets today only vary from their predecessors in side-effect profile, with negligible improvements in efficacy. Therefore, in addition to combining pharmacotherapy with psychotherapy, clinicians are often forced to initiate multiple antidepressant medications simultaneously, or rely on adjunct medications like thyroid hormone, antipsychotic agents or psychostimulants to boost the antidepressant response, with each additional medication coming at the expense of new off-target effects. From the examples discussed above, there is a diverse array of useful animal models that can expand our understanding of mechanisms in depression. Rather than advocate for a single "best" model,

investigators must realize the relative strengths and limitations of each paradigm and always aim to utilize tools that advance our understanding of the disease. While there are examples of "simple" tests that have provided key molecular insights (Berton et al. 2007; Svenningsson et al. 2006), there have been other instances when more "sophisticated" models have only provided behavioral minutiae (Avgustinovich et al. 2005). To increase the likelihood that these models will provide the next generation of effective antidepressants, the approach to the utilization of animal models must mature.

The overarching goal should be to narrow the gap between basic and clinical fields of investigation, and this can be executed at several different levels. Neuroplastic changes that reliably occur in rodents following stress or antidepressant exposures can be explored in human postmortem samples, with replications providing a further validation and increasing knowledge of biomarkers in depression. When examining genes of interest, instead of focusing on behavioral phenotypes in constitutive knockout mice, efforts should be focused on recapitulating human polymorphisms in those genes, understanding their cellular and physiological consequences and advancing models to tease out more subtle phenotypes. Moreover, given the significance of *gene* × *environment* interactions in the pathogenesis of virtually all psychiatric disorders (Caspi and Moffitt 2006; see also Lesch 2011), we can gain insight into their neurobiological basis by recapitulating such interactions in animal models (Carola et al. 2008). Rather than emphasizing the more traditional "treatment versus control" approach, focusing on *individual differences* will increase the understanding of biological mechanisms underlying such variability, including a role for epigenetic mechanisms. Finally, while clinicians continue to refine novel experimental treatments for depression such as intravenous ketamine or DBS, basic scientists must complement their efforts by exploring the neurobiological mechanisms underlying those treatments; such *translational* approaches will further narrow the gap between human depression and the theoretical formulations of its mechanisms.

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Animal Models of ADHD

A. Bari and T.W. Robbins

Contents

Abstract Studies employing animal models of attention-deficit/hyperactivity disorder (ADHD) present clear inherent advantages over human studies. Animal models are invaluable tools for the study of underlying neurochemical, neuropathological and genetic alterations that cause ADHD, because they allow relatively fast, rigorous hypothesis testing and invasive manipulations as well as selective breeding. Moreover, especially for ADHD, animal models with good predictive validity would allow the assessment of potential new therapeutics. In this chapter, we describe and comment on the most frequently used animal models of ADHD

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that have been created by genetic, neurochemical and physical alterations in rodents. We then discuss that an emerging and promising direction of the field is the analysis of individual behavioural differences among a normal population of animals. Subjects presenting extreme characteristics related to ADHD can be studied, thereby avoiding some of the problems that are found in other models, such as functional recovery and unnecessary assumptions about aetiology. This approach is justified by the theoretical need to consider human ADHD as the extreme part of a spectrum of characteristics that are distributed normally in the general population, as opposed to the predominant view of ADHD as a separate pathological category.

Keywords ADHD · Atomoxetine · Attention · Behavioural models · Fronto-striatal loops · Genetic models · Hyperactivity · Impulsivity · Psychostimulants

Abbreviations

5-CSRTT	Five-choice serial reaction time task
5-HT	Serotonin
6-OHDA	6-Hydroxydopamine
ACg	Anterior cingulate cortex
ADHD	Attention-deficit/hyperactivity disorder
CPT	Continuous performance task
DA	Dopamine
DAT	Dopamine transporter
DAT1	Dopamine transporter gene 1
DAT-KO	Dopamine transporter knock-out
dB	Decibels
DRD4	Dopamine receptor D4
DRL	Differential reinforcement of low rates of responding
DSM-IV	Diagnostic and statistical manual of mental disorders 4th edition
FCN	Fixed consecutive number
GH	Genetically hypertensive rat
ICD-10	International classification of diseases 10th revision
IL	Infralimbic cortex
LHT	Lever-holding task
ms	Milliseconds
NA	Noradrenaline
NAc	Nucleus accumbens
NHE	Naples high-excitability
OFC	Orbitofrontal cortex
PD	Post-natal day
PFC	Prefrontal cortex

PrL	Prelimbic cortex
SHR	Spontaneously hypertensive rat
SNAP-25	Synaptosomal-associated protein 25
SSRT	Stop-signal reaction time
SST	Stop-signal task
TRbeta 1	Thyroid hormone receptor beta 1
WK	Wistar-Kyoto

1 Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a complex developmental condition mainly characterized by symptoms of impulsivity, hyperactivity and inattention (American Psychiatric Association 2000). It is one of the most common psychiatric disorders diagnosed in childhood with a worldwide prevalence estimated to be 8–12% of the population (Faraone et al. 2003). Possible adverse consequences of having ADHD include, among others, drug abuse, delinquency, anxiety and depression, high risk for school failure, social rejection and poor self-esteem (Barkley 1997b; Biederman 2005). As such, this disorder places an enormous burden in terms of healthcare cost, familial stability and academic outcomes.

1.1 Aetiological Mechanisms and Pathophysiology

To date, the aetiological mechanisms of ADHD are obscure, although the genetic influence is estimated to be rather strong, probably involving small effects of several interacting genes (Faraone et al. 2005) that determine the severity of symptoms. ADHD children are often described as hyperactive, impulsive, with a short attention span and easily distractible, although the hyperactivity often disappears during adulthood. High rates of comorbidity are also characteristic of ADHD, which is often accompanied by reading disabilities, anxiety and conduct disorders (Sobanski 2006; Spencer 2006).

Children with ADHD have a much higher probability of having close relatives with psychopathology than control children even though it is difficult to separate the genetic contribution from the shared environment. For example, less educated parents are more likely to provide a poorly structured educational environment, which could exacerbate ADHD symptoms in their offspring. On the other hand, lower levels of parental education may be the consequence of ADHD symptoms during childhood, revealing the genetic origin of the disorder in their children. Environmental risk factors include brain traumas during infancy, or perinatal period, alcohol and drug abuse during pregnancy, autoimmune diseases and bacterial brain

infections, heavy metal poisoning and psychosocial adversity (Biederman et al. 1995; Mick et al. 2002; Sprich-Buckminster et al. 1993). However, ADHD research points to heritability as the most common cause of the disorder, with some environmental and familial factors influencing how the symptoms are expressed (Biederman 2005; Wallis et al. 2008).

Patients with ADHD often present brain abnormalities especially of the right prefrontal cortex (PFC), basal ganglia and cerebellum (Giedd et al. 2001). These structures are modulated by dopamine (DA) and noradrenaline (NA), which are implicated in the pathophysiology of ADHD (Faraone and Biederman 1998). DA is usually associated with reward, learning and motor functions, while NA regulates arousal, attention and mood. The most prescribed medications to ADHD patients are psychostimulant drugs, which have a "paradoxical" calming effect on these patients (Bradley 1937). Low to moderate doses of amphetamine or methylphenidate increase the synaptic availability of DA and NA by blocking degradation and reuptake, promoting release and synthesis (Oades 1987).

Broad cognitive domains that appear disrupted in children with ADHD comprise self-regulatory functions such as attention, working memory and action selection/inhibition, as well as affective decision-making and the processing of temporal information (Barkley 1997a; Nigg 2005). However, it is not yet clear what are the precise neuropsychological deficits that characterize this complex and multifactorial condition.

1.2 Single and Multiple Deficit Approaches

It is evident that a broad range of cognitive functions are affected in ADHD and debate continues as to whether the study of behaviourally defined developmental disorders should be looking for a single cognitive deficit that can explain the behavioural symptoms or follow a multi-deficit approach (Applegate et al. 1997; Barkley 1997b; Barkley and Biederman 1997; Pennington 2006; Routh and Roberts 1972; Shaffer and Greenhill 1979; Solanto et al. 2001; Sonuga-Barke 2002).

The first approach leads to the creation of different subtypes based on the main behavioural features displayed by the affected individuals and, in its extreme form, to considering them as separate disorders (Lahey and Applegate 2001; Milich et al. 2001). An example of this approach is to be found in the current criteria listed in the DSM-IV for the diagnosis of ADHD (Table 1). The usual practise is to subdivide ADHD patients into three subgroups: predominantly impulsive/hyperactive, predominantly inattentive and combined subtype (American Psychiatric Association 2000).

A multi-deficit approach, instead, should consider ADHD as being determined by the interaction of various cognitive disabilities, probably sharing overlapping pathophysiological mechanisms which affect a large range of cognitive functions, as well as different brain regions and neurotransmitter systems (Solanto et al. 2001). The distinction between single- and multiple-deficit approaches is also relevant for the treatment of ADHD which to date has not taken into account the possibility of a targeted intervention based on the particular needs of the different diagnostic

Table 1 Examples of DSM-IV criteria for ADHD and behavioural tasks used to assess inattention, hyperactivity and impulsivity in rodents

Examples of DSM-IV criteria for ADHD	Examples of tasks used to asses DSM-IV criteria in animal models of ADHD
(1) Inattention	
• Often has difficulties sustaining attention in tasks or play activities	• 5-choice serial reaction time task (accuracy)
• Often has difficulties organizing tasks and activities	• Visual timing task
• Is often easily distracted by extraneous stimuli	• Cross-modal divided attention task
(2) Hyperactivity/impulsivity	
Hyperactivity	
• Often fidgets with hands or feet or squirms in seat	• Locomotor boxes
• Often runs about or climbs excessively in situations in which it is inappropriate	• Open field
• Is often "on the go" or often acts as if "driven by a motor"	• Làt maze
• Often talks excessively	• Circular corridors
Impulsivity	
• Often blurts out answers before questions have been completed	• 5-choice serial reaction time task (premature responses)
• Often has difficulty awaiting turn	• Stop-signal task
• Often interrupt or intrudes on others (e.g. butts into conversations or games)	• Lever holding task
	• Differential reinforcement of low rates of responding (DRL)
	• Delay discounting
	• Fixed interval/extinction schedule

subtypes. The same consideration applies to the development of animal models, which often feature only one particular aspect of the pathology in the model organism or in the behavioural or cognitive construct under test. The creation of animal models featuring multiple deficits of ADHD is now possible and encouraged thanks to the advances in our understanding of the heterogeneity and polygenic nature of this condition. On the other hand, more selective approaches could be also advantageous in that they simplify the pathology under investigation through the study of specific endophenotypes and provide a parsimonious method for testing pharmacological interventions and causal hypotheses (Gottesman and Gould 2003).

1.3 Categorical and Dimensional Approaches

Another distinction that has to be made between different approaches in the study and treatment of ADHD, as well as for many neuropsychiatric conditions, is between dimensional and categorical conceptualizations of the pathology, which are fundamentally equivalent and interchangeable in theory, but not in practise (Kraemer et al. 2004). A dimensional (or trait) approach would consider ADHD individuals as representing the extreme end of a continuum of cognitive and

behavioural functioning, as opposed to a categorical view, which assumes that ADHD is a discrete disorder. The alternative view is based on a certain number of diagnostic criteria (see Table 1), which define the presence of the pathology, but without taking into account different degrees of severity (Lubke et al. 2007; Polderman et al. 2007). These two approaches are complementary in that the former can inform decisions about the need and the amount of clinical intervention, as well as help drawing conclusions from experimental evidence, while the second serves to establish some cut-off points useful for the diagnosis and inclusion criteria in scientific research.

Studies using a variety of statistical approaches (Gjone et al. 1996; Hudziak et al. 1998; Levy et al. 1997; Lubke et al. 2009) support the view of ADHD as a continuum, which justifies the emerging tendency towards the use of animals selected from a normal population as laboratory models of ADHD. These animals usually display extreme behavioural characteristics and are subsequently assigned to a separate experimental group or category for statistical analysis and treatment evaluation purposes, which, in turn, is consistent with a categorical view of the disorder (Neuman et al. 2005; Rasmussen et al. 2004; Todd et al. 2002).

2 Validity and Utility of Animal Models of ADHD

Ideally, a good animal model of ADHD should have all the symptoms present in affected patients as well as respond similarly to the same pharmacological interventions (Davids et al. 2003; Oades 1987). Although the aetiology of ADHD is not yet clear, animal models should be produced by means of experimental manipulations that are similar to already known predisposing risk factors for the development of ADHD such as chemical or physical insults in the newborn, and genetic or environmental alterations. Behavioural impairments should be evident during neuropsychological tasks, exposure to environments and developmental stages in ways that resemble the human condition.

Very few animal models of ADHD have been able to mimic multiple deficits at the same time, but nevertheless their use has proven fruitful. Animal models present many advantages in the study of human diseases because they have a relatively simple nervous system compared to human subjects, their environment is easier to control and their behaviour is easier to interpret and quantify (Sagvolden et al. 2005). Experimentation with animal models has informed ADHD theories on the neurobiological and genetic mechanisms causally underlying this pathology. Moreover, psychopharmacological studies have elucidated the mechanisms of action of various drugs used in ADHD pharmacotherapy and have highlighted the possible risks and long-term side effects of their use.

Animal models of human psychiatric diseases should conform to specific validity criteria (Sarter et al. 1992). These are face, predictive and construct validity. Face validity refers to the observable similarities between the model and the clinical manifestation of the condition modelled. Predictive validity is the capacity of the

model to predict the efficacy of new treatments and the existence of genetic and biological correlates of the disease that had not been observed before. Construct validity pertains to the theoretical status of the model (Willner 1986). The "gold standard" animal model of ADHD, however, has yet to be defined, but many of the models available have shown good face and predictive validity, at least for one or a subset of ADHD symptoms (Sagvolden et al. 2005). Laboratory models of ADHD have been developed mostly in rodents, partly because more is known about their biology and genetics and also because they are less expensive and less complex than non-human primates.

Animal models of ADHD can be grossly divided into groups according to the method used to create or select them from a normal population of animals. In the following sections, after an overview of the laboratory tests most frequently used in animal models of ADHD, we will describe and comment on *behavioural models*, selected for specific deficits on certain behavioural tasks; *genetic models*, which have been created by genetic manipulation or selective breeding and finally *chemically induced* and *physical trauma models,* which are the result of different kinds of experimental brain damage and interference with normal neural development.

3 Assessing ADHD Features in Animal Models

To date there is no physical test for ADHD, consequently the diagnosis is commonly based on behavioural criteria formally described in the diagnostic manuals DSM-IV and ICD-10. These manuals list a range of symptoms grouped by clusters of inattentive, impulsive and hyperactive behaviours, a subset of which have to be displayed by the subject in order to fulfil the criteria for a diagnosis of ADHD (Table 1).

Animal models of ADHD are usually screened for behavioural symptoms that resemble the human condition by the use of laboratory tests, some of which are directly based on those used in clinical neuropsychology and adapted for use in model organisms. Researchers use these tasks to assess the validity of the model according to the defining criteria of ADHD but also to assess behaviours related to, and often co-morbid with, ADHD (e.g. novelty-seeking, learning deficits, anxiety and memory impairments), which are also of particular interest. Moreover, neurochemical and neuromorphological assays, both in vivo and in vitro, are other important ways of validating the model at the physiological level and for gaining new insights into the neural determinants of the observed behavioural deficits.

3.1 Inattention

Attention deficits are a main feature of the ADHD syndrome. It is not clear, however, to what extent these deficits represent an independent or super-ordinate impairment, if they are a consequence of other primary deficits or they simply

co-occur with the other main behavioural symptoms, sharing the same aetiological factor. This is because although signs of inattention and deficits in the continuous performance task (CPT) of attentional performance (Corkum and Siegel 1993) have been largely replicated, it has been difficult to find specific impairments in ADHD when attention has been fractionated into sub-processes such as divided, sustained, and selective attention (Huang-Pollock and Nigg 2003; Huang-Pollock et al. 2005). However, it has been shown recently that ADHD subjects may have spatially asymmetric selective attention deficits (Chan et al. 2009) and are impaired in tasks assessing more generalized alertness levels (Johnson et al. 2008). Thus, as we will see for animals, detecting attentional impairments in human ADHD crucially depends on the nature of the task used and the specific construct under investigation.

One of the most frequently used tests of sustained attention in rodents is the five-choice serial reaction time task (5-CSRTT), which was developed partly with the aim of investigating and better understanding the deficits underlying ADHD (Carli et al. 1983; Robbins 2002). The basic task is modelled on the CPT (Beck et al. 1956; Wilkinson 1963) used to study human attentional processes. The rat version of the task requires animals to monitor a horizontal array of five apertures and to withhold from responding until the onset of a stimulus, which is a brief flash of light presented pseudo-randomly in one of the five holes. Then, the animal has to make a nose-poke response in the spatial location where the stimulus was presented in order to receive a food reward (Fig. 1). Generally, the accuracy of stimulus discrimination provides an index of attentional capacity, while premature responses – made before the presentation of the stimulus – are regarded as a form of impulsive behaviour and hence a failure in impulse control (Bari et al. 2008; Robbins 2002). This is a considerable advantage of the 5-CSRTT because the capacity to measure

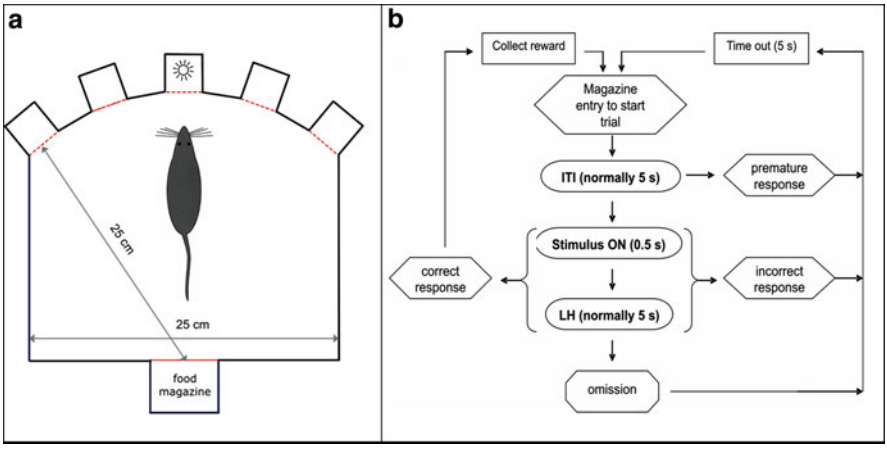

Fig. 1 (**a**) Schematic representation of a 5-choice operant chamber, (**b**) Diagram representing the sequence of events during a standard trial of the 5-CSRTT pictures are taken from (Bari et al. 2008)

both impulsivity and attentional performance in the same setting is a parsimonious way of assessing the validity of animal models on multiple deficits avoiding the possible confound of generalization between different task environments.

Attention can also be measured by visual timing tasks (Broersen and Uylings 1999; Granon et al. 1998), cross-modal divided attention paradigms (McGaughy et al. 1994) and others, although sometimes there is no clear consensus on the construct measured by a particular task (Bushnell 1998).

3.2 Hyperactivity

Hyperactivity in animal models of ADHD is usually measured by observing and quantifying motor activity levels (i.e. horizontal locomotion and vertical rearing) in disparate settings. Most commonly used apparatus for this purpose are activity chambers equipped with infrared-emitting diode/photocell arrays, which are able to automatically count beam breaks caused by the movements of the animal. Circular corridors (Le Moal et al. 1975) and the "Làt maze" (Sadile et al. 1986) have also been extensively used for the same purpose. As suggested by Solanto (1998, 2000), a good animal model should display elevated spontaneous locomotor activity over the 24 h, which returns to normal levels after low doses of psychostimulants, but without any tolerance, sensitization, long-term effects or difference between acute and chronic administration of the drug. However, locomotor hyperactivity is not expressed in novel situations by children with ADHD (Sleator and Ullmann 1981; Whalen and Henker 1976), but more often in potentially boring and non-stimulating environments.

3.3 Impulsivity

Impulsivity has perhaps been the most explored construct in the behavioural neuroscience of ADHD. Although impulsivity is generally considered to be a multifactorial trait (Evenden 1999), the behavioural dimension of inhibitory control lies at its core and is regarded by many as the cardinal feature of ADHD (e.g. Barkley 1997b; Nigg 2001). Impulsive behaviour is characterized by deficient inhibitory processes, that is, by a difficulty in withholding inappropriate, pre-potent responses or thoughts even in the face of punishment or long-term negative outcomes. In children with ADHD, impulsivity is often manifested as the inability to wait in a variety of situations and the tendency to interrupt others' conversations or games, or to respond before the end of the question. One of the most disruptive characteristics is the inability to control one's emotions resulting in exaggerated affective reactions.

In the theoretical formulation of Barkley (1997b), behavioural inhibition comprises three interrelated processes: inhibition of a pre-potent response, stopping an ongoing response and interference control. More recently, Kipp (2005) distinguished between behavioural and cognitive inhibition (plus resistance to interference) in the

context of developmental psychology, referring to the suppression of inappropriate motor responses and thoughts, respectively. The same distinction, but with different implications, can be made in a cognitive perspective between the inhibition, through executive control processes, of motor responses (e.g. withholding or stopping a response) and motivational and affectively charged processes (e.g. deferred gratification) (Castellanos et al. 2006; Nigg 2005; Zelazo and Mueller 2002). Accordingly, inhibition in humans and other animals is most commonly operationalized by tasks measuring motor inhibition and delay discounting, respectively.

Inhibitory processes subserve executive functions such as working memory, self-regulation of affect, motivation and arousal (Barkley 1997b) and are assessed by the ability to withhold or stop an on-going response, all processes that are particularly disrupted in ADHD (Adams et al. 2008; Alderson et al. 2007; Schachar et al. 2000). The stop-signal task (SST, Logan et al. 1984) and the Go/No-Go task (Pennington and Ozonoff 1996; Trommer et al. 1988) are the most commonly used behavioural paradigms for measuring the speed and efficacy of response inhibition in children. Of the various forms of impulsivity, deficits in inhibitory processes as assessed by these tasks – especially by the SST – are among the most replicated findings in children with ADHD (Alderson et al. 2007; Castellanos et al. 2006; Lijffijt et al. 2005; Oosterlaan et al. 1998; Schachar et al. 2000). Therefore, the adaptation of these two tasks for use in animals represents an enormous advance in the development and validation of animal models of ADHD (Eagle et al. 2008a; Eagle and Robbins 2003; Feola et al. 2000; Winstanley et al. 2006).

In both the SST and the Go/No-Go task, the subject is required to produce a motor response (e.g. lever or button press) upon the presentation of a "Go" signal, which is usually a visual stimulus. In a subset of trials, a "Stop" or "No-Go" signal (either visual or auditory) is presented, and the subject has to inhibit the "Go" response, which is made prepotent by its higher rate of appearance and/or by time

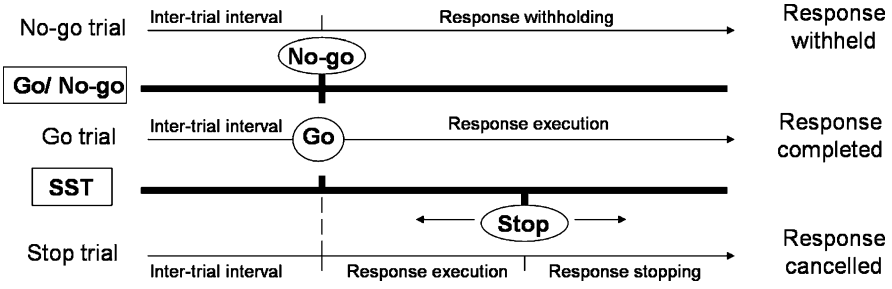

Fig. 2 Schematic diagram representing the typical course of events during correct trials in the Go/No-go task and SST. Trials are preceded by a short interval (inter-trial interval, ITI) then in Go trials (centre line, the same for the two tasks) an imperative stimulus is presented which signals the animal to start the response. Go/No-go task: during No-go trials, the No-go signal is presented concurrently, or instead of, to the Go signal, thus the animal has to withhold from responding. SST: during Stop trials, the Stop-signal can be presented at different times post-stimulus, but only after the Go signal, hence, during the execution of the action which has to be cancelled in order for the animal to obtain a food pellet

constraints. The important difference between the two tasks is in the timing of the "Stop" signal, which is presented shortly *after* the "Go" signal, therefore closer to response execution, only in the SST. By contrast, in the Go/No-Go paradigm, the "No-Go" stimulus is usually presented *concurrently* with, or instead of, the "Go" signal (Fig. 2).

This feature of the SST allows the estimation of the speed of the inhibitory process, the stop-signal reaction time (SSRT), from the distribution of the reaction times during go trials and the probability of inhibition, through the implementation of the "race" model (Fig. 3, see Logan 1994). Varying the delay at which the "Stop" signal is presented, it is possible to bias the outcome of the trial in favour of the go or the stop process, which are assumed to be mutually independent and to "race" one another: the fastest process wins the race determining whether the action is executed or not. The rodent version of the SST has shown good validity in being able to replicate certain findings from human experimentation in both the neurochemical (Bari et al. 2009; Eagle et al. 2007) and the neuroanatomical (Eagle et al. 2007; Eagle and Robbins 2003) domains, plus informing modern theoretical accounts of the neurobiological systems involved in behavioural inhibition (Eagle et al. 2009; Robbins 2007).

Delay discounting paradigms are also very important in ADHD research. According to Nigg (2005), it would be possible to distinguish a delay aversion subtype of ADHD, since some patients show deficits in the signalling of delayed reward and are strongly driven by immediate gratification (Sonuga-Barke 2002, 2005). However, the construct needs further validation in humans, while encouraging results come from animal models of ADHD (Johansen et al. 2005; Sagvolden et al. 2005) and from psychopharmacological studies of the efficacy of ADHD drugs (Robinson et al. 2008) on measures of delay aversion. In the delay discounting

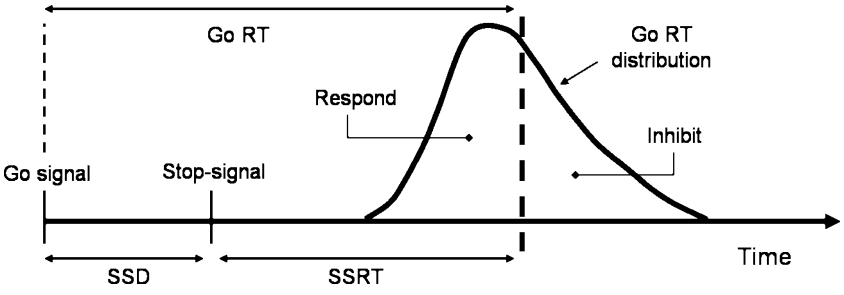

Fig. 3 The curve over the time line represents the distribution of reaction times (RT) on go trials. On stop trials, a tone occurs after the Go stimulus at a particular delay. The stop signal reaction time (SSRT) is the time necessary to cancel the ongoing action and divides the go RT distribution in two parts. The left part represents the responses that were faster than the inhibitory process and thus escape inhibition. The right part represents the probability of inhibiting the response at that particular stop signal delay (SSD). Note that by changing the delay at which the stop signal is presented, the SSRT will intersect the RT distribution at different time points increasing or decreasing the probability to inhibit/respond

task, impulsive choice is usually defined as the preference for a smaller immediate reward over a larger but delayed one (Fig. 4). Choosing the small, immediate option leads to a net loss in the long run and hence the subject has to be able to delay gratification in order to maximize the quantity of rewards obtained (see Mar and Robbins (2007) for the detailed procedure in the rat). Humans and other animals show a similar pattern of temporal discounting for rewards, which can be well represented by a hyperbolic function (Fig. 5). In rodents, drugs commonly used in ADHD pharmacotherapy have been shown to reduce impulsive choice in the delay discounting paradigm (Adriani and Laviola 2004; Robinson et al. 2008).

Other paradigms used to assess impulsivity in rodents are behavioural tasks in which the subject has to perform an action for a predetermined number of times – or to withhold from responding for a predetermined amount of time – before emitting the response that will be rewarded. Any premature behavioural output is considered

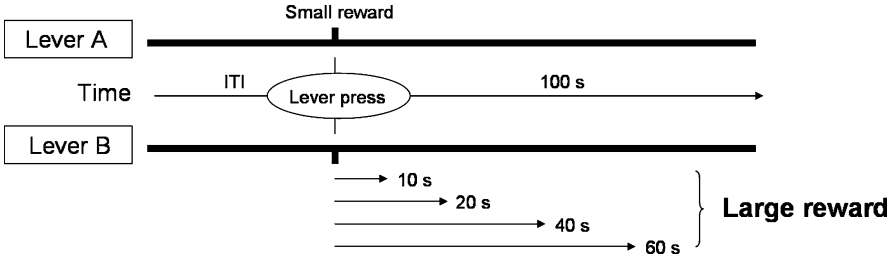

Fig. 4 Schematic diagram representing typical course of events during correct trials in the delay discounting task. Rats have to choose between a small reward delivered immediately (lever A in the example) and a large reward delivered after a certain amount of time (lever B)

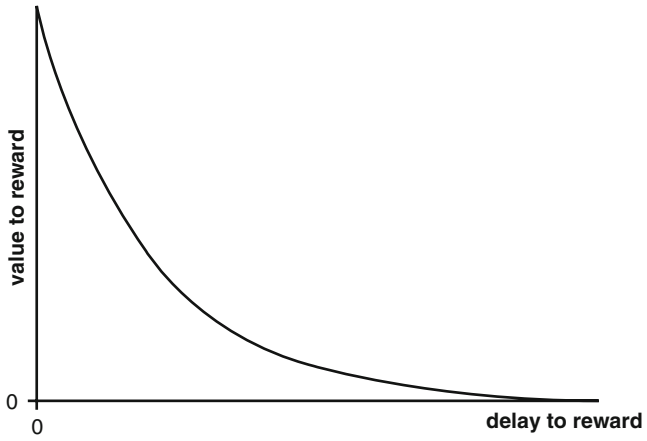

Fig. 5 Hyperbolic curve representing the rate of reward discounting as a function of the reward delay following a choice response

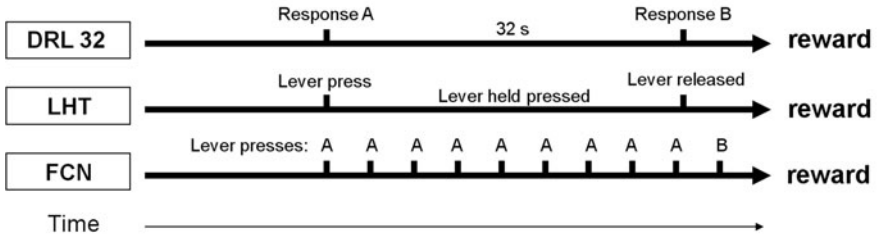

Fig. 6 Schematic diagram representing the typical course of events during correct trials in the DRL, LHT and FCN schedules

as an impulsive response. Examples of this kind of tasks are the differential reinforcement of low rates of responding (DRL) schedule, the lever-holding task (LHT) and the fixed consecutive number (FCN) schedule (Fig. 6). These tasks tax also cognitive processes that are often disrupted in ADHD such as time estimation, resistance to interference and organization of motor behaviour (Barkley 1997b; Evenden 1999; Smith et al. 2002).

In DRL schedules (Ferster and Skinner 1957), a response is rewarded only if made after a pre-determined amount of time has elapsed since the last response. Available studies on DRL yielded contrasting results in ADHD sufferers (Avila et al. 2004; Daugherty and Quay 1991; Gordon 1979) as well as in rodent models of ADHD (Bull et al. 2000; Sanabria and Killeen 2008). In the LHT (Ferguson and Paule 1996; McClure et al. 1997), the subject is required to hold a lever or a button pressed down for a pre-determined amount of time before releasing it in order to be rewarded. FCN schedules (Evenden 1998) require a certain number of responses in one location, before producing a single response in a different one, in order to receive reward. Premature interruption of these chains of responses is usually punished with a short time-out (e.g. 5 s of darkness).

Finally, Sagvolden and colleagues assess impulsivity in the spontaneously hyperactive rat by measuring bursts of lever presses with short inter-response times in the fixed interval/extinction schedule of reinforcement (Johansen et al. 2002; Sagvolden et al. 2005).

4 Dimensional (trait) Models

In animals, as in humans, inter-individual differences in the performance on a neuropsychological task can be very marked and represent different levels along a continuum. When performance levels are low compared to the mean of the population under examination, it is plausible to think of a deficit in the system responsible for the poor performance, which, in turn, represents a “biomarker” of the pathology or a pathological trait itself. A defective system can benefit from an appropriate pharmacological intervention, which may have different effects on a fully functional one, due to ceiling effects for the neuropsychological measure, or to

intrinsic limitations of the system itself. Psychostimulants, for example, modulate brain circuitries differently in ADHD compared to control individuals, according to the brain imaging studies (e.g. Vaidya et al. 1998). The different modulation of brain areas between normal and ADHD individuals after drug administration is likely to reflect the underlying neurochemical and neuroanatomical abnormalities of the patients, who presumably need different degrees of brain activation when compared to control subjects during the same task. However, some ADHD drugs such as methylphenidate and atomoxetine improve performance also in normal subjects, although this improvement often depends on task difficulty or pre-existing differences among individuals (e.g. Chamberlain et al. 2006; Clatworthy et al. 2009; Elliott et al. 1997; Turner et al. 2003b). This would be consistent with ADHD being the extreme manifestation of a condition which is distributed as a continuum in the general population (Levy et al. 1997; Lubke et al. 2009) and, at the same time, justifies the use of subjects drawn from a normal population as animal models of ADHD.

Behavioural deficits in animal models are often induced by means of invasive manipulations that are, however, unlikely to represent the aetiological factor of the pathology under examination. Especially in ADHD, for which the causal mechanisms are still not known, a valuable approach to model specific symptoms of the disease in animals would be to select from a general (outbred) population subjects that deviate negatively from the average performance of that population on a behavioural measure of interest and use them as animal models (Rivalan et al. 2009).

Rats achieving poor levels of attentional performance on the 5-CSRTT have been proposed as a model of ADHD (Barbelivien et al. 2001; Puumala et al. 1996). These rats are not hyperactive, but show impulsivity – as measured by the number of premature responses – as well as low levels of accuracy. On the other hand, rats specifically selected for high levels of premature responses (high-impulsive rats) in the 5-CSRTT have altered characteristics such as lower dopamine D_2 receptor density in the nucleus accumbens (NAc), but not in the dorsal striatum, compared to non-impulsive rats (Dalley et al. 2007). In fact, both dopaminergic and noradrenergic neurotransmission have been shown to play an important role in the expression of impulsivity in the 5-CSRTT (Cole and Robbins 1987; Pattij et al. 2007; van Gaalen et al. 2006), although NA seems more related to resistance to interference in this task (Carli et al. 1983). The differential behavioural analysis of high and low performers on the 5-CSRTT and the use of task manipulations that challenge specific executive functions (e.g. long or variable inter-trial interval, short stimulus duration, etc.; see Bari et al. 2008) have been validated in part by studies showing the sensitivity of these models to drugs used in the treatment of ADHD such as atomoxetine and methylphenidate (Bizarro et al. 2004; Blondeau and Dellu-Hagedorn 2007; Navarra et al. 2008; Puumala et al. 1996; Robinson et al. 2008) and by their susceptibility to compulsive drug self-administration (Belin et al. 2008). Also in human patients, methylphenidate and atomoxetine improve performance in tasks analogous to the 5-CSRTT, such as CPT and the rapid visual information processing task (Boonstra et al. 2005; Chamberlain et al. 2007; Kupietz and Balka 1976; Lawrence et al. 2005; Losier et al. 1996).

Rats that show slow inhibitory processes, as measured by the SST, display differential responses to drugs used in ADHD pharmacotherapy if compared to "fast stoppers" in the same task. Thus, amphetamine and modafinil speed stopping processes only in "slow stoppers" and have limited effects on fast-stopping animals; methyphenidate improves action inhibition in slow stoppers, but impairs it in fast stoppers and, finally, atomoxetine speeds SSRT in all animals independent of baseline performance (Eagle and Robbins 2003; Eagle et al. 2007; Robinson et al. 2008). Many of these effects closely resemble findings obtained in human subjects (Chamberlain et al. 2006; Turner et al. 2003a). Other drugs such as guanfacine (Muller et al. 2005) and citalopram (Chamberlain et al. 2006) do not affect SSRT speed in human volunteers, neither in rats (Bari et al. 2009), confirming the strong predictive validity of the rodent version of the SST.

Few attempts have been made in modelling DSM-IV criteria of ADHD, but promising results in this direction have been obtained by ourselves and other groups. Blondeau and Dellu-Hagedorn (2007) modelled in rats the characteristics of different ADHD subtypes using a cluster analysis based on attention (correct responses) and impulsivity (premature responses) scores on the 5-CSRTT. The clusters of animals identified by this technique showed varying levels of performance when challenged with different doses of methylphenidate and atomoxetine.

In our laboratory, we used a similar procedure to differentiate three subpopulations of rats (efficient, inattentive and impulsive-inattentive) based on their performance on two slightly different versions of the SST. Rats were tested on a version of the task where the stop-signal was very salient (~80 dB, 200 ms) and used the SSRT obtained as an index of impulsivity. In the modified version, the attentional load of the task was dramatically increased by shortening the stimulus duration down to 5 ms and, in this case, the stop accuracy (percentage stop trials inhibited) was used as the attentional correlate. Both measures (impulsivity and attention) were then introduced in a cluster analysis. The three subgroups identified in this way (efficient, inattentive and impulsive-inattentive) clearly showed different patterns of behaviour when tested for anxiety, novelty seeking and exploratory activity. When challenged with the wake-enhancing drug modafinil, only impulsive-inattentive animals showed a faster SSRT compared to the other groups (Bari and Robbins, unpublished findings). These preliminary data confirm previous findings in healthy subjects (Turner et al. 2003a) and rats (Eagle et al. 2007) giving support to the model as a valid alternative to the median split method that is used to differentiate subjects according to just one behavioural dimension.

5 Genetic Models

ADHD has been associated with polymorphisms in several genes, most of which are implicated in catecholaminergic neurotransmission such as the dopamine transporter (DAT) (Lim et al. 2006), the dopamine D_4 receptor (DRD4) (Faraone et al. 2001; LaHoste et al. 1996), and the dopamine beta-hydroxylase (Roman et al. 2002)

genes. Moreover, ADHD is one of the most heritable psychiatric disorders (Faraone et al. 2005), suggesting that good animal models of ADHD should have heritable phenotypic traits.

5.1 Inbred Strains

One of the most used animal models of ADHD is the spontaneously hypertensive rat (SHR) an inbred strain derived from the Wistar-Kyoto (WK) rat, for which may exist very different sub-lines, thus complicating its use as an appropriate control strain (Kurtz and Morris 1987). Initially selected for hypertension (Okamoto and Aoki 1963), the SHR showed unexpectedly high levels of hyperactivity and impulsivity that develop over repeated testing and are particularly evident in settings with low rates of reinforcement (Bull et al. 2000; Moser et al. 1988; Sagvolden 2000; Sagvolden et al. 2005). Similarly to ADHD patients, the SHR is more sensitive to immediate reward and less sensitive to delayed reward (Sagvolden et al. 1992; Sutherland et al. 2009), and shows learning and memory deficits (Wyss et al. 1992). These traits are present before the SHR develops hypertension and remain stable during adulthood (Adriani et al. 2003). NA concentrations and tyrosine hydroxylase gene expression have been found to be higher (de Villiers et al. 1995; Reja et al. 2002), while DA efflux is reduced in the striatum of SHR compared to WK rats (Linthorst et al. 1991), but see also (Heal et al. 2008). Moreover, SHR brain displays neuroanatomical abnormalities similar to ADHD patients (Bendel and Eilam 1992) and altered densitites of DAT (Leo et al. 2003; Watanabe et al. 1997), which can explain the reduced responsiveness to psychostimulants (Russell et al. 2005). Also serotonin (5-HT) neurotransmission seems to be altered in the SHR (Pollier et al. 2000; Stocker et al. 2003), although 5-HT is not considered to be central to the phenotype of this animal model (Russell et al. 2005). Despite its high face validity, SHR model does not show cognitive deficits across all behavioural tasks of impulsivity and sustained attention (e.g. van den Bergh et al. 2006), and the ability of drugs used in the treatment of ADHD to improve performance in this animal model has been inconsistent (Bizot et al. 2007; Hand et al. 2009; Sagvolden 2006; van den Bergh et al. 2006; Wultz et al. 1990) critically depending on the behavioural test employed and on the rat strain used as control (Alsop 2007; Heal et al. 2008; Russell 2007; Sanabria and Killeen 2008). For example, SHR are more impulsive in tasks such as DRL, LHT, fixed interval/extinction schedules and delayed reinforcement (Fox et al. 2008; Johansen et al. 2007; Orduna et al. 2009; Sagvolden 2000; Sagvolden et al. 1998), but are not impaired in tasks of sustained attention such as the 5-CSRTT and visual discrimination procedures (Thanos et al. 2010; van den Bergh et al. 2006) when compared to normotensive rats. It has been noted that the WK rat, usually employed as control strain, is hypoactive thus exaggerating the differences in activity levels compared to the SHR (van den Bergh et al. 2006), which would influence the results in a variety of tasks. Alsop (2007) demonstrated that differences in baseline response rate accounted for

differences between the two strains in tasks of impulsivity and attention. In summary, the large behavioural (Alsop 2007) and genetic (Festing and Bender 1984; Johnson et al. 1992) differences compared to the SHR make the WK rats unsuitable as a control strain for both behavioural and physiological experiments.

The genetically hypertensive (GH) rat (Smirk and Hall 1958), another hypertensive inbred strain phenotypically similar to, but genetically distinct from the SHR, has been recently described to have high levels of impulsivity in a delayed reinforcement task compared to control animals (Sutherland et al. 2009). The GH rat needs further behavioural investigation in order to be established as a model of ADHD.

Naples high-excitability (NHE) rats are selected for their high levels of activity in the Làt maze. They show DA D_1 receptor down-regulation and increased DAT and tyrosine hydroxylase in the PFC suggesting that NHE rats' impaired attention and hyperactivity may be caused by hyperfunctioning of the mesocorticolimbic DA system (Aspide et al. 1998; Sadile et al. 1993; Viggiano et al. 2002). Interestingly, Granon et al. (2000) showed beneficial effects on attentional performance after D_1 agonist (SKF 38393) microinfusion in the PFC of Lister hooded rats tested in the 5-CSRTT. Thus, NHE rats could serve as a useful model for the study of DA D_1 receptors involvement in attentional processes.

The inbred acallosal mouse strain I/LnJ is hyperactive in the open field and spends more time in the central sector of the arena (Magara et al. 2000). However, not much is known about its neurochemical profile and, although neuroimaging studies have consistently shown callosal abnormalities in ADHD (Luders et al. 2009; Valera et al. 2007), the total absence of the corpus callosum in this model is certainly an extreme phenotype.

5.2 Knockout and Transgenic Animals

Mice lacking the DAT (DAT-KO) are hyperactive in new environments and display learning and memory deficits (Gainetdinov and Caron 2000, 2001; Giros et al. 1996). DAT-KO mice are characterized by high levels of extracellular DA in the striatum and reduced phasic DA release (Gainetdinov et al. 1999a). Hyperactivity in this model is reduced by psychostimulants and serotonergic agents (5-HT transporter inhibitor fluoxetine, 5-HT receptor agonist quipazine and 5-HT precursors), but not by NA reuptake inhibitors (Gainetdinov and Caron 2001; Gainetdinov et al. 1999b). This has been considered as evidence that in this model psychostimulants do not decrease hyperlocomotion directly by enhancing catecholaminergic neurotransmission, but by modulating the serotonergic system. Although drugs targeting the 5-HT system have proven of limited use in the treatment of human ADHD, a 5-HT_{2A} receptor gene polymorphism has been associated with this condition (Quist et al. 2000) and antagonists of this receptor ameliorate DAT-KO mice deficits (Barr et al. 2004), thus it might be worth investigating the potential of 5-HT receptors knockout mice as genetic models of ADHD (e.g. Dalton et al. 2004).

Despite the high face validity of this animal model of ADHD, it also displays characteristics that do not resemble the human condition, such as growth retardation and premature death (Gainetdinov et al. 1998; Giros et al. 1996). Moreover, it is not clear yet whether ADHD is characterized by increased or decreased DAT function, and its availability in the human brain has been shown to significantly change in response to drug medication (Cheon et al. 2003; Jucaite et al. 2005; Krause et al. 2000). This latter point well exemplifies the complexity of using evidence from human genetic studies to create valid animal models.

The Coloboma mutant mouse, produced by neutron irradiation, bears a mutation of the synaptosomal-associated protein 25 (SNAP-25) gene and has been proposed as a model of ADHD (Barr et al. 2000). SNAP-25 is an important protein required for exocytotic neurotransmitter release and protein translocation, and SNAP-25 gene polymorphisms have been associated with human ADHD (Feng et al. 2005; Kustanovich et al. 2003; Mill et al. 2002). Coloboma mice display impulsivity in a delayed reinforcement task (Bruno et al. 2007) and their hyperlocomotion is decreased by amphetamine but not methylphenidate (Fan et al. 2010; Hess et al. 1996; Wilson 2000). It has been suggested that the hyperactive phenotype of this model is caused by an imbalance between noradrenergic hyperfunction and dopaminergic hypofunction (Jones and Hess 2003; Jones et al. 2001a, b; Russell 2007), and that its hyperactivity is dependent on NA neurotransmission (Jones and Hess 2003). The coloboma mouse is a promising model of ADHD and recent reports are indicating possible pathways to test its predictive value suggesting new pharmacological targets for the treatment of ADHD (Fan et al. 2010).

Although ADHD has been repeatedly associated with DRD4 gene polymorphisms (Faraone et al. 2005), DRD4-knockout mice are not often used as animal models of ADHD (Mill 2007). This is because they display an essentially hypoactive phenotype and supersensitivity to various drugs of abuse (Rubinstein et al. 1997), characteristics that do not resemble human ADHD. However, the combined use of neonatal dopamine depletion (described below) and DRD4-knockout animals yielded interesting results. Avale et al. (2004) found that DRD4-knockout mice did not show hyperactivity after dopamine depletion as did wild-type controls and that DA D_4 antagonist administration prevented wild-type dopamine depleted animals from developing the hyperactive phenotype. Taken together, these results confirm the importance of DRD4 in the expression of hyperactivity when the dopaminergic system is compromised, although it is not clear how to compare this phenotype with the human polymorphism associated with ADHD. Moreover, DRD4-knockout mice are not more impulsive than wild-type animals in the delay discounting and Go/No-go tasks, but only show increased novelty-seeking, (Helms et al. 2008) confirming the limitations of the use of these knockout mice as a model of ADHD.

Recently, a genetic mouse model based on the deletion of the beta2-subunit of the nicotinic receptor has been described as having ADHD-like behavioural inflexibility and inhibitory deficits (Granon et al. 2003). Considering a reported association between polymorphisms of nicotinic receptor subunit and ADHD (Todd et al. 2003), high incidence of cigarette smoking in individuals with ADHD (Wilens et al. 2008)

and the beneficial effects of nicotine administration on attentional function (Levin and Rezvani 2002), further investigation of this model is warranted (Granon and Changeux 2006).

Male transgenic mice expressing a mutant form of the human thyroid hormone receptor (TRbeta1) display increasing hyperactivity over time, impulsivity and inattention (McDonald et al. 1998; Siesser et al. 2006). Thyroid hormone controls the development of brain areas involved in the regulation of executive functions (Bernal 2002; Thompson and Potter 2000). This genetic model shows increased striatal dopamine turnover and hyperactivity that is reduced by methylphenidate administration (Siesser et al. 2006).

6 Chemically Induced Models

Despite its high heritability, some forms of ADHD are thought to be caused by heavy metal exposure (Braun et al. 2006; Nigg 2006; Nigg et al. 2008) or drug intoxication (Button et al. 2005; Linnet et al. 2003; Streissguth et al. 1994) during pre-, peri- or post-natal developmental. Such environmental factors may have teratogenic effects on the foetus during pregnancy, which will lead to ADHD symptoms in the offspring (Linnet et al. 2003).

6.1 Toxin Exposure

Since exposure to chemicals during pregnancy represents an independent risk factor for the development of ADHD (Biederman et al. 1995; Mick et al. 2002), it could be useful to create animal models that simulate such conditions. For example, the finding of ADHD-like cognitive deficits in animals exposed to nicotine before birth (Paz et al. 2007) gives strength to the conclusion that maternal smoking during pregnancy increases the risk of developing ADHD in children (Fung and Lau 1989; Milberger et al. 1998; Richardson and Tizabi 1994).

Prenatal ethanol exposure (Fahlke and Hansen 1999; Hausknecht et al. 2005) causes ADHD-like symptoms in animals. Rats prenatally exposed to ethanol display dysregulation of dopaminergic neurons in the ventral tegmental area, which is normalized by amphetamine and methylphenidate administration (Shen and Choong 2006; Xu and Shen 2001).

Heavy metal exposure in early ontogeny causes hyperactivity, which improves after acute amphetamine or methylphenidate administration. In animals exposed to lead, 5-HT and DA turnover is decreased in PFC and striatum, respectively (Silbergeld and Goldberg 1974, 1975). Other heavy metals such as manganese and cadmium are believed to cause similar effects as lead exposure in producing hyperactive rats (Kostrzewa et al. 2008; but see Zimering et al. 1982).

6.2 Brain Lesion Models

Neuroimaging studies have repeatedly shown brain morphological and functional abnormalities in patients with ADHD (Seidman et al. 2005). Fronto-striatal networks are thought to be dysfunctional in ADHD, probably as a consequence of altered monoaminergic transmission. Most common structural abnormalities have been found for the cerebellum, the splenium of the corpus callosum, the right striatum and cortical structures mainly in the right hemisphere (Valera et al. 2007). The prevalent idea is that hypofunctional cortical areas do not adequately regulate subcortical structures, causing a dysregulation of behavioural and cognitive functions (Fineberg et al. 2009). Thus, interfering with structures and neurotransmitter systems that are dysfunctional in ADHD individuals is a powerful tool for testing the behavioural consequences of these interventions. The use of this method of creating animal models of ADHD can (1) inform on the cognitive functions subserved by the lesioned area, (2) help develop a better understanding of this disorder by parcelling the ADHD syndrome into more discrete deficits and (3) test the effects of ADHD drugs on a compromised system for which the pathogenetic process is known. However, animal models created by the use of neurotoxins do not inform at all about the causes of ADHD, but are useful tools for studying the contribution of specific brain areas or circuits to cognitive processes that are affected by this pathology.

The most widely used neurodevelopmental model of ADHD created by lesioning brain systems is the neonatal 6-hydroxydopamine (6-OHDA) lesioned rat. Shaywitz et al. (1976a, b) showed for the first time that rats lesioned 5 days after birth with intracerebroventricular infusion of 6-OHDA apparently display hyperactivity and other cognitive deficits that are attenuated by acute administration of amphetamine. Further research demonstrated that the behavioural phenotype also improves after methylphenidate administration (Davids et al. 2003), and that these animals have decreased striatal DAT density, increased DRD4 expression and 5-HT system alterations resembling the human condition (Dougherty et al. 1999; Hanna et al. 1996; Kostrzewa et al. 2008; LaHoste et al. 1996; Zhang et al. 2001). These rats show patterns of hyperactivity similar to childhood ADHD in that their hyperlocomotion is less evident in novel environments and then increases with repeated exposures to the testing apparatus (Archer et al. 1988a; Luthman et al. 1989), but are not impulsive. Thus, the 6-OHDA model has a good face validity, but lacks construct validity because is unlikely that an almost complete destruction of the dopaminergic system accurately mirrors the neurochemical pathology of ADHD. Nevertheless, this model could provide important insights into the relationship between monoaminergic alterations in ADHD and the mechanisms underlying hyperactivity. For example, the lack of effect of selective DAT blockers on hyperactivity suggests that psychostimulants may exert their action mainly on other monoamine transporters in this model (Russell et al. 2005; Zhang et al. 2002). Accordingly, 5-HT and NA transporter inhibitors, as well as DRD4 receptor antagonists, greatly reduce hyperactivity in 6-OHDA lesioned rats (Davids et al. 2002; Zhang et al. 2002).

Another developmental ADHD model is based on effects of lesioning the cerebellum at post-natal day 1–12 which causes cerebellar stunting and different levels of behavioural deficits depending on the method used (Archer et al. 1988b; Ferguson 1996). Early exposure to methylazoxymethanol acetate, dexamethasone or trans-retinoic acid all produce cerebellar damage and hyperactivity partly mimicking the human condition (Ferguson et al. 1996, 2001; Holson et al. 1997). However, these models have not been extensively used in ADHD research.

Lesions of specific brain areas have also been used in conjunction with behavioural tests of impulsivity and inattention. The effects of such lesions are consistent with the different involvement of partially segregated fronto-striatal "loops" (Alexander et al. 1990), which subserve executive functions and are thought to be dysfunctional in ADHD (Fig. 7). In the 5-CSRTT, lesions in rat anterior cingulate (ACg) and prelimbic (PrL) cortex impaired sustained attention (Muir et al. 1996), while infralimbic (IL) lesions increased impulsive premature responding: damage to the orbitofrontal cortex (OFC) caused perseverative responding (Chudasama et al. 2003). In the same task, lesions of the medial striatum impair response accuracy and increase both premature and perseverative responses (Rogers et al. 2001), while NAc core lesions increase impulsivity without effects on response accuracy (Christakou et al. 2004). Moreover,

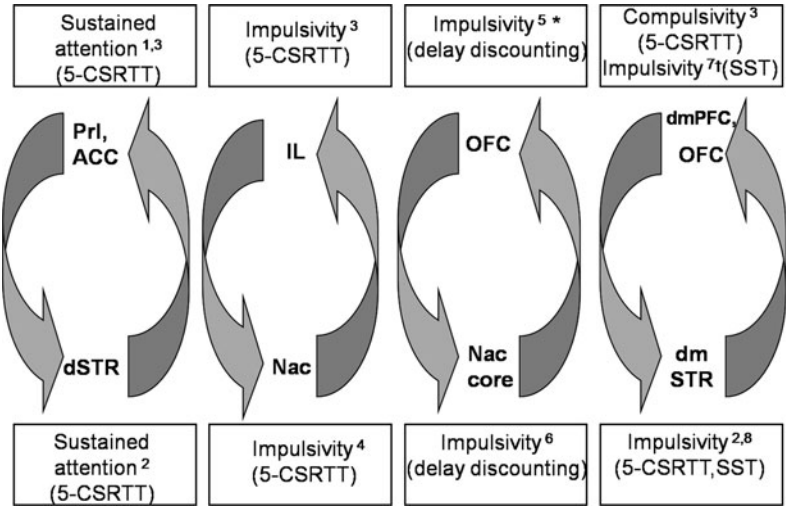

Fig. 7 Hypothesized cortico-striatal circuits subserving different cognitive functions and the processes affected by lesioning specific cortical and subcortical structures in the rat. References: *1* Muir et al. (1996); *2* Rogers et al. (2001); *3* Chudasama et al. (2003); *4* Christakou et al. (2004); *5* Mobini et al. (2002); Winstanley et al. (2004) *asterisk* indicates depending on procedure used; *6* Cardinal et al. (2001); *7* Eagle et al. (2008b), *Dagger* denotes Bari and Robbins unpublished; *8* Eagle and Robbins (2003). Abbreviations: *ACC* Anterior cingulate cortex, *d* Dorsal, *dm* Dorsomedial, *IL* Infralimbic, *Nac* Nucleus accumbens, *OFC* Orbitofrontal cortex, *STR* Striatum

depleting brain 5-HT by intracerebroventricular infusion of 5,7-dihydroxytryptamine during adulthood produces long-lasting hyperactivity and impulsivity in the 5-CSRTT, but not attentional impairments (Harrison et al. 1997; Robbins 2002). Finally, attentional deficits with no increase in impulsivity have been obtained by globally interfering with cholinergic neurotransmission and, under certain conditions, with the noradrenergic system (Carli et al. 1983; Cole and Robbins 1987; Muir et al. 1994; Robbins et al. 1989). Using the SST, Eagle et al. (2008b) showed that lesions of the OFC impair stopping performance in the rat, while Bari et al. (unpublished) found that reversible inactivation of dorso-medial PFC by local infusion of muscimol selectively slowed SSRT. Moreover, SSRT is slower in rats with medial striatal lesions (Eagle and Robbins 2003), a region in receipt of OFC afferents, probably homologous to the caudate nucleus in humans, which has been shown to be smaller or dysfunctional in ADHD patients (Filipek et al. 1997; Rubia et al. 1999). In a delay discounting paradigm, Cardinal et al. (2001) showed that lesions of the NAc core render animals less tolerant to delays, leaving intact the preference for the large reward when no delays are interposed, while OFC lesions increased impulsivity in one study (Mobini et al. 2002), but not in another using a similar paradigm (Winstanley et al. 2004). In the latter study, basolateral amygdala lesions were shown to induce impulsive choice similar to that of the NAc core lesion.

Collectively, these results confirm the hypotheses of discrete neuroanatomical substrates being involved in different kinds of impulse control (Fig. 7). Dorsal sectors of the striatum (served by the nigrostriatal DA pathway) and medial portions of the PFC are involved in action restraint/inhibition, while more limbic aspects of the striatum (i.e. NAc, served by the mesolimbic pathway) and of the PFC (i.e. OFC, probably lateral) are important for affectively charged types of behavioural inhibition, such as delay of gratification, as well as the expression of compulsive behaviour.

7 Physical Trauma Models

Physical trauma models of ADHD include neonatal hypoxia and X-ray exposure (Kostrzewa et al. 2008). Neonatal hypoxia is a risk factor for human ADHD (Lou 1996) and causes long-lasting monoaminergic and behavioural dysregulations in the rat brain (Dell'Anna et al. 1991, 1993; Iuvone et al. 1996). Exposure to X-radiation during development damages the hippocampus and causes hyperactivity and learning deficits in rats (Kostrzewa et al. 2008). These models both possess predictive validity since they respond to acute amphetamine administration (Highfield et al. 1998; Speiser et al. 1983), but are created by means of physical insults that are unlikely to be the cause of the majority of ADHD cases in humans.

8 Overview: Comparisons and Future Directions

An ideal animal model should be able to mimic all the main features of the disease, both at the behavioural and at the physiological level (McKinney and Bunney 1969). Although models expressing only one or two symptoms are of great interest and utility, they will not elucidate the full aetiopathology of the ADHD syndrome.

Lesion studies have been able to assign specific roles in the regulation of behaviour to discrete brain areas, circuits and neurotransmitter systems, and to mirror the effects of dysfunctional anatomical loci as seen in humans with ADHD. However, brain structures do not work in a vacuum, and ADHD symptoms are the result of several dysfunctional loci interacting within a neural network to produce the observable behaviour. Nevertheless, the behaviour of the lesioned animal, although induced by an artificial intervention, is functionally equivalent or, in other words, has comparable behavioural consequences to the symptom under investigation and hence has important implications for research on the specific hypothetical construct (Gorenstein and Newman 1980). Not only is important to take into account the interactions between different brain regions and neurotransmitter systems, but also between the behaviour and the context where it is expressed. Juvenile hyperactivity, for example, is situation specific and usually is not expressed in novel or unstructured settings (Whalen and Henker 1976). Hyperactivity is also a very general construct and potentially caused by very different pathophysiological mechanisms. Therefore, we should be critical of models that superficially mimic ADHD symptoms, which probably derive from very different underlying causes (although that may well also be the case for human ADHD). The fact that so many of the symptoms of ADHD can be simulated in so very many different ways highlights the possibility that the syndrome may have multiple aetiologies, which however may impinge on common neural systems, which respond to a common pharmacological treatment, in the case of psychomotor stimulants.

Translational neuropsychological tasks such as the 5-CSRTT, delay discounting task, Go/No-Go and SST are powerful tools for comparing the performance of ADHD patients and animal models in analogous standardized settings and, possibly, after comparable pharmacological treatments. In this regard, animal models have been very useful in understanding the role of the major ascending neuromodulatory systems in the regulation of cognitive processes that are disrupted in ADHD. Both human and animal research has shown that boosting noradrenergic, but not serotonergic, activity by inhibiting its reuptake improves behavioural inhibition as measured by the SST (Bari et al. 2009; Chamberlain et al. 2006; Robinson et al. 2008), while DA and 5-HT preferentially modulate behaviour when there is a need of delaying gratification (Rogers et al. 1999; Winstanley et al. 2006).

Physical trauma and toxin-induced models have not yet been thoroughly investigated. These models are able to mirror adequately only rare cases of ADHD, namely those caused by pre- and peri-natal insults such as maternal substance abuse or hypoxia during delivery, and those caused by environmental toxin contamination.

ADHD has a strong genetic component, but is probably characterized by the products of several interacting genes. Animal models of ADHD offer the unique opportunity to investigate epigenetic factors such as the effects of different environmental manipulations on genetically identical individuals or the effects of repeated pharmacological treatment on gene expression. Measures of hyperactivity, impulsivity and inattention are normally distributed in the general population, and ADHD is regarded by some authors as the extreme end of these quantitative traits (e.g. Levy et al. 1997). It is worth noting that ADHD drugs such as methylphenidate and atomoxetine, as well as the wakefulness-promoting agent modafinil, can improve cognition also in normal subjects (e.g. Chamberlain et al. 2006; Elliott et al. 1997; Robinson et al. 2008; Turner et al. 2003a) (both rodents and humans), suggesting that these drugs affect the same neuropsychological processes in ADHD and healthy individuals, normalizing the performance in patients and boosting cognitive processes in normal subjects. Thus, the effect of these drugs is not qualitatively different in the two groups, confirming the view of ADHD as the extreme manifestation of a continuum which includes "normal" and probably hypoactive individuals as well, at the other extreme.

Modern approaches to modelling ADHD in animals should be directed towards the attempt to isolate the relevant genotype by cross-breeding individuals that show ADHD-like characteristics, after selecting them from a normal outbred population. In order to select the relevant phenotype, several behavioural correlates of the disease should be considered in the same subject. This is not an easy task, but the same problem is present in the clinic also and is exemplified by the poor diagnostic selectivity, which at the moment is based only on behavioural observation. Even the use of standardized neuropsychological tasks tapping into a single behavioural construct has proven somewhat challenging. For example, in children with ADHD, impulsivity on a delay discounting task did not correlate with SSRT (Solanto et al. 2001) – a result recently replicated on rats in our laboratory (Robinson et al. 2009) – but both measures, taken together, are able to accurately classify almost 90% of children with ADHD (Sonuga-Barke et al. 2003).

This evidence points to the existence of different aetiopathological pathways for the development of ADHD symptomatology. Abnormalities in one or several cortico-striatal circuits subserving different cognitive processes could lead to an ADHD phenotype, characterized by either difficulties in deferring gratification or serious problems in behavioural inhibition or both. This could explain the large heterogeneity present in ADHD patients in terms of behaviour and development of comorbid disorders as well as the poor correlation between laboratory measures of impulsivity. A more detailed classification of the disorder not only would be important for choosing the appropriate therapeutic intervention (especially behavioural), but would also help increasing the reliability of laboratory tests and neuroimaging data. To obtain a valid animal model, the ADHD phenotype should be carefully selected through the use of several translational paradigms assessing the main features of the pathology, and then the frequency of gene polymorphisms of subjects showing the desired characteristics can be compared to that of control

animals (Mill 2007). Animal models of ADHD created by these means would possess strong construct validity and a great heuristic potential for ADHD research.

In conclusion, research approaches to animal models of ADHD should begin to accommodate the multiple facets of this syndrome that have thus far been considered mostly in isolation. A strong conceptual background in neurocognitive functions, more refined diagnostic criteria and carefully described clinical subtypes must continue to guide these efforts.

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Mouse Models of Autism: Testing Hypotheses About Molecular Mechanisms

Florence I. Roullet and Jacqueline N. Crawley

Contents

Abstract Autism is a neurodevelopmental disorder that is currently diagnosed by the presence of three behavioral criteria (1) qualitative impairments in reciprocal social interactions, (2) deficits in communication, including delayed language and noninteractive conversation, and (3) motor stereotypies, repetitive behaviors, insistence on sameness, and restricted interests. This chapter describes analogous behavioral assays that have been developed for mice, including tests for social approach, reciprocal social interactions, olfactory communication, ultrasonic vocalizations, repetitive and perseverative behaviors, and motor stereotypies. Examples of assay applications to genetic mouse models of autism are provided.

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Robust endophenotypes that are highly relevant to the core symptoms of autism are enabling the search for the genetic and environmental causes of autism, and the discovery of effective treatments.

Keywords Autism · Behavior · Candidate genes · Communication · Genetics · Mice · Mouse models · Olfactory · Repetitive · Social · Vocalization

Abbreviations

5Htt	Serotonin transporter mutant line of mice
Avpr1b	Arginine vasopressin receptor 1b null mutant line of mice
B6	C57BL/6J inbred strain of mice
BTBR	BTBR T+tf/J inbred strain of mice
Fmr1	Fragile X Fmr1 null mutant line of mice
Nlgn2	Neuroligin 2 mutant line of mice
VPA	Valproic acid [Di-*n*-dipropylacetic acid]

1 Introduction

Autism is a neurodevelopmental disorder that is defined by three behavioral criteria (1) qualitative impairments in social interactions, (2) deficits in communication, including delayed and noninteractive language, and (3) motor stereotypies, repetitive behaviors, insistence on sameness, and restricted interests (Kanner 1943; American Psychiatric Association 1994; Piven et al. 1997; Bodfish et al. 2000; Lord et al. 2000; Dawson et al. 2002; Cuccaro et al. 2003; Frith 2003; Volkmar and Pauls 2003; South et al. 2005; London 2007; Tager-Flusberg and Caronna 2007; Happe and Ronald 2008; Baron-Cohen 2009). Prevalence is currently estimated at 1:100 to 1:150 for autism spectrum disorders, a dramatic increase over the past decade that appears to be related primarily to improved detection (Fombonne 2009; Hertz-Picciotto and Delwiche 2009; King and Bearman 2009). The essential needs, for early behavioral intervention through special education programs for children and social services support for adults, place a high financial burden on society, as well as heavy personal demands on the families of autistic individuals.

No consistent biological markers for autism have been identified across individuals to date. Evidence from small numbers of autistic individuals suggests a variety of indicators, including cortical connectivity abnormalities (Williams and Minshew 2007), minimal activation of the fusiform gyrus and amygdala during social tasks (Pelphrey et al. 2002), insufficient GABAergic inhibitory neurotransmission (McDougle et al. 2005), mitochondrial dysfunctions (Zecavati and Spence

2009), high platelet serotonin (Anderson 2002), autoantibodies (Wills et al. 2009), and cognitive disabilities following prenatal exposure to valproate (Ornoy 2009) and environmental toxins (Halladay et al. 2009).

The causes of autism remain unknown; however, the strongest evidence is genetic. Concordance between monozygotic twins is as high as 90% for autism spectrum disorders, as compared to 5–10% concordance in dizygotic twins and siblings (Abrahams and Geschwind 2008; Lintas and Persico 2009). Male-to-female ratios are above 4:1 (Abrahams and Geschwind 2008). No single gene mutation has been implicated as uniformly causal. Rather, a variety of de novo and familial mutations have been documented, including a large number of genetic mutations and copy number variants, each in a small number of autistic individuals (Abrahams and Geschwind 2008; Cook and Scherer 2008; Levitt and Campbell 2009; Lintas and Persico 2009). Particularly interesting are clusters of candidate genes with similar functions, such as the synaptic cell adhesion protein families of neurexins, neuroligins, shanks, contactins, cadherins, and integrins (Jamain et al. 2003; Laumonnier et al. 2004; Jeffries et al. 2005; Lise and El-Husseini 2006; Autism Genome Project Consortium 2007; Durand et al. 2007; Garber 2007; Moessner et al. 2007; Alarcon et al. 2008; Arking et al. 2008; Jamain et al. 2008; Kim et al. 2008; Lawson-Yuen et al. 2008; Sudhof 2008). Several other neurodevelopmental disorders display comorbidity with autism. Syndromes caused by known single gene mutations, in which a significant proportion of individuals meet the diagnostic criteria for autism, include Fragile X (*FMR1*), Rett (*MECP2*), tuberous sclerosis (*TSC*), Angelman syndrome (*UBE3A*), and Phelan–McDermid syndrome (22q13.3) (Abrahams and Geschwind 2008). Hypotheses which focus on environmental causes, such as immune dysfunction and environmental toxins, are often conceptualized in terms of susceptibility genes and gene × environment interactions (Fombonne 2009; Halladay et al. 2009; Zecavati and Spence 2009).

Biomedical research has benefited from animal models of diseases, which provide translational systems to test hypotheses about causes and to develop treatments. The autism research field is at early stages in the development of appropriate assays with definitive relevance to the features of autism, and appropriate model systems for testing the many hypotheses about genetic and environmental causes of autism. As described in the sections below, several useful assays and model systems are now available. These preclinical research tools offer translational opportunities to test compounds for their ability to reverse autism-relevant behaviors in mouse models. Potential treatment targets include cell adhesion proteins that regulate the formation and development of synapses, intracellular signaling mechanisms mediating synaptic plasticity and pharmacological manipulation of neurotransmission through receptors for GABA, glutamate, serotonin, and oxytocin (Ehninger et al. 2008b).

As in other fields of biomedical research, mouse models for autism are being perfected across the required criteria for use in treatment development. *Face validity* refers to highly analogous endophenotypes in the human disease and the animal model. *Construct validity* refers to the induced cause being nearly identical in the animal models and the human disease. The first step is to introduce the hypothesized

cause of the disease in the model organism. The consequences of the mutation, lesion, toxin, etc. are evaluated with assays that maximize similarities for the human disease and the model organism. *Predictive validity* refers to therapeutic efficacy. Treatments that reverse symptoms in the human disease should similarly reverse symptoms in the model organism.

To evaluate the roles of mutations in candidate genes for producing the symptoms of autism, the same gene that is mutated in an autistic individual is similarly mutated in the mouse genome (see Gondo et al. 2011 for further discussion). Molecular geneticists have generated thousands of lines of mice with targeted single gene mutations or humanized knock-in mutations relevant to human diseases, including autism spectrum disorders and comorbid neurodevelopmental disorders. Given the behavioral criteria for the diagnosis of autism, and the lack of consistent biomarkers, mouse behavioral assays with high face validity to the behavioral symptoms of autism provide the best tools to evaluate the functional outcomes of candidate gene mutations. The same assays can be used to evaluate environmental hypotheses. The fundamental challenge is to design mouse behavioral tasks with sufficient face validity to the core symptoms within each of the three diagnostic behavioral categories of autism (Crawley 2004, 2007a, b).

This chapter describes strategies from our laboratory and others to model the diagnostic and associated symptoms of autism in mice. Phenotypes obtained in mouse models with genetic and environmental manipulations are presented, relevant to several of the proposed molecular causes of autism spectrum disorders. Preliminary findings from mouse models of autism spectrum disorders that use hypothesis-driven treatments, which effectively reversed relevant phenotypes, are discussed.

2 Mouse Behavioral Tasks with Face Validity for the First Diagnostic Symptom of Autism, Qualitative Impairments in Reciprocal Social Interactions

Mice are a social species, which engage in easily scored social behaviors including approaching, following, sniffing, allogrooming, aggressive encounters, sexual interactions, parental behaviors, nesting and sleeping in a group huddle (Grant and MacIntosh 1963; Hofer et al. 2001; Miczek et al. 2001; Carter et al. 1992; Young et al. 2002; Winslow 2003; Terranova and Laviola 2005; Wersinger et al. 2007; Keller et al. 2006; Panksepp et al. 2007; Yang et al. 2007a, b, 2009; Chadman et al. 2008; McFarlane et al. 2008; Paylor et al. 2008; Scattoni et al. 2008a, b). Behavioral assays using dedicated equipment have been developed by our laboratory and others to quantify the types of social interactions that are unusual in autistic individuals. These include low spontaneous seeking of interactions with others, lack of social reciprocity, and failure to develop peer relationships appropriate to developmental ages.

We invented a simple social approach task in an automated three-chambered apparatus, illustrated in Fig. 1a, which compares the time that the subject mouse spends with a novel mouse versus the time that the subject mouse time spent with a nonsocial novel object (Moy et al. 2004, 2007, 2008b; Nadler et al. 2004; Kwon et al. 2006; Mineur et al. 2006; Crawley et al. 2007; Yang et al. 2007a, b, 2009; Chadman et al. 2008; McFarlane et al. 2008; Jamain et al. 2008; Ryan et al. 2008; Page et al. 2009; Radyushkin et al. 2009). If the group of mice spends more time in the side chamber with a novel mouse than time spent in the side chamber with the novel object, then sociability is demonstrated. If time spent in the two side chambers is not statistically different, or time with the novel object is greater than time with the novel mouse, then lack of sociability is demonstrated. A second parameter, time engaged in sniffing the novel object versus time sniffing the novel mouse, is scored by an observer to provide an independent corroborating measure of true social interactions. Most strains of mice spend more time with the novel mouse, representing normal sociability (Moy et al. 2007, 2008a, b; McFarlane et al. 2008; Yang et al. 2009). Equal time spent with the novel mouse and the novel object would represent impaired sociability in a mouse model of autism. Less time spent with the novel mouse than with the novel object may be analogous to the tendency of autistic children and adults to engage in nonsocial activities such as playing exclusively with a favorite toy train, rather than with the other children or adults in the room.

The social approach task has been applied to investigate many lines of mice with mutations in candidate genes for autism, ranging from synaptic genes such as neuroligins to cancer genes such as *Pten*, and to genes associated with neurotransmission, including the serotonin transporter, GABA receptor subunits, vasopressin receptors, oxytocin, and vasoactive intestinal peptide, as well as to inbred strains of mice such as BTBR T+tf/J and BALB/c that display low social approach (Bolivar et al. 2007; Brodkin 2007; Crawley et al. 2007; Chadman et al. 2008; DeLorey et al. 2008; Jamain et al. 2008; Moy et al. 2008a, b, 2009; Stack et al. 2008; Page et al. 2009; Radyushkin et al. 2009; Zhou et al. 2009). Table 1 summarizes findings with this task from selected examples.

Reciprocal social interactions between two or more freely moving mice offer more sensitive assays for some of the specific types of social reciprocity deficits seen in autism spectrum disorders. Interactive sessions between two mice of the same sex and age are conducted in an arena, such as the Noldus Phenotyper 3000 illustrated in Fig. 1c, d. Sessions are videotaped and later scored by an observer, or by videotracking software (Cheh et al. 2006; Bolivar et al. 2007; Yang et al. 2007a, b, 2009; McFarlane et al. 2008). Measures that are reliably scored by human observers include following another mouse, sniffing each other, grooming each other, crawling over and under each other, sitting together in close physical contact, and sleeping together in a huddle. Investigators first define the behavioral parameters of interest, then quantify the number of bouts and/or cumulative time engaged in each behavior, across a test session as short as 10 min. Either reduced interactions or unusual interactions can thus be detected. Dyads of partners can be tested at any age postweaning, and pairs can be composed of any combinations of genotypes, strains, and genders. Representative examples are described in Table 1.

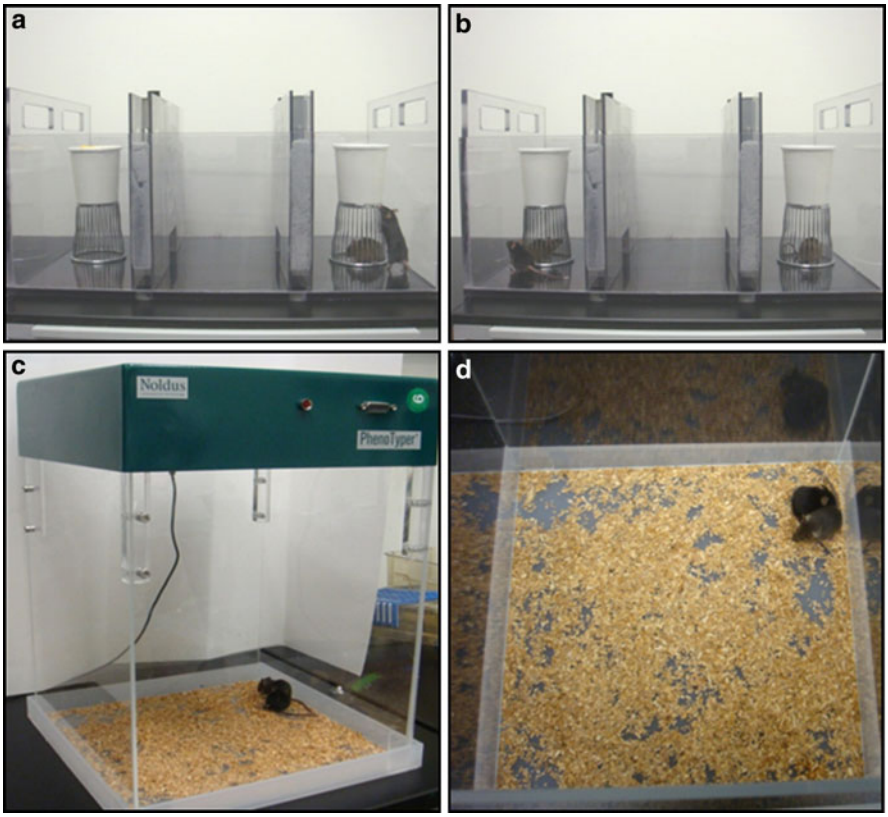

Fig. 1 *Social approach and juvenile play*. *Panel A* illustrates the automated three-chambered apparatus, designed as an initial simple assay for sociability in mice (McFarlane et al. 2008; Moy et al. 2004, 2007; Nadler et al. 2004; Ryan et al. 2008; Yang et al. 2007a, b, 2009). Sets of photocell beams across the entrances between compartments are sequentially broken when the mouse moves between compartments. Software tallies the time spent in each chamber and number of entries into each chamber. The social approach test measures the sociability of the subject mouse as a simple yes-or-no parameter, defined as significantly more time spent in the side chamber containing a novel mouse than in the side chamber containing a novel object during a 10-min test session. The equipment can be used to assess preference for social novelty in a subsequent 10-min test session. Preference for social novelty is defined as significantly more time spent in the side chamber with a second novel mouse than in the side chamber with the first, now familiar, mouse (*Panel B*). Time spent sniffing the novel mouse versus the familiar mouse, or time spent sniffing the second novel mouse versus a familiar mouse, provides a corroborative measure of actual social interaction. Number of entries offers a control measure of general exploratory locomotion. Habituation to the empty test chamber takes place during a 10-min test session immediately before the sociability session. Innate side preference is evaluated during the habituation phase. If the group spends significantly more time on one side during habituation, the room configuration is changed to equalize time in each side chamber during general exploration. *Panels C* and *D* illustrate reciprocal social interactions between two freely moving mice. Reciprocal social interaction parameters provide more sensitive measures of relevant components of interactive sociability. Two juvenile mice, or two adult mice, usually of the same sex, are videotaped during a 10- to 30-min test session in the Noldus Phenotyper 3000 open field apparatus

Social recognition and social memory in mice are evaluated by amount of time spent sniffing a novel mouse upon repeated exposures, to induce familiarity, and reinstatement of high levels of sniffing when a novel stimulus animal is introduced (Bielsky et al. 2004, 2005; Bielsky and Young 2004; Richter et al. 2005; Sanchez-Andrade et al. 2005; Wersinger et al. 2007; Wanisch et al. 2008). Social memory is assayed from videotapes in three-chambered environments, using a time delay of up to 30 min between presentations (Winslow and Insel 2002; Winslow 2003; Young et al. 2002). In the automated three-chambered apparatus illustrated in Fig. 1b, preference for social novelty is assayed by replacing the novel object with a second novel mouse for a 10 min social choice session following the 10 min sociability session (Moy et al. 2007, 2008a, b; Chadman et al. 2008; McFarlane et al. 2008). Representative examples are described in Table 1.

3 Mouse Behavioral Tasks with Face Validity for the Second Diagnostic Symptom of Autism, Communication Deficits

The second category of core symptoms involves language delays, low ability to maintain interactive conversations, strictly literal use of words, and inability to understand nuances such as humor, sarcasm, facial expressions, and body language (Lord et al. 2000; Frith 2003; Tager-Flusberg and Caronna 2007). It will be difficult to design mouse tasks with high face validity to these uniquely human modes of communication. Communication in mice is primarily through olfactory cues (Bowers and Alexander 1967; Doty 1986; Schellinck et al. 1993; Isles et al. 2001; Brennan and Keverne 2004; Keverne 2004; Kavaliers et al. 2005; Wang et al. 2008; Arakawa et al. 2009; Restrepo et al. 2009; Roullet et al. 2010). Ultrasonic vocalizations emitted by mice in some social situations may represent another mode of communication (Maggio and Whitney 1985; White et al. 1998; Branchi et al. 2001; D'Amato and Moles 2001; Hofer et al. 2001; Gourbal et al. 2004; Panksepp et al. 2007; Scattoni et al. 2008a, b, 2009; Wöhr et al. 2010). Social olfactory tasks for mice generally measure sniffing of urinary pheronomones (Humphries et al. 1999; Hurst et al. 2001; Bakker 1994; Brennan and Keverne 2004; Hurst and Beynon 2004; Hurst et al. 2005; Arakawa et al. 2007; Yang and Crawley 2009) and behavioral responses toward scent marks (Cheetham et al. 2007; Arakawa et al. 2007, 2009; Roullet et al. 2010). Olfactory habituation/dishabituation involves presenting a sequence of nonsocial and social cues on cotton swabs and measuring time spent sniffing to same and to novel odors (Chadman et al. 2008;

Fig. 1 (continued) containing a thin layer of clean bedding. The invesigator scores the videotape using Noldus Observer event software for number of bouts and time spent in each behavioral category. Event categories include social behaviors such as sniffing, allogrooming, pushing past with physical contact, and crawling under and over. Control nonsocial behaviors are simultaneously scored, such as locomotion and self-grooming. Publications using this task are described in the text. Photographs contributed by the authors

Table 1 Description of behavioral and biological phenotypes for five mouse models of autism

Mouse model	Phenotypes	References
Fragile X	Fragile X syndrome is the most common genetic cause of mental retardation. *Fmr1* mutant mice deficient in the FMRP protein displayed endophenotypes relevant to components of Fragile X. Phenotypes included elevated locomotor activity in an open field, anxiety-like behaviors in the mirrored chamber test, audiogenic seizures, macroochidism, and low prepulse inhibition of acoustic startle. Attentional deficits and impairment in inhibitory control (impulsivity) were detected in the five choice serial reaction time task. *Fmr1* mutant mice engaged in more sniffing of a familiar partner in the partition test, and won fewer social dominance challenges. Impairments in spatial learning of the Morris water maze were accompanied by resistance to change during the reversal phase. Increased spine density and length were reported for the somatosensory cortex, while reduced dendritic spine density and length were reported for the hippocampus. Long-term potentiation was found to be impaired, and long-term depression enhanced, in *Fmr1* mutant mice.	Bakker (1994), Beckel-Mitchener and Greenough (2004), Bilousova et al. (2009), D'Hooge et al. (1997), Dolen et al. (2007), Errijgers et al. (2008), Lauterborn et al. (2007), Moon et al. (2006), Paylor et al. (2008), Peier et al. (2000), Spencer et al. (2005)
Chr 15q11-13	Deletions and duplications in the 15q11-13 chromosomal region are associated with Angelman's syndrome, Prader–Willi syndrome, and autism. Mice with a paternally transmitted duplication on the homologous region of mouse chromosome 7 displayed impaired sociability in the three-chambered social approach task. Anxiety-like phenotypes were detected on the elevated plus-maze. More ultrasonic vocalizations were emitted by pups separated from their dams, while fewer vocalizations were emitted by adults in the resident-intruder test. PatDp/+ paternal duplication mice were normal on acquisition but failed on the reversal phase in both the Morris water and the Barnes maze, indicating an inability to reverse a spatial habit. Higher freezing in the altered context was reported for fear conditioning, indicating impaired learning and memory.	Nakatani et al. (2009)

Pten	Mutations in phosphatase and tensin homolog deleted on chromosome ten (*PTEN*) are associated with cancers, seizures, macroencephaly, mental retardation, and autism. Mice with a conditional *Pten* mutation on a neuron-specific promoter exhibited seizures, macroencephaly, and dendritic hypertrophy. Increased acoustic startle and lower prepulse inhibition indicated hypersensitivity to sensory stimuli. Social interaction deficits were detected during juvenile reciprocal interactions, sociability in the three-chambered social approach task, preference for social novelty, and nest building. Impaired spatial learning with high thigmotaxis was found in *Pten* mutants in the Morris Water Maze test. Many of the neuroanatomical and behavioral abnormalities in *Pten* mutant mice were reversed by treatment with the mTOR inhibitor rapamycin.	Kwon et al. (2006), Zhou et al. (2009)
BTBR	BTBR T+ tfJ (BTBR) is a genetically homogenous, commercially available inbred strain of mice, included in the top tier of the Mouse Phenome Project (http://phenome.jax.org/). BTBR juveniles and adults display low levels of reciprocal social interactions, lack of sociability in the automated three-chambered social approach task, and reduced social transmission of food preference, as compared to social strains such as C57BL/6J and FVB/NJ. BTBR displays high levels of spontaneous self-grooming both as juvenile and as adults. More frequent and louder ultrasonic vocalizations were emitted by BTBR pups separated from their dams, while fewer vocalizations are emitted by adults in response to female urinary pheronome cues, as compared to C57BL/6J. Measures of general health, motor functions, and sensory abilities including olfaction are normal in BTBR, supporting an interpretation of highly selective abnormalities in traits relevant to the diagnostic symptoms of autism.	Bolivar et al. (2007), McFarlane et al. (2008), Moy et al. (2007), Scattoni et al. (2008a, b), Yang et al. (2007a, b, 2009), Wöhr et al. (2010)

(*continued*)

Table 1 (continued)

Mouse model	Phenotypes	References
Prenatal valproic acid	A small number of cases of autism were linked with valproic acid (VPA) medication taken by mothers during pregnancy. Rats from dams treated with VPA during pregnancy demonstrate reduced social interactions as juveniles, and low sociability as adults on the social approach task. VPA-treated rat pups display delays in olfactory discrimination during nest-seeking behavior. Repetitive/stereotyped patterns of behavior were observed in VPA rats placed in an open field. Lower sensitivity to painful stimuli as measured by tail flick and thermal paw withdrawal tests, but increased sensitivity to nonpainful stimuli as measured in tactile test (von Frey filaments) have been reported. VPA-treated rats display enhanced responses on eye blink conditioning, and impaired spatial learning in the Morris Water maze. Cerebellar pathology, increased complexity of apical dendritic arborization, and reduced excitatory synaptic responses have been observed in VPA-treated rats, as well as increased numbers of NMDA receptors and hyperconnectivity. Altered levels of monoamines, elevated brain serotonin, and disturbance of sleep patterns have been described. Mice treated prenatally with VPA have reduced expression of neuroligin-3, a cell adhesion protein involved in synapse formation, in hippocampus and somatosensory cortex.	Ingram et al. (2000), Kolozsi et al. (2009), Narita et al. (2002), Rinaldi et al. (2007), Rodier et al. (1996), Schneider and Przewlocki (2005), Snow et al. (2008), Stanton et al. (2007), Tsujino et al. (2007), Wagner et al. (2006)

Stack et al. 2008; Yang et al. 2009; Yang and Crawley 2009). Scent marking involves measuring the number of urinary spots deposited by the subject mouse in proximity to urinary spots deposited by another mouse, or to a urine sample placed in an arena by the investigator (Hurst and Beynon 2004; Arakawa et al. 2007, 2008; Wang et al. 2008; Roullet et al. 2010). Open field arenas fitted with specialized urine-absorbing paper and ultrasonic microphones and representative scent markings by a male mouse in response to a spot of female urine in the center of the arena, are illustrated in Fig. 2.

Ultrasonic vocalizations are emitted by mouse pups when separated from the nest, and detected by the parents to locate the straying pup and retrieve it to the nest (Zippelius and Schleidt 1956; Hofer et al. 2001; D'Amato and Moles 2001; Winslow and Insel 2002; Shu et al. 2005; Scattoni et al. 2008a, b). Pup calls in the range of 40–90 kHz are a robust, easily replicated phenomenon in mice, which represent communication in the sense that they elicit a response from the adult parents. However, intentionality on the part of the pup has not been demonstrated. Another issue is face validity. It is not obvious that low numbers of pup vocalizations represent the type of communication deficits seen in autism. Infants later diagnosed with autism display less crying in some cases, but louder and more frequent and inconsolable crying in some cases, and normal crying in other cases (Sheinkopf et al. 2000; Zwaigenbaum et al. 2005). Nevertheless, the number of ultrasonic vocalizations by separated pups has been widely used as an assay for communication in mouse models of autism, as described for some of the examples in Table 1.

Ultrasonic vocalizations in juvenile and adult mice may provide better models for intentional communication in mice. Vocalizations during social interactions by pairs of previously isolated juvenile mice, calls between pairs of adult females and vocalizations emitted by adult male mice sniffing female urine, are being analyzed for call numbers and call properties in mouse models of autism and other neuropsychiatric disorders (D'Amato and Moles 2001; Panksepp et al. 2007; Wang et al. 2008; Scattoni G2B paper if in press). In addition, vocalizations have proven to be a useful measure in mouse models of human speech disorders such as mutations in the *FOXP2* gene (Shu et al. 2005; Fujita et al. 2008; Enard et al. 2009).

4 Mouse Behavioral Tasks with Face Validity for the Third Diagnostic Symptom of Autism, Repetitive Behaviors with Restricted Interests

Stereotyped, repetitive behaviors, and the restricted range of interests and activities that characterize autism (Bodfish et al. 2000; Lord et al. 2000; Cuccaro et al. 2003; Frith 2003; South et al. 2005) are amenable to modeling with available rodent tasks that incorporate reasonable face validity. Mice engage in motor stereotypies including vertical jumping, backflipping, circling, digging, marble burying rearing, repeated sniffing of one location or object, barbering, excessive self-grooming, and excessive running (Creese and Iversen 1975; Turner et al. 2001; Lee et al. 2002;

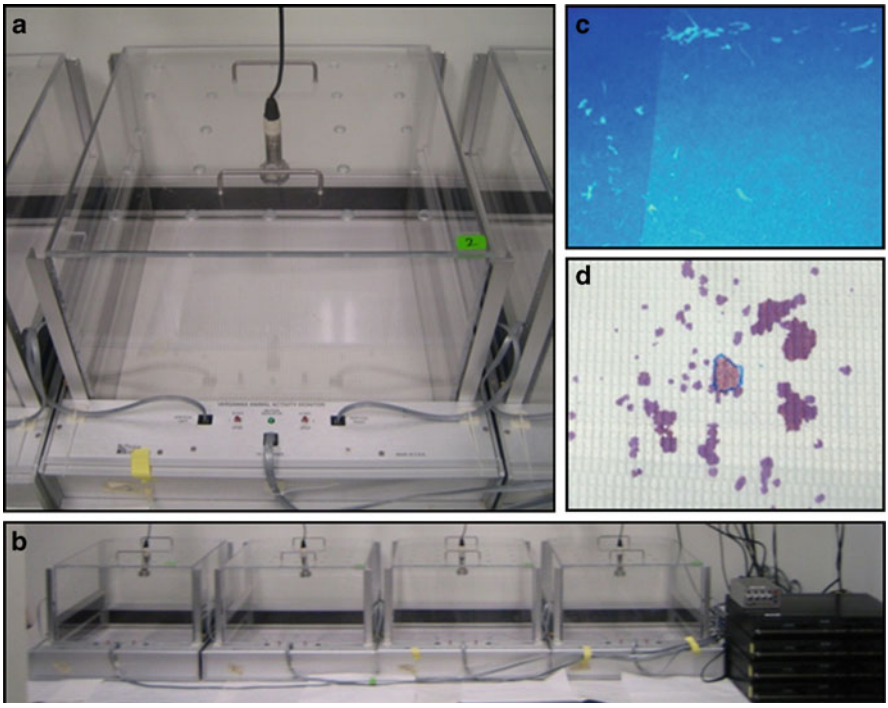

Fig. 2 *Social olfactory scent marking and countermarking*. Figure 2 illustrates our apparatus for measuring urinary scent marking and countermarking behavior in an open field by adult mice. Scent marking in mice is often used as a mechanism for social dominance in marking territory and access to females, and may reflect the ability of mice to interpret and respond correctly to social cues. A sheet of paper with absorbent properties is placed in the bottom of the open field. The male mouse explores an open field in which a drop of urine from an estrus female has been placed in the center of the paper. The male will deposit his urine in spots located in proximity to the female urine sample and throughout the open field. Countermarking is another scent marking test that measures the scent marks deposited by an adult male in the presence of scent marks previously deposited by another male on the paper lining. As shown in *Panels A* and *B*, ultrasonic microphones positioned above each of four open fields simultaneously record ultrasonic vocalizations emitted by the subject mice during the session. At the end of the 5-min test session, the paper is placed under an ultraviolet lamp for visualization and quantitation of the deposited urinary traces (*Panel C*). Alternatively, the sheet of paper is treated with Ninhydrin spray. Scent marks appear as purple spots (*Panel D*). Number and location of scent marks are scored and analyzed for factors including proximity to a female urine spot (*circled in blue*) by the male subject mouse. See text for further descriptions. Photographs contributed by the authors

Pogorelov et al. 2005; Korff and Harvey 2006; Lewis et al. 2007; Crawley 2007b; Welch et al. 2007; Yang et al. 2007a, b; McFarlane et al. 2008, Moy et al. 2008a). Excessive stereotyped grooming was reported for several mouse lines with mutations in developmental genes. *Sapap3* (Welch et al. 2007) and *Hoxb8* knockout mice (Greer and Capecchi 2002) exhibit extreme self-grooming that leads to hair loss and skin injuries. The inbred strain BTBR T+tf/J exhibits the normal pattern of

grooming but at very high levels, representing a prolonged repetitive behavior (Yang et al. 2007a, b, 2009; McFarlane et al. 2008).

Assays for repetitive behaviors include perseverations, such as the inability to change to a spatial habit (Ralph et al. 2001; Brigman et al. 2006; Chen et al. 2007; Moy et al. 2008a). Spatial habits in mice can be induced by first locating the reinforcer consistently in one place, e.g., the food pellet is always in the left arm of a T-maze, or the hidden platform is always in the northwest quadrant of a water maze, during the initial training sessions. If the food pellet is later moved to the right arm of the T-maze, or the hidden platform is later moved to the southeast quadrant of the water maze, most mice can learn to change their spatial habit during the reversal training sessions. A mouse model of autism is predicted to acquire the initial habit but not the reversal. Examples are described in Table 1. An interesting report with a Fragile X model engaged in an operant task indicates a resistance to change, in that general performance was disrupted after an error was made (Moon et al. 2006).

Restricted interest assays for mice are under development. One is a holeboard array, in which mice usually nosepoke into all of the holes in the floor of an open field, including holes baited with various objects and odors (Moy et al. 2008a). A mouse model of autism would be predicted to show nosepoke activity into only one or a small subset of holes, representing restricted interest in one location or type of bait. Spontaneous alternation in a Y-maze, which represents a common exploratory strategy in mice, could be used to measure restricted exploration to only one arm of the Y-maze. Another approach is an attentional task, in which a mouse model of autism would be predicted to excel at maintaining focused attention, and ignoring distractors.

5 Mouse Behavioral Tasks with Face Validity to Associated Symptoms of Autism

Associated symptoms of autism that occur in varying percentages of cases include mental retardation, seizures, anxiety, hyperreactivity to sensory stimuli, and sleep disturbances. Endophenotypes with face validity to these associated symptoms may be easier to model in mice. However, analogs of associated symptoms raise questions about the extent to which they are essential to include in a mouse model of autism. For example, standard anxiety-related tasks for mice include the elevated plus-maze, elevated zero maze, light/dark exploration, emergence test, and Vogel thirsty lick conflict test (Cryan and Holmes 2005; Crawley et al. 2007). Anxiety-related phenotypes have been reported for the *Nlgn2*, *5Htt*, *Fmr1*, *Avpr1b*, and other lines of mice with mutations that may be relevant to autism (Wersinger et al. 2002; Holmes et al. 2003; Spencer et al. 2005; Blundell et al. 2009). Seizures in mice are scored with tonic–clonic rating scales and electroencephalogram recordings. High levels of seizures and seizure susceptibility have been reported for some lines of mice with mutations relevant to neurodevelopmental disorders, e.g., GABA receptor *Gabrb3* subunit and *Pten* knockouts (DeLorey et al. 1998; Zhou et al. 2009).

Compelling findings relevant to associated symptoms offer important leads to pursue, in defining genes underlying a broad set of neurodevelopmental abnormalities that may converge in the etiology of autism. However, at a practical level, traits such as seizures, high anxiety-like behaviors, or circadian disruptions produce confounds in the interpretations of other behavioral findings such as social deficits. For example, mice with high anxiety-like tendencies will remain in the center chamber of the three-chambered automated social approach apparatus and will not explore either the side chamber with the novel mouse or the side chamber with the novel object. Mice experiencing frequent seizures or lack of sleep may similarly be generally inactive in all tasks requiring exploratory locomotion. The role of learning deficits on social behaviors remains to be determined in mice, as well as in humans.

6 Biological Assays

As described above, clinical studies have reported unusual neuroanatomical features in some autistic individuals as compared to typically developing children and adults. Reported differences include larger head circumference at young ages, larger or smaller volumes of cortical gray matter and white matter, in thickness of long cortical connectivity pathways and intrahemispherical connections including the corpus callosum, loss of cerebellar Purkinje cells, reduced amygdala size, and less activation of brain regions during social tasks in fMRI studies (Herbert et al. 2003; Minshew and Williams 2007: Pelphrey et al. 2002; Spezio et al. 2007; Amaral et al. 2008; McAlonan et al. 2008). Analogous morphometric analyses are beginning to be applied to mouse models (Radyushkin et al. 2009). Hypotheses about impairments in synaptic connections, dendritic spines, and electrophysiological measures of synaptic plasticity are being tested in mouse models of neurodevelopmental disorders (Beckel-Mitchener and Greenough 2004; Dolen et al. 2007; Lauterborn et al. 2007; Ehninger et al. 2008a; Sudhof 2008; Zhou et al. 2009). Table 1 and the section below include descriptions of biological and behavioral phenotypes that have been reported in mutant mouse models of autism spectrum disorders.

7 Comprehensive Phenotypes of Selected Mouse Models of Autism Spectrum Disorders

7.1 Fragile X

Fragile X syndrome, the major form of mental retardation with a known genetic basis, is caused by highly expanded CGG trinucleotide repeats within the X-linked *FMR1* gene, an RNA binding protein (Bassell and Warren 2008). Approximately 25% of individuals with Fragile X syndrome also meet the diagnostic criteria for

autism (Abrahams and Geschwind 2008). *Fmr1* knockout mice display several behavioral phenotypes relevant to autism spectrum disorders, depending on the genetic background into which the mutation is bred (Errijgers et al. 2008; Moy et al. 2009). As shown in Table 1, well-replicated phenotypes include hyperactivity, high anxiety-like behaviors, low prepulse inhibition of acoustic startle and mild impairments on water maze learning (Bakker 1994; D'Hooge et al. 1997; Peier et al. 2000; Spencer et al. 2005; Errijgers et al. 2008). *Fmr1* knockout mice display abnormally high densities of long, thin, immature dendritic spines and impaired long-term potentiation (Beckel-Mitchener and Greenough 2004; Lauterborn et al. 2007). Gene therapy with normal human *FRM1* rescued the hyperactivity, prepulse inhibition deficit, anxiety-like behaviors, and the social anxiety-like behaviors in *Fmr1* knockout mice (Peier et al. 2000; Paylor et al. 2008; Spencer et al. 2008). Treatments with minocycline, brain-derived neurotrophic factor, and mGluR5 receptor antagonists reduced the dendritic spine abnormalities and long-term potentiation deficits in *Fmr1* knockout mice (Lauterborn et al. 2007; Dolen and Bear 2008; Bilousova et al. 2009).

7.2 Chromosome 15q11–13 Duplication

Chromosomal duplications and deletions, termed copy number variants, appear with higher frequencies in autism than in the general population (Sebat et al. 2007). The most frequent duplication appears to be at the 15q11–13 locus, and is usually maternally transmitted (Cook et al. 1997; Kwasnicka-Crawford et al. 2007). Mice with 15q11–13 duplications displayed lack of sociability in the three-chambered social approach task, and normal acquisition but failure to reverse a spatial habit in the Morris water maze (Nakatani et al. 2009), as shown in Table 1. Comprehensive analyses of general health, sensory abilities, and motor functions confirmed normal physical abilities, including olfactory (Nakatani et al. 2009). It is interesting to note that paternal transmission of the 15q11–13 duplication in mice produced these autism-relevant phenotypes to a great extent than the maternal transmission of the duplication, in contrast to the maternal duplication of 15q11–13 more frequently causing autism in humans.

7.3 Pten

Phosphatase and tensin homolog on chromosome ten (*PTEN*) is a tumor suppressor gene implicated in cancers (Diaz-Meco and Abu-Baker 2009). *PTEN* mutations additionally result in macrocephaly, and some individuals meet the diagnostic criteria for autism (Varga et al. 2009). Mice with *Pten* null mutations generated with a conditional neuronal promoter displayed macroencephaly, neuronal hypertrophy, poor spatial learning, higher anxiety-like behaviors, higher open field

activity, elevated acoustic startle, and lack of preference for social novelty (Kwon et al. 2006). Signaling proteins downstream from *Pten* include PI3K, AKT, TSC, and mTOR (Zhou et al. 2009). Remarkably, long-term treatment with the mTOR inhibitor rapamycin reversed the neuronal soma hypertrophy, dentate gyrus enlargement, and social interaction deficits in 2-month-old *Pten* knockout mice (Zhou et al. 2009).

7.4 BTBR

A broad survey of inbred strains of mice from the top tier of The Mouse Phenome Project (http://phenome.jax.org/) revealed an obscure strain, BTBR T+tf/J (BTBR), which displayed specific deficits on social approach and high levels of repetitive self-grooming, described in Table 1, along with normal scores on measures of general health, sensory abilities, and motor functions (Moy et al. 2007). Failure to display sociability in the automated three-chambered social approach task has been replicated in multiple cohorts of BTBR, across three laboratories, and in both the light and the dark phases of the circadian cycle (Moy et al. 2007; Bolivar et al. 2007; McFarlane et al. 2008; Yang et al. 2007a, b, 2009). Low reciprocal social interactions have been found in pairs of juvenile BTBR as compared to pairs of juvenile C57BL/6J, a commonly used inbred strain with high social approach (McFarlane et al. 2008; Yang et al. 2007a, b, 2009). Relevant to the second diagnostic symptom of autism, unusual patterns of ultrasonic vocalizations have been reported for BTBR (Scattoni et al. 2008a, b). Relevant to the third diagnostic symptom of autism, normal patterns but very long bouts of repetitive self-grooming in BTBR have been detected in multiple cohorts in various environments (McFarlane et al. 2008; Yang et al. 2007a, b, 2009). Similar social approach deficits and unusual vocalizations have been detected in another inbred strain, BALB/cJ (Brodkin 2007; Panksepp et al. 2007). While inbred strains do not test specific hypotheses about autism candidate gene mutations, they provide opportunities to discover background genes mediating social, communication, and repetitive behaviors. Robust phenotypes in BTBR offer translational tools to evaluate treatments for low sociability and high repetitive behaviors. For example, repetitive self-grooming in BTBR was reduced by acute treatment with an mGluR5 antagonist, MPEP (Silverman et al. 2010).

7.5 Prenatal Valproic Acid

Valproic acid (VPA) is a drug used in the treatment of epilepsy and mood disorder (Ornoy 2009). Administered during pregnancy, VPA can induce fetal valproate syndrome in the offspring, characterized by neural tube defects such as spina bifida, craniofacial abnormalities (Ardinger et al. 1988; Arpino et al. 2000; DiLiberti et al. 1984;

Wide et al. 2004), and behavioral and cognitive dysfunctions associated with autism (Christianson et al. 1994; Moore et al. 2000; Rasalam et al. 2005; Williams et al. 2001; Williams and Hersh 1997). In rodents, prenatal exposure to VPA results in deficit in social interaction, repetitive/stereotyped patterns of behavior, a lower sensitivity to pain but increased sensitivity to nonpainful stimuli, (Schneider and Przewlocki 2005), disturbed sleep pattern (Tsujino et al. 2007), and alterations in eye blink conditioning (Stanton et al. 2007). In addition, rats treated in utero with VPA show cerebellar pathology (Ingram et al. 2000; Rodier et al. 1996), increased complexity of apical dendritic arborization (Snow et al. 2008), elevated levels of brain serotonin (Tsujino et al. 2007), and enhanced hyperconnectivity (Rinaldi et al. 2008). Adult mice previously treated in utero with VPA have reduced neuroligin 3 mRNA expression in some brain areas (Kolozsi et al. 2009). Interestingly, prenatal exposure to VPA produced greater behavioral and physiological abnormalities in male rats than in female rats (Schneider et al. 2008). Behavioral deficits relevant to autism (abnormalities in social behavior, stereotypy) were reversed when VPA-treated rats were exposed to an enriched environment (Schneider et al. 2006).

8 Conclusions

Considerable progress has been made in the translational use of mouse models to investigate molecular hypotheses about the causes of the behavioral symptoms of autism spectrum disorders. The nascent field of mouse models of autism will mature in concert with the clinical field in diagnosing specific symptoms and subcategories of autism. Evaluation instruments for both the mouse models and the human syndrome are in place, but considerably more development and refinement is needed. Interactive conversations between clinical investigators and behavioral neuroscientists will aid the process of generating mouse assays most relevant to the core endophenotypes of autism. Synergistic discoveries of genetic mutations in autistic individuals, genes underlying social, communicative, and repetitive behaviors in mice, and the functional consequences of autism candidate gene mutations in mice, are likely to move the field forward significantly in the next few years.

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Advances in Animal Models of Drug Addiction

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Contents

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Abstract Drug addiction is a syndrome of impaired response inhibition and salience attribution, which involves a complex neurocircuitry underlying drug reinforcement, drug craving, and compulsive drug-seeking and drug-taking behaviors despite adverse consequences. The concept of disease stages with transitions from acute rewarding effects to early- and end-stage addiction has had an important impact on the design of nonclinical animal models. This chapter reviews the main advances in nonclinical paradigms that aim to at model (1) positive and negative reinforcing effects of addictive drugs; (2) relapse to drug-seeking behavior; (3) reconsolidation of drug cue memories, and (4) compulsive/impulsive drug intake. In addition, recent small animal neuroimaging studies and invertebrate models will be briefly discussed (see also Bifone and Gozzi, Animal models of ADHD, 2011). Continuous improvement in modeling drug intake, craving, withdrawal symptoms, relapse, and comorbid psychiatric associations is a necessary step to better understand the etiology of the disease and to ultimately foster the discovery, validation and optimization of new efficacious pharmacotherapeutic approaches. The modeling of specific subprocesses or constructs that address clinically defined criteria will ultimately increase our understanding of the disease as a whole. Future research will have to address the questions of whether some of these constructs can be reliably used as outcome measures to assess the effects of a treatment in clinical settings, whether changes in those measures can be a target of therapeutic efforts, and whether they relate to biological markers of traits such as impulsivity, which contribute to increased drug-seeking and may predict binge-like patterns of drug intake.

Keywords Animal models · Craving · Drug addiction · Drug-seeking · Drug-taking · Impulse control disorders · Relapse · Substance use disorders · Withdrawal

Abbreviations

5-CSRT	5-Choice serial reaction time
6-OHDA	6-Hydroxydopamine
AA	Alko alcohol rats
ADE	Alcohol deprivation effect
ANA	Alko non alcohol rats
APA	American Psychiatric Association
BSR	Brain stimulation reward

CPA	Conditioned place aversion
CPP	Conditioned place preference
CS	Conditioned stimulus
DA	Dopamine
DSM-IV	Diagnostic and statistical manual fourth edition
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
fMRI	Functional magnetic resonance imaging
FR	Fixed ratio
HAD	High alcohol drinking rats
HAP	High alcohol preferring mice
ICD-10	International classification of diseases and related health problems tenth revision
ICSS	Intracranial self-stimulation
LAP	Low alcohol preferring mice
LgA	Long-access escalation
MK-801	(5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclo hepten-5, 10-imine maleate
NAc	Nucleus accumbens
NMDA	*N*-methyl-D-aspartate
OFC	Orbitofrontal cortex
PET	Positron emission tomography
phMRI	Pharmacological magnetic resonance imaging
PR	Progressive ratio
SD	Discriminative stimulus
sP	Sardinian alcohol-preferring rats
VTA	Ventral tegmental area
WHO	World Health Organization

1 Introduction

According to the World Health Organization (WHO), there are about 200 million users of illegal drugs worldwide, which represent 3.4% of the world population. Among other neuropsychiatric diseases, alcohol and drug abuse cost the United States economy an estimated $544.11 billion per year including costs related to crime, loss in productivity, health care, incarceration, and drug enforcement operation (e.g., Uhl and Grow 2004). Recent figures indicate societal costs of about €57.274 billion per year in the European Community (Andlin-Sobocki and Rehm 2005; Andlin-Sobocki et al. 2005). Most recent information from the National Survey on Drug Use and Health (NSDUH 2008) reveals that an estimated 20.1 million Americans aged 12 or older were current (past month) illicit drug users (including marijuana/hashish, cocaine/crack, heroin, hallucinogens, inhalants, or

prescription-type psychotherapeutics used nonmedically), which represents 8.0% of that population. Among Americans aged 12 or older, an estimated 129.0 million (51.6%) reported being current drinkers of alcohol, 58.1 million (23.3%) participated in binge drinking, whereas heavy drinking was reported by 6.9% of that population, or 17.3 million people. Finally, an estimated 70.9 million Americans aged 12 or older (28.4%) were current users of a tobacco product. Overall, an estimated 22.2 million persons (8.9% of the population aged 12 or older) were classified with substance dependence or abuse in the past year based on criteria specified in the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV).

The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) celebrated 15 years of collecting information and reporting on the European drug problem in 2009. With sales of illicit drugs estimated to generate more than €100 billion, Europe has also now become a laboratory to study drug use across culturally different countries by using a transversal drug monitoring system that encompasses 30 countries with a combined population of more than half a billion people. The 2009 estimates of drug use in Europe in the adult population (15–64 years old) show a lifetime prevalence of cannabis of at least 74 million (22.1%), with last-year use and last-month use reported by about 22.5 million (6.8%) and 12 million (3.6%) European adults, respectively. For cocaine, EMCDDA reports a lifetime prevalence of about 13 million (3.9%), with last-year use and last-month use reported by about 4 million (1.2%) and 1.5 million (0.4%) European adults, respectively. Lifetime prevalence of ecstasy use is about 10 million (3.1%), with last-year use and last-month use of about 2.5 million (0.8%) and less than one million European adults, respectively. In addition, lifetime prevalence of amphetamines use is about 12 million (3.5%), with last-year use and last-month use of about two million (0.5%) and less than one million European adults, respectively. Finally, problem opioid users are estimated at between 1.2 and 1.5 million Europeans. Drug-induced deaths in 2009 accounted for 4% of all deaths of Europeans aged 15–39, with opioids being found in around three quarters. Another defining factor in Europe's substance use problem is the concomitant consumption of alcohol across age groups.

Evidence-based addiction medicine originates from different types of studies conducted in various groups or populations of subjects, which may or may not apply to a particular drug user with a particular drug history (e.g., mono- or polydrug use), a specific genetic makeup, and a set of cultural values. Attempting to model addiction-related symptomatology therefore requires a careful examination of the criteria that are currently used to define substance use disorders. Two major versions of formal diagnostic systems have been developed and regularly updated by the American Psychiatric Association (APA) and WHO. The most recent set of diagnostic guidelines published by these organizations are the Diagnostic and Statistical Manual, Fourth Edition (DSM-IV; APA 1994a, b) and the International Classification of Diseases and Related Health Problems, Tenth Revision (ICD-10; WHO 1991). Both of these systems rely on a syndrome-based approach (Edwards 1986) to diagnosing substance use disorders. These diagnostic systems conceptualize substance dependence as a maladaptive pattern of substance use leading to

significant impairment or distress (Table 1). Addiction then can be defined as a reward-seeking activity that, through experience, comes to be given such a high priority that it is maladaptive. Addiction is an acquired chronic disposition to experience powerful motivation to engage in a reward-seeking activity and/or a weakened disposition to exercise inhibition. As such, drug addiction has aspects of impulse control disorders (i.e., increasing sense of tension or arousal before committing an impulsive act; pleasure, gratification, or relief at the time of committing the act, and presence or not of regret, self-reproach, or guilt following the act) and compulsive disorders (i.e., anxiety and stress before committing a compulsive

Table 1 Criteria of substance dependence according to DSM-IV and ICD-10

DSM-IV criteria[a]	ICD-10 criteria[b]
Tolerance, as defined by either: – Need for markedly increased amounts of the substance to achieve intoxication or desired effect – Markedly diminished effect with continued use of the same amount of the substance	Evidence of tolerance, such that increased doses of the psychoactive substance are required to achieve the effects originally produced by lower doses
Withdrawal, as manifested by either: – Characteristic withdrawal syndrome for the substance – The same (or a closely related) substance is taken to relieve or avoid withdrawal symptoms	Physiological withdrawal state when substance use has been reduced or ceased
The substance is often taken in larger amounts or over longer periods than intended	A strong desire or sense of compulsion to take the substance
Persistent desire or unsuccessful efforts to cut down or control substance use	Difficulties in controlling substance-taking behavior in terms of its onset, termination or level of use
A great deal of time is spent in activities necessary to obtain the substance (e.g., visiting multiple doctors or driving long distances), use the substance (e.g., chain-smoking), or recover from its effects	
Important social, occupational, or recreational activities are given up or reduced because of substance use	Progressive neglect of alternative pleasures or interests because of psychoactive substance use, increased amount of time necessary to obtain or take the substance or to recover from its effects
Continued substance use despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance	Persisting with substance use despite clear evidence of overly harmful consequences, depressive mood states consequent to heavy use, or drug-related impairment of cognitive functioning

[a] To meet a diagnosis of *dependence*, an individual would have to endorse three or more of the seven criteria below, with at least three criteria having been experienced within the same 12-month period

[b] To meet a diagnosis of *dependence*, three or more of the six following criteria must have been experienced or exhibited at some time during the previous year

repetitive behavior, and relief from the stress by performing the act). As an individual moves from an impulsive disorder to a compulsive disorder, there is also a shift from a positive to a negative reinforcement process that drives the motivated behavior.

The extent to which nonclinical paradigms can actually model specific DSM-IV or ICD-10 criteria is arguable, but the following sections in this chapter will show that some associations have recently emerged. There are, however, three important concepts that should be mentioned prior to discussing these associations: (1) the validity of the nonclinical models; (2) the too often neglected importance of pharmacokinetics–pharmacodynamic (PK/PD) relationships, and (3) the limitations of the diagnostic classification systems.

First, understanding the underlying nature of addiction in clinical settings is contingent upon the actual validity of the nonclinical model and which aspect of the addiction process is being modeled (Willner 1991). The face validity of nonclinical models typically refers to whether the overt behavioral qualities of the human condition are similar to what is observed in the model (Willner 1991). In other words, the face validity is based on a phenotypic comparison of the animal's behavior with what is seen in human addicts. For example, drug self-administration in nonhuman species is usually referred to as one of the models with the highest degree of face validity because it mimics voluntary drug-taking behavior in humans. Predictive validity refers to whether the behavioral outcomes in the model predict performance in the human condition being modeled (Willner 1991). For example, drug self-administration models are extremely valuable in assessing whether a novel drug possesses abuse liability in humans; these models will then be referred to as having a high degree of predictive validity. Finally, perhaps the most difficult aspect of nonclinical models relates to construct validity that refers to whether there is a sound theoretical rationale linking the human condition and the animal model (Willner 1991). Construct validity is extremely challenging for a disease such as addiction whose etiology is still only partially understood.

Second, animal modeling requires a constant balance between validity, throughput, and reliability. However, the extent to which nonclinical models will further increase our confidence in a potential therapeutic drug target or decrease its associated risks will also depend on the integration of a PK/PD strategy. The latter is essential to aid dose selection for transition to human studies. Thus, the selection of a nonclinical paradigm should also be contingent upon the feasibility of implementing a PK/PD strategy to investigate pharmacological activity in humans that is similar in type and magnitude to that observed in preclinical efficacy models (PD and/or disease model). This strategy should aim at answering the following questions: What is the expected clinical response for the treatment strategy? What is the level of certainty surrounding the predicted response? How do different treatment strategies impact response? What dose is required to achieve a target response? What is the probability of achieving a specific efficacy target while keeping probability for adverse events below a certain level? What is the optimal strategy to balance safety and efficacy?

Third, challenges do also reside on the clinical side of the translational seesaw. In addition to the validity of nonclinical models, one should recognize the limitations of current diagnostic classification systems. For example, criteria for classification only reflect consensus at the time a system of classification is published, and are therefore constrained by the state of scientific knowledge at any given time. Furthermore, there might be considerable heterogeneity among individuals within diagnostic classes, and the prescribed threshold (i.e., dependence defined as the presence of three or more features) for determining the presence versus absence of a disorder is potentially an arbitrary cut-off along a continuous dimension of diagnostic symptomatology. Finally, there is no evidence that all criteria should be given equal weight for a diagnosis, and each criterion may not apply equally across all drug categories.

2 Animal Models of the Positive and Negative Reinforcing Effects of Addictive Drugs

The following sections review the main advances in animal models of drug addiction and discuss how they relate to specific aspects of the disease and the degree to which they serve as a valid index of the human condition (i.e., aspects of validity). It will also be argued that if one takes addiction as a reward-seeking behavior that has become out of control, then its assessment, measurement, and/or modeling should also be undertaken using manifestations of that lack of control.

2.1 Brain Stimulation Reward

Brain stimulation reward (BSR), also referred to as intracranial self-stimulation (ICSS), is a procedure during which steady rates of lever-pressing behavior in rats can be maintained if brief electrical stimulation of the medial forebrain bundle or other rewarding brain sites follows the operant behavior (for a thorough review, see Gardner 2005). The rate–frequency procedure typically involves the generation of a stimulation–response function and provides a frequency threshold measure. The frequency of the stimulation is varied, and the subject's response rate is measured as a function of frequency. The relationship between response rate and pulse frequencies yields a sigmoidal rate–frequency curve. Lateral shifts along the pulse frequency axis in the rate–frequency curve are a selective measure of reward, while vertical shifts provide information on motor/performance capacity. Thus, an increase in the level of BSR current that is required to maintain lever-pressing (i.e., a right-shift along the pulse frequency axis in the rate–frequency curve paradigm) is taken as evidence of decreased sensitivity to brain reward. Conversely, almost all drugs of abuse will lower the BSR threshold or produce a

leftward shift in the rate–frequency curve paradigm. The affective properties of drug withdrawal can also be assessed using ICSS procedures. For example, withdrawal from chronic nicotine produces an increase in BSR threshold (Epping-Jordan et al. 1998; Panagis et al. 2000; for a review, see O'Dell and Khroyan 2009). Similarly, nicotine-dependent mice and rats show an increase in current intensity thresholds during withdrawal (Johnson et al. 2008; Stoker et al. 2008). Thus, by measuring the threshold for BSR, one can either assess the degree to which drugs are rewarding or measure the degree of dysphoria produced by withdrawal from those drugs.

2.1.1 Notes on the Validity of the BSR Paradigm

BSR has relatively low face validity: the paradigm requires refined surgical intervention and does not overtly mimic any component of drug use in humans. Predictive validity is moderate: BSR has the advantage of directly interfacing with brain reward circuits and eliminates any interference with consummatory-like behaviors. In addition, the paradigm is a reliable measure of brain reward: all reinforcing drugs lower the threshold for ICSS and the rewarding value of the drug is predictive of relapse to drug-seeking in humans. However, some brain regions support higher rates of BSR than others and different circuits might be activated by different sites. Furthermore, animals must be trained for several weeks to obtain stable rates of responding or stable thresholds. Finally, construct validity would also appear to be moderate. First, changes in threshold values are a valid measure of drug reinforcement. Second, dopaminergic systems play an important role in drug-induced changes in ICSS. Third, the paradigm mimics negative aspects associated with drug abstinence.

2.2 Behavioral Sensitization and Cue-Conditioned Locomotion

Behavioral sensitization or reverse tolerance to a drug refers to the progressive and persistent increase in that drug effect produced by its repeated, intermittent administration. For example, it has been shown that both direct and indirect dopamine (DA) agonists can elicit a progressive increment of some of their acute behavioral responses (Castro et al. 1985; Hoffman and Wise 1992; Silverman 1991; Stewart and Badiani 1993; Szechtman et al. 1993). The long-term behavioral, neurochemical, and molecular adaptive responses to the repeated administration of these drugs are thought to underlie the addiction process as well as stimulant-induced psychoses (for preclinical evidence, see Paulson et al. 1991; Post and Rose 1976; Robinson and Becker 1986; for clinical evidence, see Lieberman et al. 1997; Sato 1986). Although the neural basis of behavioral sensitization to DA agonists still remains to be completely elucidated, considerable progress has been achieved in the phenomenological characterization of the sensitization phenomenon at both the behavioral and

the neurochemical levels. The repeated administration of psychostimulants produces persistent adaptations within the mesolimbic DA system (Kalivas and Stewart 1991; Robinson and Becker 1986), and the resulting molecular and neurochemical changes may lead to behavioral sensitization, which in turn has been linked theoretically with the addiction process (for thorough reviews, see Robinson and Berridge 1993, 2001, 2008). It is believed that an increase in extracellular DA in the somatodendritic region of the DA neurons in the ventral tegmental area (VTA) (Cador et al 1995; Kalivas and Stewart 1991; Kalivas and Weber 1988; Vezina 1993) initiates the necessary pre- and postsynaptic processes for behavioral sensitization to the systemic administration of DA agonists to be expressed at terminal regions of the DA neurons in the nucleus accumbens (NAc) (Kalivas and Duffy 1990, 1993; Parsons and Justice 1993; Patrick et al. 1991; Pettit et al. 1990; Robinson et al. 1988; Segal and Kuczenski 1992a; but for time-dependent changes in DA levels in response to the acute vs. repeated administration of DA agonists, see Heidbreder et al. 1996; Kuczenski and Segal 1989; Segal and Kuczenski 1992b; Weiss et al. 1992). Mesolimbic DA activity also underlies the reinforcing properties of DA agonists, which can be sensitized when these drugs are either injected repeatedly or self-administered (Horger et al 1990; Lorrain et al. 2000; Shippenberg and Heidbreder 1995; Vezina et al. 1999, 2002). Recent evidence suggests that behavioral sensitization can be observed in humans (for reviews, see Leyton 2007; Sax and Strakowski 2001; Vezina and Leyton 2009). For example, drug-associated behaviors are sensitized following repeated exposure to amphetamine (Strakowski and Sax 1998; Strakowski et al. 1996). Furthermore, expression of neurochemical sensitization is observed following repeated administration of amphetamine (Boileau et al. 2006; Cox et al. 2009; but see Narendran and Martinez 2008).

Cue-conditioned locomotion is based on Pavlovian conditioning. In this procedure, rats are typically injected with a drug and immediately placed in individual locomotor activity cages (experimental environment), which the animals presumably perceive as an environment distinct from their home environment. The procedure is repeated once a day for 4–5 days and locomotor responses are recorded during each session. Upon repeated administration of the drug in the experimental environment, a progressive behavioral sensitization develops, which is typically reflected by a progressive enhancement of the locomotor response to the drug (see above). This phenomenon is also referred to as context-dependent behavioral sensitization and may have a role in the development of compulsive drug-seeking behaviors (Robinson and Berridge 2001, 2008).

2.2.1 Notes on the Validity of the Behavioral Sensitization and Cue-Conditioned Locomotion Paradigms

The extent to which these paradigms have translational value to the design of clinical studies is questionable. First, few controlled human studies of this process have been published in the literature. Second, in those published studies, some self-reported subjective responses might be sensitized whereas others seem to

exhibit tolerance; the reasons underlying this differential response are currently unknown. In contrast, sensitization of objective endpoints such as eye-blink rate, elevated mood, and talkativeness lacks replication in controlled studies. Third, sensitized responses in humans can be associated with personality traits, indicating that certain individual characteristics may alter the human response to repeated stimulant administration. Fourth, in subjects diagnosed with new-onset and mild-to-moderate psychotic symptoms or with a recent history of intravenous cocaine use, sensitization to behavioral responses may not be observed. Fifth, raters in these studies typically observe the same individuals following both placebo and stimulant administrations, which could influence results based on rater expectations. Sixth, human studies have reported that women are more likely than men to show differences in stimulant response based on the number of previous stimulant exposures. Finally, in contrast with nonclinical studies, a complex interaction exists between measured and expected drug effects in humans. The subjects' ability to guess what they have received, coupled with their expectations of what psychostimulant-induced effects actually are, is likely to influence their subjective response to drug ingestion.

2.3 Conditioned Place Preference

The conditioned place preference (CPP) paradigm relies on the phenomenon of secondary conditioning in which a neutral stimulus that has been paired with a reward acquires the ability to serve as a reward itself. Consequently, a drug treatment and its presumed internal effects (interoceptive cues) are paired with the external neutral stimuli of a particular environment. If during a subsequent test the animal increases the time that it spends approaching and maintaining contact with the stimuli in that environment, it is inferred that the drug treatment was rewarding. In a so-called biased CPP procedure, the animal receives repeated drug administration in their initially nonpreferred environment (if examining the rewarding effects of a drug), or the preferred side (if examining the aversive effects of a drug). In an unbiased CPP experiment, the animals are randomly assigned without regard to initial bias for either side of the conditioning apparatus (for reviews see Tzschentke 1998, 2007).

The CPP paradigm can also be used to evaluate aversive effects and is then typically referred to as conditioned place aversion (CPA). For example, one may use place conditioning to measure aversive effects induced by nicotine withdrawal (Krishnan-Sarin et al. 1999; Malin et al. 1992, 1993; O'Dell and Khroyan 2009). In these studies, animals typically receive chronic nicotine via osmotic minipumps for 5–7 days. During conditioning, the animal receives a nicotinic receptor antagonist (e.g., mecamylamine) to precipitate withdrawal and is confined to one side of the apparatus. On alternating days they receive saline in the other compartment. Following conditioning, nicotine-dependent adult rats reliably display a CPA for

the compartment where they experienced withdrawal (O'Dell and Khroyan 2009; O'Dell et al. 2007; Suzuki et al. 1996).

2.3.1 Notes on the Validity of the CPP Paradigm

The CPP paradigm has a low-to-moderate degree of face validity: the drug is administered noncontingently and there is evidence that the behavioral and neurochemical effects of abused drugs differ depending on whether drug administration is controlled by the subject. However, in this paradigm environmental cues are repeatedly paired with the drug and eventually become a conditioned stimulus. Thus, when animals express a CPP, the environment is said to have acquired secondary reinforcing properties much like drug-related cues elicit conditioned responses and craving in humans. The predictive validity of the CPP model is also moderate: one of the main advantages of this paradigm is its ability to assess both rewarding (CPP) and aversive (CPA) effects of drugs, a similarity with the subjective effects of drugs in humans. The CPP/CPA model also seems to be predictive of the efficacy of some pharmacotherapies (e.g., mecamylamine and Rimonabant® can decrease nicotine-induced CPP, whereas Bupropion® attenuates physical signs of withdrawal and CPA produced by nicotine withdrawal). However, one should be aware that route of drug administration, duration of conditioning sessions and number of environmental pairings can significantly affect place conditioning. Furthermore, because tests of conditioning are conducted in the absence of drug, the issue of state-dependency must be addressed: memories acquired during exposure to a drug may be forgotten when the drug wears off and not remembered until re-exposure to the drug. Conversely, material learned in an undrugged state may be forgotten when a drug is taken; and material learned under one drug may be forgotten when another drug is used. In addition, a lack of a conditioned response may indicate a loss of the reinforcing effects of a drug or a generalized impairment of learning or memory processes required for the acquisition or performance of a conditioned response. Finally, genetic studies using CPP must take into account possible genotypic differences in the saliency of environmental cues used for conditioning.

2.4 Subjective Effects of Drugs: Drug Discrimination

Drug discrimination refers to the perception of the effects of drugs, and assesses whether or not the experimental subject perceives the drug as the pharmacological equivalent of another drug (for a thorough review, see Colpaert 1999). Pharmacological equivalence refers to the fact that drugs from different chemical classes may produce similar pharmacological effects that collectively constitute their pharmacological profile. The primary hypothesis underlying drug discrimination research is that the ability to perceive and identify the particular subjective effects

of drugs and their withdrawal syndromes promotes drug-seeking behaviors (Falk and Lau 1995). In a typical drug discrimination experiment, animals are trained to discriminate the injection of a training dose of a drug (the training drug; D) from the injection of saline (S). Food-deprived rats are trained to press one of two levers for food in daily sessions before which the animals are injected with either D or S. After D injection, the animal is required to press the drug lever (DL) to obtain food, and presses on the other lever do not yield food. After S injection, the animal now is required to press the saline lever (SL) and presses on the DL are without consequences. Training is pursued until the animal reliably selects the appropriate lever after injections of either D or S. Once trained, the animals undergo tests of stimulus generalization. Before the test session, the animal receives S, the training dose, any other dose of D, or any dose of any other agent. If the test treatment makes the animal select DL, then it is considered that stimulus generalization occurred, and it is inferred that the test treatment produced a discriminative stimulus that is qualitatively similar to that produced by D. If the test treatment makes the animal select SL, then it is considered that it did not produce stimulus generalization.

2.4.1 Notes on the Validity of the Drug Discrimination Paradigm

Generalization gradients are dependent on the dose of drug used for training and, therefore, the use of multiple training doses is essential. Different test procedures may yield different results depending on the variable used to measure generalization. Species and strain differences as well as the experimental history of the animals used in drug discrimination may alter the discriminative stimulus effects of a drug. Finally, differences in the discriminative stimulus effects of a drug may occur depending on whether appetitive or aversively maintained responding is employed.

2.5 Withdrawal

In a typical drug withdrawal model, animals are chronically treated with a drug, and then abruptly withdrawn (for a review on animal models of nicotine withdrawal, see Malin and Goyarzu 2009). For example, in the case of nicotine, the frequency of withdrawal signs are observed and coded from a checklist which includes writhes and gasps, wet shakes and tremors, ptosis, bouts of teeth chattering and chewing, as well as less frequent signs such as foot licks, scratches, yawns, and episodes of freezing behavior (Malin et al. 1992). Emotional and motivational correlates of withdrawal signs have also been described, such as shifts in ICSS reward thresholds (see above) or aversion as assessed by place preference conditioning (see above). Withdrawal models have been shown to be predictive of withdrawal signs in humans (Krishnan-Sarin et al. 1999; Malin 2001; Malin and Goyarzu 2009; Malin et al. 1993) and as such have a fair face and predictive validity.

3 Intravenous Drug Self-Administration

More than 20 psychoactive drugs that are abused by humans have also been found to act as reinforcers in rodents and nonhuman primates, thus supporting the hypothesis that drug self-administration in animals may be a reliable predictor of abuse liability in man. The self-administration model has probably one of the highest face validities as virtually all drugs of abuse, but also natural reinforcers, such as food or sucrose are self-administered by laboratory animals. Nonclinical drug self-administration studies performed over a relevant range of doses are typically characterized by an inverted U-shaped function between drug dose and a measure of behavior (e.g., response rate or number of self-administered injections). The ascending portion of this inverted U-shaped curve, which is typically studied in clinical self-administration studies, may provide the most informative data on the reinforcing effects of a drug. A major focus of preclinical research on drug self-administration has been to examine the variables (behavioral and pharmacological) that modify this behavior. Consequently, different reinforcement contingencies have led to variants of the core self-administration model (for reviews, see Haney and Spealman 2008; Oleson and Roberts 2008).

3.1 Measurement of Patterns of Rate of Drug Intake (Low Fixed-Ratio Schedules)

In low fixed-ratio (FR) schedules of reinforcement, the response requirements for each drug infusion are set at a fixed number. Within a range of drug doses that maintain stable responding, animals will typically increase their response rate as the unit dose is decreased, but will reduce their rate of self-administration when the unit dose is increased. It has been argued (Yokel and Wise 1975, 1976) that because animals compensate by increasing their rate of self-administration (under low FR reinforcement conditions) following decreases in unit amount of self-administered drug, such increased rates of FR self-administration must reflect decreased reinforcer efficacy. Yet, 6-hydroxydopamine (6-OHDA) lesions of the meso-accumbens DA system (Roberts 1989) confound that interpretation as partial depletion of DA in the NAc produces partial inhibition of cocaine self-administration. Since the reinforcing efficacy of cocaine is believed to correlate with its enhancement of DA in the NAc (de Wit and Wise 1977; Ettenberg et al. 1982; Gardner 2000, 2005; Spyraki et al. 1987; Wise and Rompré 1989), the decreased FR drug self-administration seen during recovery from the 6-OHDA-induced DA depletion has been interpreted as reflecting decreased reinforcer efficacy. The main issue, however, is that the same alteration in the reinforcing efficacy of cocaine manifests itself by opposite patterns of low FR drug self-administration (i.e., by either increased or decreased FR drug-self-administration) (Roberts and Zito 1987).

3.2 *Measurement of the Relative Strength of a Reinforcer Independent of Response Rate (Progressive Ratio Schedules)*

During progressive ratio (PR) schedules of reinforcement rats must complete increasing FR response requirements to obtain a reinforcer (here the drug). The essential feature of the PR schedule is that the response requirement continues to increase until responding ceases altogether and the reinforcer is no longer obtained. The final ratio completed is termed "breaking point" or "break point". Since the PR break-point is an index of the relative strength of a reinforcer to sustain responding, independent of response rate, one assumes that a shift in PR break point produced by a pharmacological agent indicates that the latter decreases the reinforcing value of the drug (Arnold and Roberts 1997; Morgan and Roberts 2004; Richardson and Roberts 1996; Rowlett 2000; Stafford et al. 1998). The escalation in final ratios, which translates into a leftward and upward shift in the cocaine dose–response curve (also referred to as "reverse tolerance" or "sensitization" – see above) is affected by the unit injection dose of cocaine and by the speed of drug infusion (Liu et al. 2005; Morgan et al. 2006).

3.3 *Measurement of the Impact of the Conditioned Reinforcing Properties of the Drug-Paired Stimulus (Second-Order Schedules)*

To address the potential issue of sequential drug build-up inherent to the PR paradigm, conditioned reinforcement paradigms have been developed. These models typically combine instrumental and Pavlovian (classical) conditioning procedures. Second-order schedules of drug reinforcement have examined how a response sequence is maintained by intermittent reinforcement of instrumental behavior (typically a lever press) by the environmental stimulus that has acquired conditioned reinforcement properties (Everitt and Robbins 2001; Goldberg 1973; Goldberg and Tang 1977). One of the main advantages of second-order schedules of reinforcement is that responding for the drug can be maintained for extended periods prior to actual drug infusion. Furthermore, this model is not contaminated by cumulative drug effects. Decreased responding during the first and second intervals produced by treatment with a pharmacological agent is typically explained as an attenuation of the impact of the conditioned reinforcing properties of the drug-paired stimulus. A concomitant increase in latency to the first presentation of the contingent conditioned stimulus and the first cocaine infusion may suggest that the decrease in drug intake under the second-order schedule is related to a decreased motivation to respond for that drug. This paradigm may potentially dissociate brain mechanisms underlying drug-seeking vs. drug-taking behaviors (Whitelaw et al. 1996).

3.4 Additional Models of Intravenous Drug Self-Administration

3.4.1 Runway Model

In this model, the time required for an animal to run down a straight alley from a start compartment to a goal compartment where a drug is administered is a reliable index of the animal's motivation to seek that drug (for a review, see Ettenberg 2009). Changes in the animals' motivation to seek the drug produce shifts in the run times required to reach the goal compartment. This method incorporates aspects of both drug self-administration and CPP, and is close to the second-order schedules of reinforcement model (see above).

3.4.2 Long-Access Training and Drug Intake Escalation

The phenomenon of long-access escalation (LgA), which models the increased drug intake observed in human addicts, has been systematically demonstrated by showing that providing extended access (i.e., at least 6 h) during daily low FR self-administration sessions results in an increase, or "escalation" in the rate of drug intake over weeks (Ahmed and Koob 1998, 1999; for a review, see Zernig et al. 2007).

3.5 Notes on the Validity of the Intravenous Drug Self-Administration Paradigm

Self-administration under low FR schedules of reinforcement typically results in an inverted U-shaped curve, and both leftward and rightward shifts in the dose–response function will decrease self-administration, but will also simultaneously increase self-administration of other doses. Thus, the interpretation of downward shifts in the dose–response function might be problematic, and the generation of full dose–response functions is absolutely essential in the design of such studies. Few publications actually provide such information and contribute to the lack of clarity around the use of low response-cost FR reinforcement schedules. In addition, drug self-administration is quite sensitive to nonspecific effects of pharmacological treatments on behavior, and the effect of this treatment on non-drug reinforcers should always be carefully assessed. Thus, low FR schedules of reinforcement are useful for exploring patterns of rate of drug intake, which are an ambiguous measure of drug efficacy unless full dose–response functions are generated, but are less appropriate to assess changes in the reinforcing efficacy of drugs of abuse. The PR paradigm, however, seems to benefit from translational value into clinical trials as the reinforcing effects of cocaine, D-amphetamine, caffeine, and methylphenidate are influenced by behavioral demands following drug administration in humans (Rush et al. 2001; Stoops et al. 2004, 2007).

The increased final ratios observed after PR training seems to address the DSM-IV criterion of increased time and energy spent to obtain a drug. Although the PR break-point shift paradigm measures the reinforcing efficacy and motivation to self-administer drugs, one should be aware that the sequential build-up of the drug following repeated self-administration may lead to unwanted results including impaired instrumental responding that might be misinterpreted as impairment of reinforcement or incentive motivation. To palliate this potential confounding factor, it would appear that the first, drug-free interval of responding under a second-order schedule of reinforcement has particular translational value in that it provides (1) a measure of drug-seeking behavior and reinforcing efficacy that are not affected by the pharmacological effects of recently administered drug, and (2) a means of investigating the role of drug-paired stimuli in drug-seeking behavior. Finally, the escalation in drug intake observed after LgA training appears to address the DSM-IV criterion of increased consumption over time.

One of the issues in self-administration paradigms, as also observed in the majority of nonclinical models, relates to the duration of exposure to a potentially new pharmacotherapeutic agent. Acute or subchronic administration regimes are still the rule and rodent as well as nonhuman primate models may have significantly higher predictive validity by adopting protocols that include longer periods of medication treatment. It is also worth noting that the great majority of nonclinical drug self-administration studies do not provide alternative reinforcers as part of the experimental design. One may hypothesize that increasing the relative availability of alternative reinforcers may actually result in reduced drug choice, and that medications that decrease the reinforcing effects of drugs might shift choice from these drugs to nondrug alternatives. More research is needed to understand the impact of nondrug reinforcers on drug choice.

3.6 Oral Alcohol Self-Administration

The intravenous self-administration of alcohol is difficult to sustain in rodents. An alternative method to measure alcohol consumption in rodents is to provide the animals with a choice between bottles containing a given percentage of alcohol and a bottle containing water (for a review, see Spanagel 2009). The proportion of alcohol intake relative to total fluid intake is then calculated as a preference ratio. More recently, selected high alcohol-preferring rat and mice lines have been used in alcohol preference models. For example, the Finnish model also referred to alko alcohol (AA) and alko nonalcohol (ANA) rats consists of two strains of rats that were selectively bred since 1963 based on their selection or rejection of a 10% alcohol solution and water (Eriksson 1968). The AA and ANA lines also show relevant behavioral traits that are abnormal in human alcoholics (e.g., high impulsivity) (Möller et al. 1997). A second line of rats, typically referred to as the Sardinian alcohol-preferring (sP) rat line, has also been selectively bred for high alcohol preference and consumption for more than 20 years (Colombo et al. 1997).

A third line of alcohol-preferring rats is the alcohol-preferring (P) and the high-alcohol-drinking (HAD) lines from Indiana University (Li et al. 1993). High and low alcohol-preferring mice (HAP and LAP, respectively) were also generated by the same Group (Grahame et al. 1999). The relevance of alcohol preference models in alcohol-preferring rat and mice lines to chronic alcoholism in humans is, however, questionable since these animals significantly decrease their alcohol intake when they are offered sugar-enhanced diets.

A variant of the oral alcohol self-administration model is the persistent alcohol intake following alcohol vapor exposure. Continuous alcoholization by inhalation of alcohol vapors can result in a blood alcohol concentration greater than 150 mg/dl and typically leads to high alcohol intake and preference in experimental animals (Roberts et al. 2000). One of the shortcomings of this model is that drinking behavior following exposure to alcohol vapors is transient and declines after approximately a week. To palliate this limitation, a new model using repeated cycles of intoxication and mild withdrawal has been developed (Rimondini et al. 2002). The validity of this model is supported by data showing (1) an increase in severity of alcohol withdrawal symptoms following repeated detoxification cycles (Maier and Pohorecky 1989; McCown and Breese 1990), and (2) an increase in operant responding for alcohol on a PR schedule of reinforcement following repeated withdrawal cycles from chronic alcohol intake (Brown et al. 1998).

4 Models of Relapse to Drug-Seeking Behavior

One of the main challenges in drug addiction treatment is the high rate of relapse to drug-seeking and drug-taking after detoxification. This challenge actually reflects the complexity of long-term and persistent neuroadaptations produced by drugs of abuse. Nonclinical modeling of these neuroadaptations may, therefore, lead to a better understanding of the behavioral, environmental, and neural mechanisms underlying relapse and to the development of new pharmacotherapies to prevent reinstatement of drug-seeking and drug-taking behaviors. In the following paragraphs, we briefly review some of the nonclinical reinstatement models, which study factors that underlie relapse in humans.

4.1 Reinstatement Models of Relapse Using Operant Drug Self-Administration

The propensity for relapse during abstinence can be modeled in the so-called reinstatement paradigms using operant intravenous drug self-administration (for thorough reviews, see Fuchs et al. 2008; Shaham et al. 2003; Yahyavi-Firouz-Abadi and See 2009). In reinstatement models, animals are typically trained to self-administer a drug in an operant conditioning chamber where drug delivery is

paired with the presentation of a conditioned stimulus (CS) (e.g., light or tone). After self-administration training, the animals are subjected to behavioral extinction in the same chamber. During extinction, the animals are tested under conditions of nonreinforcement (i.e., responses no longer result in drug reinforcement or CS presentations) until drug-seeking behavior is extinguished. Drug-seeking behavior (i.e., the instrumental response that previously yielded the drug) is then measured in response to either a "priming" injection of the drug itself (drug-induced reinstatement) (de Wit and Stewart 1981), re-exposure to the CS (CS-induced reinstatement) (Erb et al. 1996), or exposure to stressors (stress-induced reinstatement) (See 2002, 2005). Most reinstatement studies with stressors typically use intermittent electric shock pulses, applied to the tail or feet, as well as restraint and forced-swim stress. There is, however, increased need for stressors (e.g., maternal separation, food deprivation or social defeat stress) that translate more validly to clinical conditions (Miczek et al. 2008; Yap and Miczek 2008). In the runway version of the reinstatement model, rats are trained to run an alley once each day for drug reinforcement (for a review, see Ettenberg 2009). After the initial training period, the drug reinforcer is removed and the operant running is progressively extinguished. A single unexpected reinforced trial reliably reinstates operant responding (i.e., running) on the next trial (24 h later). In contrast to lever-press procedure, re-exposure to the drug reinforcer occurs following the emission of the operant runway response, as opposed to a noncontingent experimenter-administered injection of the drug.

The lack of robust and enduring behavioral effects of cocaine-associated cues in typical "reinstatement" paradigms (de Wit and Stewart 1981; Fuchs et al. 1998; Tran-Nguyen et al. 1998; Weissenborn et al. 1995) appears to be inconsistent with the presumed strength and persistence of motivating effects of drug cues in humans (Childress et al. 1993). An important consideration in the stimulus control of drug-seeking behavior involves the role of discriminative stimuli (Weiss et al. 2000). Discriminative stimuli signal the availability of a reinforcer and thereby set the occasion to engage in behavior that brings the organism in contact with the reinforcing substance. A condition often associated with drug craving in humans is cognitive awareness of drug availability (Meyer and Mirin 1979). It has been argued, therefore, that the manner in which drug-associated contextual cues attain their incentive properties is likely to involve the predictive nature of these stimuli rather than only classically CS-response associations (McFarland and Ettenberg 1997). This hypothesis is supported by the results obtained in a model of extinction/reinstatement in which cocaine-associated cues induce a robust and enduring drug-seeking behavior in abstinent rats as measured by the recovery of extinguished responding at a previous drug-paired lever (Cervo et al. 2003; Weiss et al. 2000, 2001). In this procedure, a discriminative stimulus (SD) predictive of drug availability to discrete cues is usually paired to each drug self-infusion. The animals first receive extensive stimulus discrimination training, during which drug reinforced and non-reinforced training sessions alternate (Ciccocioppo et al. 2002). During the reinforced sessions, drug availability is contingent upon instrumental responding in the presence of a passively presented SD (CS+) and a response-contingent "time-out" stimulus. During nonreinforced sessions, drug reinforcement is withheld in the

presence of a different SD (S−) and time-out stimulus (CS−). After this stimulus discrimination training, subjects undergo extinction in the absence of the discriminative and time-out stimuli. On the reinstatement test days, drug seeking is assessed in the presence of the S+/CS+ and the S−/CS− using a repeated testing design, thus allowing the study of dose–response curves of the effects of pharmacological manipulations on drug-seeking behavior.

Variants of the cue-triggered reinstatement model include the contextual, renewal, abstinence, incubation of craving, and conflict models. In the contextual reinstatement model, animals are trained to self-administer a drug in a distinct environmental context (context A), in which CS presentations are not programmed to occur (Fuchs et al. 2005). After self-administration training, animals' responding is extinguished in yet a different context (context B). On the reinstatement test day, the subjects are re-exposed to the previously drug-paired context (context A), which results in reinstatement of responding.

In the renewal paradigm, the animals undergo self-administration training after which their responding is extinguished in a different context, but in the presence of the previously drug-paired CS. On the test day, subjects are given response-contingent access to the CS in the previously drug-paired context (Crombag et al. 2002, 2008; Bossert et al. 2006). Renewal of drug seeking has been argued to reflect the context's ability to predict drug availability contingent upon responding.

In the abstinence model, subjects are first trained to self-administer a drug in the absence of a CS. After training, subjects undergo a forced drug-free period (abstinence). On the test day, subjects are re-exposed to the drug-paired context in the absence of a CS, which results in drug seeking behavior (Grimm et al. 2002). In contrast to the other reinstatement procedures described above, subjects do not undergo extinction training before testing.

In the case of craving incubation, animals typically self-administer a drug under a low FR schedule of reinforcement, and then undergo several periods of abstinence before returning to the operant chamber where they acquired self-administration. Following extinction, cue-induced reinstatement is triggered by re-introducing the conditioned cue. Incubation refers to the observation that the amount of cue-reinforced responding increases proportionally to the duration of the abstinence period (Grimm et al. 2001, 2003; Lu et al. 2004).

Finally, in the conflict model, adverse consequences (e.g., painful foot shock) eliminates drug-seeking behavior, which is then reinstated by presentation of relapse "triggers" as described in the other reinstatement models. Evidence seems to suggest that drug-paired environmental cues can trigger relapse in this model (Cooper et al. 2007).

4.2 Reinstatement Models Using CPP

In the CPP version of the reinstatement model, extinction typically involves exposing animals to the previously drug-paired context while in a drug-free state

(Epstein et al. 2006; Shalev et al. 2002; for a review, see Aguilar et al. 2009). The CPP response is considered to be extinguished when (1) there is no significant difference between the time spent in the drug-paired compartment in the extinction session and the time spent in the same compartment during preconditioning; (2) there is no significant difference between the time spent in the drug-paired compartment by the drug and vehicle groups, or (3) animals spent less than 50% of the total time in the drug-paired compartment on two consecutive days (Li et al. 2002; Shoblock et al. 2005).

In the drug priming-induced reinstatement an extinguished CPP response is recovered after a priming injection of the conditioning drug or, in some cases, of a different drug to that administered during conditioning. In the stress-induced reinstatement an extinguished CPP response is recovered following exposure to stressful events such as intermittent footshock (Lu et al. 2000; Wang et al. 2006), immobilization (Ribeiro Do Couto et al. 2006), restraint stress (Sanchez et al. 2003), continuous tail-pinch (Ribeiro Do Couto et al. 2006), single forced swim (Ma et al. 2007), conditioned withdrawal (Lu et al. 2005), conditioned fear (Sanchez and Sorg 2001), and defeat in social interaction with a conspecific (Ribeiro Do Couto et al. 2006).

4.3 Reinstatement Models of Alcohol-Seeking Behavior

The first alcohol reinstatement study in rats was reported by Chiamulera and colleagues in 1995 (Chiamulera et al. 1995). Since then, other research groups have shown that intermittent foot-shock stress with or without response-contingent presentation of an alcohol-associated cue (Lê et al. 1998; Liu and Weiss 2002) as well as alcohol-associated olfactory and other cues (Katner et al. 1999) can reinstate previously extinguished responding for alcohol.

Long-term alcohol self-administration procedures with repeated deprivation phases produce alcohol-experienced animals showing a transient increase in alcohol consumption and alcohol preference after a period of forced abstinence, which is also referred to as the alcohol deprivation effect (ADE). The ADE reflects alcohol craving, and can be observed in long-term alcohol-drinking rats that have developed alcohol dependence (Hölter et al. 1998; Spanagel and Hölter 1999) as well as in nondependent rats (Heyser et al. 1997; Hölter et al. 2000). The ADE is also prolonged and enhanced in alcohol preferring P- and HAD-rat lines after repeated deprivation phases (Rodd-Henricks et al. 2001).

Finally, in the point-of-no-return model of alcohol self-administration rats have access to alcohol for 9 months, followed by a long-term abstinence period of 9 months. After this period the rats again have access to alcohol and exhibit high alcohol intake and preference over several weeks (Wolffgramm and Heyne 1995).

5 Models of Reconsolidation of Drug Cue Memories

In the previous section it has been shown that, with the exception of a few models, reinstatement paradigms typically use extinction training. We have also reported evidence that responses to an extinguished cue can re-emerge by changing contexts (renewal), or by re-exposure to the unconditioned stimulus (reinstatement). In contrast to extinction, reconsolidation is the process of restabilizing the memory trace after it is retrieved or "reactivated" (Tronson and Taylor 2007; for a review, see Taylor et al. 2009). From a translational perspective, there are two possible strategies in order to reduce the motivational impact of drug cues: enhance the extinction of drug cue memories (Quirk and Mueller 2008) or disrupt reconsolidation of these memories (Nader et al. 2000). Extinguishing drug cue memories does not appear to be efficacious in reducing relapse in either humans (Conklin and Tiffany 2002) or rats (Crombag and Shaham 2002). Recent studies have focused on the disruption of reconsolidation of fear memory (Debiec and LeDoux 2006; McCleery and Harvey 2004; Tronson and Taylor 2007) and evidence suggests that cue-induced alcohol-seeking behavior is reduced by disrupting reconsolidation of ethanol-associated memories (Von der Goltz et al. 2009). Both the protein synthesis blocker anisomycin and the *N*-methyl-D-aspartate (NMDA) receptor antagonist MK-801 ((5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclo hepten-5,10-imine maleate) given immediately after re-exposure of animals to alcohol-paired conditioned stimuli were shown to impair the ability of these stimuli to induce alcohol-seeking behavior in subsequent test sessions (von der Goltz et al. 2009).

6 Models of Compulsive/Impulsive Drug Intake

6.1 Impulsivity as a Multifactorial Construct

Impulsivity is a multifactorial construct encompassing several processes that include: (1) an inability to delay gratification; (2) an inability to withhold a response; (3) acting before all of the relevant information is provided, and (4) decision making that is risky and inappropriate (for a thorough review on impulsivity, see Duka and Crews 2009; see also Bari and Robbins 2011). Delay of gratification and inability to withhold a response are also often referred to as cognitive and motor impulsivity, respectively. Cognitive impulsivity may result from deficits in attention, an inability to discriminate reward magnitude, disruptions in time perception, a misunderstanding of response contingencies, an inability to consider future events, or a distortion in the value of long-term consequences. Similarly, apparent deficits in motor impulsivity may result from disruptions in sensory,

motor, or timing abilities, even if the ability to withhold an automatic (prepotent) response is intact.

6.2 Measurement of Impulsivity

There are typically three broad classes of tests to measure impulsivity: (1) measures of response inhibition based on the suppression of a prepotent response (Logan et al. 1997); (2) measures of delay discounting, which define impulsivity in terms of choice preference for a small reward available immediately (or after a short delay) over a larger reward available in the future (Bickel and Marsch 2001; Reynolds 2006; Bari and Robbins 2011), and (3) measures of cognitive impulsivity that refers to decision-making processes. One component of cognitive impulsivity is referred to as "reflection impulsivity" or the tendency to collect and assess information before making complex decisions (Kagan 1966). Reflection impulsivity may be related to what other authors have referred to as "nonplanning impulsivity" (Patton et al. 1995) or "lack of premeditation" (Whiteside and Lynam 2001). Additional measures to assess cognitive impulsivity also include tasks during which the subject has to select between a conservative option and a more risky option that offers a "superficially seductive" gain (Bechara 2003; Knoch and Fehr 2008).

6.3 Impulsivity Constructs in Drug Addiction Research

Drug addicts' behaviors have commonalities with patients suffering from damage to the orbitofrontal cortex (OFC). The OFC is thought to moderate impulsive choice (Mobini et al. 2002; Rudebeck et al. 2006), and to represent subjective value during decision-making (Izquierdo et al. 2004; Padoa-Schioppa and Assad 2006; Roesch and Olson 2004; Schoenbaum and Roesch 2005). Damage to the human OFC increases the tendency to choose immediate rewards over larger, delayed rewards (Berlin et al. 2004) and impairs the ability to refrain from responding to formerly rewarding cues that are no longer reinforced (Dias et al. 1996; McAlonan and Brown 2003; Mishkin 1964; Ostlund and Balleine 2007; Rahman et al. 1999; Rolls et al. 1994; Schoenbaum and Roesch 2005; Schoenbaum et al. 2007; Tait and Brown 2007).

Abnormal OFC function has been associated with substance abuse (Boettiger et al. 2007; Crews and Boettiger 2009; Dom et al. 2005; Ersche et al. 2005; Everitt et al. 2007; London et al. 2000; Volkow and Fowler 2000), and the impairment in reversal learning in cocaine addicts and animals that have chronically self-administered cocaine (Schoenbaum and Shaham 2008) or alcohol (Obernier et al. 2002) is consistent with the loss of control that is characteristic of addiction. Finally, reduced OFC activity during decision-making among abstinent alcoholics is correlated with their tendency to choose immediate over delayed rewards (Boettiger

et al. 2007), further suggesting that a dysfunction of the OFC may contribute to the persistence of addictive disorders.

6.4 *Animal Models of Impaired Response Inhibition and Impulsive Choice*

Animal models provide a means to prospectively evaluate the influence of both impaired response inhibition and impulsive choice on several aspects of drug abuse. For example, rats selected for poor inhibitory control on the 5-choice serial reaction time (5-CSRT) task make more responses during the acquisition of nicotine self-administration (Diergaarde et al. 2008) and self-administer larger amounts of cocaine (Dalley et al. 2007) compared to those selected for good inhibitory control. Rats screened for high levels of impulsive choice on a delay-discounting task subsequently self-administer more ethanol (Poulos et al. 1995) and intravenous nicotine (Diergaarde et al. 2008), acquire cocaine self-administration faster (Perry et al. 2005, 2008), and show greater reinstatement of cocaine-seeking (Perry et al. 2008) than those with low levels of impulsive choice (Perry and Carroll 2008). Furthermore, heightened impulsivity on two different measures, impulsive choice (delay discounting) (Anker et al. 2009) and impaired inhibition (5-choice serial reaction time) (Dalley et al. 2007) is associated with greater escalation of cocaine self-administration in rats, a finding that relates to clinical studies suggesting that self-reported impulsivity is a significant risk factor in the occurrence of binge-like patterns of crack/cocaine intake in women (Lejuez et al. 2007). Taken together, these results support the hypothesis that impaired response inhibition and impulsive choice are associated with increased drug-seeking and may predict binge-like patterns of drug intake.

7 Neuroimaging Models

The DA $D_{2/3}$ receptor antagonist [11C]-raclopride, a widely used radioligand for positron emission tomography (PET) studies of DA $D_{2/3}$ receptors in the brain, competes with DA for receptor-binding sites. Therefore, its displacement can be used as an indirect assay of DA release or reuptake inhibition in drug addicts compared to normal controls. Over the last 15 years, PET studies in drug addicts have shown that: (1) cocaine addiction translates into decreased DA $D_{2/3}$ receptor availability in the striatum compared to drug-naïve controls (Volkow et al. 1993) and reduced DA release in the striatum in response to intravenous administration of amphetamine (Martinez et al. 2007; Volkow et al. 1997); (2) chronic use of other drugs of abuse such as methamphetamine, alcohol, and heroin is also associated with decreased DA $D_{2/3}$ receptors in the striatum (Volkow et al. 2002; Volkow and

Wise 2005); (3) decreased and increased DA $D_{2/3}$ receptors in the striatum seems to predict the subjective effects (i.e., pleasantness and unpleasantness, respectively) of intravenous psychostimulants in normal healthy human volunteers (Volkow et al. 1999); (4) increased DA release in the left ventral striatum, dorsal putamen and dorsal caudate nucleus is associated with ratings of a positive response to intravenous amphetamine in drug-naïve subjects (Oswald et al. 2005), and (5) cue-induced craving is associated with increased DA $D_{2/3}$ receptor occupancy by DA in the striatum in human alcohol and stimulant abusers (Heinz et al. 2004; Volkow et al. 2006; Wong et al. 2006). The latter finding also concurs with functional imaging studies showing activation of the anterior cingulate cortex and OFC by drug-associated stimuli that elicit craving (Childress et al. 1999; Garavan et al. 2000; Goldstein et al. 2007; Grant et al. 1996). In line with these clinical studies, recent nonclinical PET studies have shown that low DA $D_{2/3}$ receptors associated with high impulsivity may predict elevated rates of intravenous cocaine self-administration and a vertical shift in the cocaine dose–response function relative to nonimpulsive control rats (Dalley et al. 2007, 2009). These findings, in turn, are compatible with previous findings in rats screened for impulsivity on a delay-discounting paradigm (Perry et al. 2005; Poulos et al. 1995).

In addition to nonclinical PET paradigms, the recent combination of pharmacological treatment with functional magnetic resonance imaging (fMRI), also referred to as pharmacological MRI (phMRI), in rodents provides a new method to identify functional changes sequentially over real time in response to the acute, subchronic, or chronic administration of drugs (see Bifone and Gozzi 2011). The classical univariate analysis of phMRI data emphasizes the specialization of function within different brain areas (also referred to as functional segregation). However, brain functional processes rely on efficient information flow within widely distributed and highly integrated neuronal networks, and drug effects may be partly explained by disrupted connectivity. The application of multivariate approaches to the analysis of phMRI time-series can provide insight into the functional integration of the brain and into changes in inter-regional interactions in response to drug treatment. For example, a recent preclinical phMRI study that used inter-subject functional connectivity analyses of phMRI responses to d-amphetamine showed that selective antagonism at DA D_3 receptors produced a reversed correlation between responses in the VTA and the dorsal thalamus, and a reduced correlation between the VTA and ventral striatum (Schwarz et al. 2007). These findings suggest that a modified functional connectivity within a circuit that is a key substrate in the so-called reward system may play an important role in the efficacy of selective DA D_3 receptor antagonists in attenuating drug-seeking behavior.

Functional neuroimaging in translation, however, faces several challenges. First, despite the growing number of publications assuming that there is a tight coupling between the measured blood oxygen-level-dependent (BOLD) signal and neural processes, there is still limited understanding of the BOLD response in terms of neuronal activation. For example, there are situations in which neural activation is not reflected in a BOLD increase or situations in which BOLD responses can be identified in the absence of neural activation (Schummers et al. 2008; Sirotin and

Das 2009). Second, statistical analyses typically focus on the voxel producing the maximal signal, also referred to as "peak voxel," with a correction for multiple comparisons. However, a "peak-voxel" approach may lead to overestimation of the size effect and the predictive value of brain responses might be significantly inflated. Third, functional patterns of brain activation eventually depend on a deep understanding of brain anatomy and its functional specialization. This is to say that a lack of precise localization will inevitably yield a lack of precision in our understanding of the underlying processes. The commonly used anatomical localization techniques are based on brain atlases that do not take into account inter-subject variability and represent only a small portion of the human population. Even more challenging is the paucity of information about the variability of brain function per se. In other words, the translational value of functional imaging techniques will ultimately rely on the identification of stable patterns of activation that represent certain functions, which also take into account both anatomical and functional variability within and between subjects. In contrast with the language used by many neuroscientists to explain their brain imaging findings, the fundamental limitations described above are calling for caution. Diagnosing those limitations is essential to further enhance the translational value of neuroimaging approaches.

8 Unbiased Phenotype-Driven Genetic Model Systems: Invertebrate Models with *Drosophila* and *Caenorhabditis elegans*

Drosophila and the nematode *Caenorhabditis elegans* (*C. elegans*) have been intensively studied organisms in biology, and have provided insight into developmental and cellular processes that are conserved with mammals, including humans (see O'Kane 2011 for further discussion). Adult flies have a relatively sophisticated nervous system (approximately 250,000–300,000 neurons), and genes implicated in the actions of drugs of abuse are, for the most part, conserved (for reviews, see Heberlein et al. 2009; Wolf and Heberlein 2003). *C. elegans* is also one of the most amenable organisms with a completely sequenced genome consisting of approximately 19,000 genes, and a nervous system of 302 neurons (Schafer 2004). Most impressive is the completion of a series of refined characterizations of each neuron and neuronal connections using electron microscopy (Schafer 2004). Furthermore, *C. elegans* shows sensitivity to drugs of abuse such as alcohol and nicotine (Davies et al. 2003; Feng et al. 2006). For example, acute and chronic exposure to nicotine has shown a key role of the conserved transient receptor potential canonical (TRPC) channels in modulating the activity of nicotinic acetylcholine receptors (Feng et al. 2006). In addition, activation of a BK potassium channel, SLO-1, was shown to mediate the acute behavioral effects of alcohol (Davies et al. 2003).

Although studies of addiction in *Drosophila* and *C. elegans* are still in their infancy, the high-throughput behavioral paradigms that have been developed thus far together with genetic studies may provide valuable information on the effects of drugs of abuse at the level of specific neurons and functional neural circuitry (see O'Kane 2011). Furthermore, unbiased genetic screens have already been used to identify novel potential candidate genes regulating drug-related behaviors. However, one potential caveat is to assume that because a gene associated with a specific function in humans also shows a behavioral effect in flies or worms, then that fly or worm behavior is necessarily relevant to that function in humans. This type of association is clearly questionable in the light of between-species variation in the genetic architecture of complex traits.

9 Conclusions

The previous sections presented the addiction process as comprised of multiple stages and sources of reinforcement including positive reinforcement, negative reinforcement, conditioned positive reinforcement, and conditioned negative reinforcement. The nonclinical paradigms that were described aim at modeling those sources of reinforcement and must, therefore, achieve the highest standards of validity and meet essential criteria if they are to translate ultimately into clinical relevance. First, drug delivery should be active (i.e., the experimental subjects must have full control over drug delivery), dose–response effects should be systematically observed, and drug exposure should be chronic or subchronic rather than acute. The first point is one of the shortcomings of some models such as the CPP paradigm, i.e., passive drug administration, lack of systematic dose–response studies, and relatively low exposure to the drug. Second, typical reinstatement experiments in rodents are conducted under drug-free conditions; by definition, true relapse in humans can only be observed when compulsive drug consumption follows a period of abstinence. Third, most reinstatement models include extinction training. Although the latter isolates the influence of the CS on reinstatement from that of the context, response habit or stress, it unfortunately reduces the face validity of the model given that humans rarely undergo extinction. Thus, models that assess drug seeking after a drug-free abstinence period as opposed to instrumental extinction training may better capture the nature of cue-induced relapse in humans. In the case of abstinence models, the fact that subjects do not undergo extinction training improves the face validity of this model, but restricts data interpretation as drug-seeking may actually reflect response habit, novelty-induced stress, exploratory behavior, and/or innate motivation in addition to context-induced incentive motivation for drug. Fourth, evaluations of potential pharmacotherapies for drug addiction in animal models most often use acute medication pretreatment paradigms. Predictive validity of those models would actually improve if they were to adopt protocols that include longer periods of medication treatment. Fifth, improved predictive validity also lies in the efficacy of pharmacological manipulations that affect drug-seeking behavior

in specific animal models, and how this nonclinical efficacy profile translates into alleviation of craving and relapse propensity in clinical populations.

The extent to which each of these nonclinical paradigms models a specific DSM-IV criterion is arguable, but some associations have emerged. First, the concept of addiction as a progressive transition from a positive to a negative reinforcement process that drives the motivated behavior somehow reaffirms the importance of withdrawal in addiction (i.e., criterion 2) (Table 1). In that respect, measuring the degree of dysphoria produced by drug withdrawal using the BSR paradigm is highly relevant. Second, the escalation in drug intake observed after long-access training and drug intake escalation mimic increased consumption over time (i.e., criterion 3) (Table 1). Third, the increased final ratios observed in PR paradigms appear to model the increased time and energy expended to obtain the drug (i.e., criterion 5) (Table 1). Fourth, we have reviewed evidence suggesting that loss of control is one of the key features of both drug and behavioral addictions. However, despite their potential translational value, few of the "classical" paradigms described in the previous paragraphs do actually address dysfunctional decision-making processes or alterations in impulsive behavior. Recent advances in animal models have exploited the translational value of behavioral economics models to address the notion of discounting of delayed rewards to provide a readout of impulsivity and its related corollary of loss of control (i.e., criterion 6) (Table 1 and see Bari and Robbins 2011 for further discussion). Animal studies investigating the link between abnormal information processing in the mesocorticolimbic system and changes in responding for delayed or intermittent reinforcement are thus extremely valuable. Similarly, procedures examining choice responding under concurrent schedules of reinforcement may provide valuable insight into drug-seeking because the impact of competing reinforcers, and the work required to obtain each, can be measured simultaneously. Finally, significant work remains to be done to explore the mechanisms involved in animal models of craving and relapse and to relate these mechanisms to vulnerability to addiction.

Beyond validity-related issues, the choice of a nonclinical paradigm should also be based upon a good understanding of what needs to be achieved for the target patient population and how PD data can be reliably linked with PK data. This can only be done by answering questions as follows: what exactly is the disease indication for the target compound; what is the proposed treatment response profile; what is the proposed clinical route and frequency of dosing; what is the expected efficacious concentration in a physiological fluid (i.e., concentration–effect relationship); how long should that concentration be maintained to obtain the desired pharmacological response; what, if any, are the biological markers to monitor toxicity and/or therapeutic effects; do changes in route or delivery rate alter the course of effect; is response to treatment time-dependent (e.g., onset mechanism, disease progression)? If a valid PK/PD strategy is in place and if a strong PK/PD relationship is characterized, then efficacy and tolerability can be reliably predicted from the PK data and relevant scenarios can be simulated for decision-making or clinical purposes.

The continually evolving knowledge base of the neurobiological underpinnings of drug addiction using the relevant nonclinical paradigms to address the right clinical questions in the context of a well-designed translational strategy will provide a heuristic framework that will help in developing the treatment of drug addiction.

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When the Serotonin Transporter Gene Meets Adversity: The Contribution of Animal Models to Understanding Epigenetic Mechanisms in Affective Disorders and Resilience

Klaus-Peter Lesch

Contents

Abstract Although converging epidemiological evidence links exposure to stressful life events with increased risk for affective spectrum disorders, there is extraordinary interindividual variability in vulnerability to adversity. The environmentally moderated penetrance of genetic variation is thought to play a major role in determining who will either develop disease or remain resilient. Research on genetic factors in the aetiology of disorders of emotion regulation has, nevertheless, been complicated by a mysterious discrepancy between high heritability estimates and a scarcity of replicable gene-disorder associations. One explanation for this incongruity is that at least some specific gene effects are conditional on environmental cues,

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i.e. *gene-by-environment interaction* (G × E) is present. For example, a remarkable number of studies reported an association of variation in the human serotonin (5-HT) transporter gene (*SLC6A4*, *5-HTT*, *SERT*) with emotional and cognitive traits as well as increased risk for depression in interaction with psychosocial adversity. The results from investigations in non-human primate and mouse support the occurrence of G × E interaction by showing that variation of 5-HTT function is associated with a vulnerability to adversity across the lifespan leading to unfavourable outcomes resembling various neuropsychiatric disorders. The neural and molecular mechanisms by which environmental adversity in early life increases disease risk in adulthood are not known but may include epigenetic programming of gene expression during development. Epigenetic mechanisms, such as DNA methylation and chromatin modification, are dynamic and reversible and may also provide targets for intervention strategies (see Bountra et al., Curr Top Behav Neurosci, 2011). Animal models amenable to genetic manipulation are useful in the identification of molecular mechanisms underlying epigenetic programming by adverse environments and individual differences in resilience to stress. Therefore, deeper insight into the role of epigenetic regulation in the process of neurodevelopmental programmes is likely to result in early diagnosis of affective spectrum disorders and will contribute to the design of innovative treatments targeting neural pathways that foster resilience.

Keywords Cognition · Depression · Emotion · Environment · Epigenetics · Gene · Mouse · Primate · Resilience · Serotonin transporter

Abbreviations

5-HIAA	5-Hydroxyindoleacidic acid
5-HT	Serotonin
5-HTT	Serotonin transporter
5-HTTLPR	5-HTT gene-linked polymorphic region
ACTH	Adrenocorticotropic hormone
BDNF	Brain-derived neurotrophic factor
CRH	Corticotropin-releasing hormone
CSF	Cerebrospinal fluid
G × E	Gene-by-environment interactions
GABA-A	Gamma-aminobutyric acid-A receptor
GR	Glucocorticoid receptor
HDAC	Histone deacetylase inhibitor
HPA	Hypothalamic–pituitary–adrenal
MRI	Magnetic resonance imaging
MS	Maternal separation
OFC	Orbitofrontal cortex
rh5-HTTLPR	Rhesus macaque 5-HTT gene-linked polymorphic region

1 Introduction

Research on genetic factors in the aetiology of neuropsychiatric diseases has been complicated by a mysterious discrepancy between high heritability estimates and a scarcity of replicable gene-disorder associations. This "missing heritability" is now either euphemized as the "dark matter" of gene-trait association or aggravated as the "looming crisis in human genetics". Several explanations for this incongruity have been suggested (Manolio et al. 2009; Yang et al. 2010). These include larger numbers of variants with smaller effect size which are yet to be found; rarer variants with larger effects that are not detected by available single-nucleotide polymorphism arrays which ignore variants present in <5% of the population; other structural variations such as repeat length variants or short copy number variations which are not detected by existing arrays; inadequate power to detect gene-by-gene interactions (G × G) or, finally, inadequate accounting for the shared environment to be found among relatives.

Another plausible explanation for the missing heritability is that at least some specific gene effects are conditional on environmental cues, i.e. *gene-by-environment interactions*. Particularly stressful childhood experiences such as trauma, abuse, neglect or other forms of early life adversity are important risk factors for multiple diseases, including obesity, cardiovascular disease and neuropsychiatric illness later in life. Why one individual develops cardiovascular disease while another develops depression following early life stress is thought to be influenced by genetic susceptibility factors. Although converging epidemiological evidence links exposure to stressful life events with increased risk for neuropsychiatric disorders, there is remarkable interindividual variability in vulnerability to environmental cues. The environmentally moderated penetrance of genetic variation is therefore thought to play a major role in determining who will either develop disease or remain resilient. In this context, the ability to recover from trauma or crisis as well as resistance against biological and psychosocial risks – commonly called *resilience* – is not conceptualized as an innate trait but comprising a variable capacity initially acquired during development in the context of individual–environment interaction. A consolidated state of resilience is therefore rooted in specific resources of both individual genetic framework and social circumstances. There is considerable demand for research on the molecular mechanisms of genetic and epigenetic programming of motivational, attentional and emotional neural circuits underlying the development and consolidation of resilience.

Gene × environment interactions (G × E), involving specific gene polymorphisms, such as are found with the serotonin (5-HT) transporter gene (*SLC6A4*, synonyms: *5-HTT*, *SERT*) for example, have been identified and replicated in humans and animal models. Research on the role of the 5-HTT in the pathophysiology of stress-linked disorders accompanied by emotional dysregulation has a history spanning more than half a century. Following the influential discovery of presynaptic neurotransmitter uptake by Hertting and Axelrod (1961) and shortly after its identification as the initial target of antidepressant drug action

(Raisman et al. 1979), the 5-HTT was first linked with the pathogenesis of depression by Langer and associates (Langer et al. 1981). After cloning of the rat 5-HTT gene (*Slc6a4*) (Blakely et al. 1991), the era of molecular genetic studies of emotion regulation began with three seminal papers in 1996 that reported associations between *5-HTT* variation and anxiety-related traits (Lesch et al. 1996) as well as depression (Collier et al. 1996; Ogilvie et al. 1996).

In the years following the first reports linking *5-HTT* variation with anxiety- and aggression-related traits, numerous clinical entities have been studied for association with disorders characterized to a large extent by emotional dysregulation, including depression, bipolar affective disorder, attention-deficit/hyperactivity disorder, alcohol dependence, suicide, eating disorders and autism or disorders related to morphogenetic actions of 5-HT in other organ systems such as heart, blood vessels, bowel, and bone (Lesch and Mössner 2006; Murphy et al. 2004, 2008). Modest effect sizes typical of complex traits, polygenic patterns of inheritance, epistatic and epigenetic interactions and sample heterogeneity across studies are all factors which have led to inconsistent replication and have confounded attempts to reach agreement regarding the role of 5-HTT in the pathophysiology of all these diseases. Nevertheless, the impact of 5-HTT on complex traits in humans, non-human primates and genetically modified mice has become a model *par excellence* in cognitive, biosocial, and psychiatric neurosciences (Lesch 2007; Murphy and Lesch 2008; Suomi 2003).

The eye-opener that early life stress and other modes of G × E uniquely reinforce or even uncover links between *5-HTT* variation, behaviour and psychopathology in humans and non-human primates has heralded a new era of behavioural genetics. Several recent studies suggest that 5-HTT variation interacts with deleterious early rearing experience in rhesus macaques and in mice to influence attentional, emotional and (social) cognitive processing (Canli and Lesch 2007). Thus, the identification of *5-HTT* as a modifier of emotionality, and its interaction with environmental adversity, was a first step *en route* to an explanation of the molecular dimension of personality, emotion, (social) cognition and behaviour; suggests strategies to identify physiologic pathways and mechanisms that lead to other disorders of cognitive function and emotion regulation; provides tools to dissect the interactive effects of genes and environment in the development of affective disorders and holds the potential to predict response to treatment. It is anticipated that controlling environmental factors will eventually improve the reliability of genetic approaches.

This chapter focuses, from an *epigenetic perspective*, on the nature of an innate variability in brain 5-HTT function that predisposes to a wide spectrum of psychiatric disorders in which emotional dysregulation is a common denominator. The various psychobiological facets of *5-HTT* variation and resulting phenomes will be critically reviewed with emphasis on neurodevelopmental programming. The relevance of G × E in emotional and (social) cognitive processes is also highlighted and an appraisal of *morphofunctional imaging* of G × E in emotionality is provided. Evidence for neural modularity of cognition and emotion is also taken into consideration. Finally, views of *developmental programming* by epigenetic

mechanisms will be discussed in the perspective of the complex genetic architecture of emotional behaviour and social interaction in non-human primates and rodents. Better understanding of the role of epigenetic programming, particularly with respect to genome-wide modification of DNA and chromatin structure in complex tissues such as the brain, in the context of adverse life events, ageing processes, and resilience is likely to have far-reaching consequences for health, and mental health in particular.

2 *5-HTT* × Environmental Adversity Interaction in Humans

It is now well established that much of the impact of genetics on emotionality, including anxiety and depression, depends on interactions between genes and the environment. Such interactions imply that the expression of environmental effects occurs only in the presence of a permissive genetic background. There are several established environmental risk factors for disorders of emotional regulation. For example, numerous studies of G × E in humans have assessed the influence of childhood maltreatment and abuse, stressful events across the lifespan, socioeconomic status and chronic somatic illness. Cumulative, repeated, or protracted exposures to adversity appear to exert a stronger effect than discrete acute events (Uher and McGuffin 2008, 2010 for reviews).

The work by Caspi and co-workers (Caspi et al. 2003) indicated that individuals carrying the low-expressing, short variant of a repetitive sequence in the upstream transcriptional control region of 5-*HTT* (Lesch et al. 1996), now commonly referred to as the *5-HTT*-linked polymorphic region (5-HTTLPR), are up to twofold more likely to get depressed after stressful events such as bereavement, romantic disasters, illnesses or losing their job. Moreover, early trauma inflicted by childhood maltreatment significantly increased the probability of developing depressive syndromes in later life in individuals with the short allele of the 5-HTTLPR. A remarkable body of evidence suggests that emotionality and stress reactivity can be influenced by experiences early in life, and it has long been supposed that severe early life trauma may increase the risk for anxiety and affective disorders (Brown and Harris 2008). For example, adults experiencing four out of a possible seven severe early traumatic events showed a more than fourfold increased risk for depressive symptoms and about a 12-fold increased risk for attempted suicide (Felitti et al. 1998). No direct correlation between any specific childhood trauma and specific adult anxiety or mood disorder could be made, however, suggesting that other, possibly genetic, factors determine the precise pathology that is precipitated by the traumatic event. The observation that during early development individuals are particularly susceptible to adverse environmental influences is currently being confirmed by studies in non-human primates and mouse models that have demonstrated influential effects of the quality of maternal care on life-long emotional behaviour and brain functioning (see Sects. 3 and 4).

These results further support the notion that a combination of genetic disposition and specific life events may interact to facilitate the development of mental illness. What went largely unnoticed, however, were the implications for the genetics of personality. Depression is strongly associated with anxiety- and depression-related traits, the personality dimensions that have been linked with allelic variation of 5-HTT function. Given the high co-morbidity between anxiety and depression and the evidence for their modulation by common genetic factors (Kendler et al. 1993, 1995; Lesch 2003), it is likely that predisposition to disorders of emotional regulation will also be determined by environmental adversity whose impact on the brain is under genetic control.

Converging evidence from a large number of studies on the influence of *5-HTT* × E established that stressful life events specifically contribute to the pathogenesis of anxiety and depression as well as of disorders in which anxiety and depression are co-morbid condition (Uher and McGuffin 2010) (Fig. 1). The effect however is variable, being closely related in time to the onset of disease and having more impact on first onset than on recurrences. Several caveats have to be kept in mind when G × E interaction is investigated in clinical cohorts. The assessment of stressful life events is generally retrospective and it is crucial to minimize recall bias, distortions and inaccuracies. As the temporal relationship between stressful life events and onset of anxiety disorders and depression is incompletely understood, a cohort of patients need to be followed over a sufficient period of time following an objectively recorded stressful event to establish the time course of G × E interactions. Moreover, there is evidence that experience and recall of stressful life events are partially under the control of both genes and Neuroticism as well as a personal history of anxiety and depression that predict reporting of life stress (Uher and McGuffin 2010). Although genetic factors that

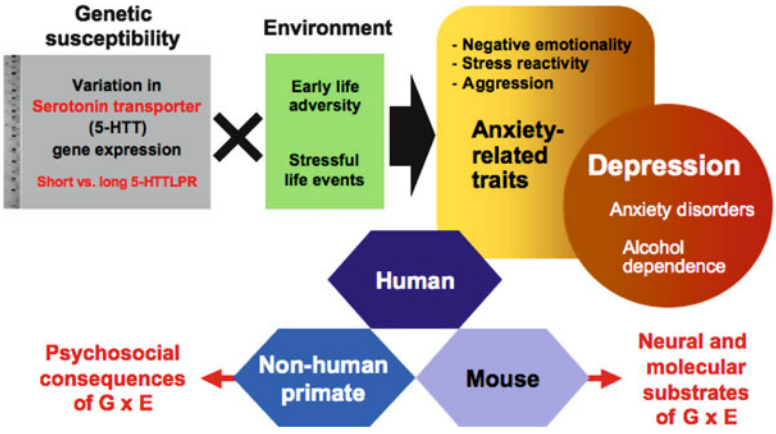

Fig. 1 Neither genes nor environment act alone: interaction between serotonin transporter gene (*5-HTT*) variation and environmental adversity (G × E) in the susceptibility of depression and related disorders. *5-HTT* × E has been demonstrated in humans as well as in non-human primate and mouse models

influence retrospective report of environmental cues overlap with those that affect personality traits, a causal relationship between adversity and affective symptomatology has, nevertheless, been demonstrated independent of individual differences in personality and recall.

While clinical evaluation and self-report of life events revealed that the effect of psychosocial adversity on depression and suicidal behaviour is modified by allelic variation of 5-HTT function, which renders carriers of the 5-HTTLPR short variant more vulnerable to depression, the neural mechanisms underlying this moderator effect are poorly understood. Although human and non-human primate functional imaging studies have begun to elucidate the neural circuits involved in the *5-HTT* × E risk factor (see Sect. 5), a molecular understanding of this phenomenon is essentially lacking (Canli et al. 2006; Kalin et al. 2008).

In summary, these findings point toward molecular and cellular mechanisms by which early stressful experiences induce persistent changes in gene expression and neuronal function whose initiation or maintenance is influenced by 5-HTT. The molecular mechanisms by which early life stressors increase risk for disorders of emotional regulation in adulthood is not known but it is presumed to include epigenetic programming of gene expression during development and throughout the entire lifespan.

3 Interaction of *5-HTT* and Maternal Separation in Rhesus Macaques

Animal models have become indispensable tools for studying the biological function of genes that are involved in the pathogenesis of neuropsychiatric disorders (Fig. 1). Since the neural and genetic basis of emotional and behavioural traits is already laid out in all mammalian species and may reflect selective forces among our remote ancestors, research efforts have recently been focussed on non-human primates, especially *Macaca mulatta*. Following the complete sequencing of the rhesus genome, this macaque species has become the "workhorse" of behavioural genetics of non-human primates. In this primate model environmental influences, while as complex as in humans, can be more easily controlled for and thus are less likely to confound gene-behaviour associations. In rhesus monkeys, all forms of emotionality and cognitive processing are moderated by environmental cues and marked disruptions to the mother–infant relationship confer increased risk for emotional dysregulation and cognitive impairment (Suomi 2003).

In rhesus monkeys, maternal separation (MS) and replacement of the mother by an inanimate surrogate mother during the first months of life results in long-term consequences for the functioning of the central 5-HT system, defects in peer interaction and social adaptation and is associated with increases in anxiety and depression-related behaviours such as rocking and excessive grooming (Higley et al. 1991). These behavioural studies already indicate that early life adversity

can directly induce long-term neuroplastic changes in emotion circuits that alter anxiety and depression-related responses in adulthood.

One of the most replicated findings in psychobiology is the observation of lower 5-hydroxyindoleacidic acid (5-HIAA), the major metabolite of 5-HT, in the brain and cerebrospinal fluid (CSF) in impulsive aggression and suicidal behaviour. In rhesus monkeys brain 5-HT turnover, as measured by concentrations of 5-HIAA in the cisternal CSF, shows a strong heritable component and is trait-like, with confirmed stability over an individual's lifespan (Higley et al. 1992; Kraemer et al. 1989). Early experiences have long-term consequences for the function of the central 5-HT system, as indicated by robustly altered CSF 5-HIAA levels, as well as anxiety, depression- and aggression-related behaviours in rhesus monkeys deprived of their mother at birth and raised only among peers. This animal model of MS was therefore used to study G × E by testing for associations between central 5-HT turnover and allelic variation of 5-HTT function based on a repeat length variation (rh5-HTTLPR) structurally and functionally orthologous to the *5-HTT*-linked polymorphic region in humans (Lesch et al. 1997) (Fig. 2). The findings suggested that the rh5-HTTLPR genotype is predictive of CSF 5-HIAA concentrations, but that early experiences make unique contributions to variation in the functioning of the 5-HT system in later life and thus provides evidence of an environment-dependent association between the *5-HTT* and a direct measure of brain 5-HT function (Bennett et al. 2002). The consequences of deleterious early experiences of MS seem consistent with the notion that the 5-HTTLPR may influence the risk for disorders of emotion regulation.

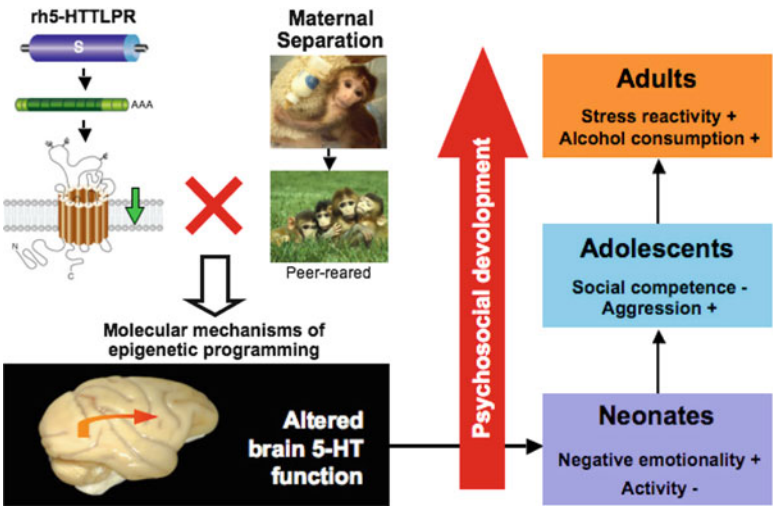

Fig. 2 Effect of interaction between maternal separation and rh5-HTTLPR genotype on psychosocial development, including brain 5-HT function, emotion regulation, social competence, stress reactivity, behaviour, and psychopathology across the lifespan of rhesus macaques

Studies were extended to the neonatal period of rhesus macaques, a time in early development when environmental influences are modest and least likely to confound gene-behaviour associations. Between postnatal days 7–30 mother-reared and maternally deprived neonates were assessed on a standardized neurobehavioural test designed to measure orienting, motor maturity, reflex functioning and temperament. Main effects of genotype and, in some cases, MS × genotype interactions, were demonstrated for items indicative of orienting, attention and temperament. In general, infants with the s form of the rh5-HTTLPR displayed higher behavioural stress-reactivity compared to one variant homozygote, as shown by diminished orientation, lower attentional capabilities and increased affective responding (Champoux et al. 2002). However, the genotype effects were more pronounced for animals raised in the neonatal nursery than for animals reared by their mothers. These results demonstrate the contributions of MS and genetic background, and their interaction, in a model of behavioural development during the neonatal phase.

Beyond the neonatal period, particularly during adolescence of rhesus monkeys, evidence for a complex interplay between inter-individual differences in the functions of the 5-HT system and social success has also been accumulated. The interactive effect of rh5-HTTLPR genotype and early rearing environment on social play and aggression was explored in infants and adolescents (Barr et al. 2003). Rhesus monkeys homozygous for the long variant were more likely to engage in rough play than those with the long/short variants, with a significant interaction between 5-HTT genotype and MS. Peer-reared infants carrying the short variant were less likely to play with peers than those homozygous for the long allele, whereas the rh5-HTTLPR genotype had no effect on the incidence of social play among mother-reared monkeys. Socially dominant mother-reared monkeys were more likely than their peer-reared counterparts to engage in aggression. In contrast, peer-reared but not mother-reared monkeys with the low-activity short allele exhibited more aggressive behaviours than their long/long variant counterparts. This genotype by rearing interaction for aggressive behaviour indicates that peer-reared subjects with the short allele, while unlikely to win in a competitive encounter, are more inclined to persist in aggression once it begins.

Since allelic variation of 5-HTT function is associated with anxiety-related traits as well as an increased risk for developing depression in the face of adversity, the impact of rh5-HTTLPR × MS interaction on stress-elicited endocrine responses was determined in infant rhesus macaques. Adrenocorticotropic hormone (ACTH) and cortisol plasma concentrations in monkeys reared with their mothers or in peer-only groups were determined at baseline and during separation stress at 6 months of age. Cortisol increased during separation and there was a main effect of rearing condition with decreased cortisol among peer-reared macaques. Monkeys carrying the rh5-HTTLPRs variant had higher ACTH. ACTH increased during separation, and there was a maternal deprivation × rh5-HTTLPR interaction, such that peer-reared short allele carriers had higher ACTH during separation than long variant homozygotes. A confirmatory study further revealed that this interaction is sexually

dichotomous and the interactive effect may underlie the increased incidence of certain stress-related disorders of emotional regulation in women (Barr et al. 2004b). These findings confirm the data from studies in human populations that allelic variation of 5-HTT function affects hypothalamic–pituitary–adrenal (HPA) axis activity and that the influence of rh5-HTTLPR on hormonal responses during stress is modulated by early life adversity and displays sex specificity (Mannie et al. 2009; O'Hara et al. 2007; Wust et al. 2009).

Previous research also revealed that peer-reared primates display a higher preference for alcohol compared to other young adults. Furthermore, maternally deprived female rhesus macaques show exaggerated HPA axis responses to alcohol (Higley and Linnoila 1997). Because their environments can be controlled, use of the macaque model permits investigation of independent influences as well as potential interactions between 5-HT signalling pathway-related genes, MS, and other stressors in the aetiology of alcohol dependence. Given that 5-HT signalling and HPA axis hormones are involved in the reinforcement of alcohol intake and contribute to the risk for symptoms of withdrawal and relapse in alcohol dependence in a gender-specific manner, the interactive effect of rh5-HTTLPR genotype and early rearing environment on the patterns of preference and consumption across a 6-week alcohol consumption paradigm was examined (Barr et al. 2004a). Female rhesus macaques were reared with their mothers in social groups or in peer-only groups. As young adults, they were then given the choice of an alcohol solution or vehicle. Interactions between rearing condition and rh5-HTTLPR genotype, with dramatically higher levels of ethanol preference, were demonstrated in s variant carriers. An effect of rearing condition on alcohol consumption during the 6 weeks was found as well as a phase × MS interaction, such that peer-reared animals progressively increased their levels of consumption. This was especially evident for peer-reared females carrying the rh5-HTTLPR s variant. Thus, the high composite scores for alcohol intake and alcohol-elicited aggression associated with the low-expressing short rh5-HTTLPR variant in female rhesus monkeys confirm an interaction between the 5-HT system activity and early aversive experience in the vulnerability to alcohol dependence and represent a clinically valid model for type II alcoholism.

Taken together, these findings provide substantial evidence of an environment-dependent association between allelic variation of 5-HTT expression and central 5-HT function and illustrate the possibility that specific genetic factors play a role in regulating behaviours in primates which are modulated by 5-HT signalling pathways. Because rhesus monkeys exhibit temperamental and behavioural traits which parallel anxiety, depression- and aggression-related trait dimensions in humans associated with the low activity short 5-HTTLPR variant, it may be possible to search for evolutionary continuity in this genetic mechanism for inter-individual differences. Non-human primate studies may also be useful to help identify environmental circumstances that compound the vulnerability conferred by a particular genetic makeup or, conversely, act to improve behavioural outcomes associated with a distinct genetic disposition.

4 5-HTT-Deficient Mouse: A Model for Epigenetic Programming of Development

Quantitative genetic research on rodent models is based primarily on inbred strain and selection studies. While comparisons between different inbred strains of mice expose remarkable differences in behavioural measures, differences within strains can be attributed to environmental influences. Inbred and recombinant inbred strain studies are highly efficient for dissecting genetic influences, for investigating interactions between genotype and environment and for testing the disposition-stress model. Furthermore, investigations in rodents have shown that intra- and extra-uterine maternal signals have long-lasting consequences on anxiety-like behaviour in the offspring and can synergistically induce long-term plastic changes in anxiety- and depression-related neural circuits.

Since humans and mice have almost the same genome size and share many orthologous genes mapped to syntenic chromosomal regions, it is conceivable that gene variations which influence a behavioural trait in humans may be modelled in the mouse. Based on the close similarity in the genomes between the two species and the extensive knowledge derived from the sequencing of the murine genome, mouse mutants have become the standard model. With the introduction of efficient gene targeting techniques, the mouse is the only mammal uniting the top-down and bottom-up genetic approach, from phenotype to gene and from gene to phenotype, respectively (see also Gondo et al. 2011; O'Tuathaigh et al. 2011 for discussion). Moreover, these mouse models provide efficient ways to control and manipulate environmental factors and allow dissection of the molecular mechanisms of G × E interactions.

In mouse, there is no analogue to the human and macaque 5-HTTLPR, but it is possible to either inactivate the *5-HTT* (Bengel et al. 1998) or to use transgenic approaches to increase its expression (Jennings et al. 2006). Inactivation of the murine 5-Htt and the resulting disturbance of brain 5-HT system homeostasis has considerably advanced our understanding of the neurobiological basis of anxiety and depression-related behaviour in mice (Lesch 2005; Murphy and Lesch 2008). 5-Htt$^{-/-}$ knockout mice show behaviours consistent with anxious and depressive traits; they appear reluctant to explore brightly lit spaces or elevated open platforms and give up struggling early when put in a stressful situation (Holmes et al. 2003). Remarkably, certain types of depression-like behaviour (e.g. behavioural despair) are manifest only after repeated exposure to stressors, which may be analogous to repeated or chronic stressful life events in humans (Wellman et al. 2007). 5-Htt$^{-/-}$ mice also show exaggerated neuroendocrine reactions to acute stress, similar to the increased HPA reactivity reported in some depressed patients. The effect of 5-Htt deficiency on anxiety and depression-like behaviours in mice may be mediated by reduced 5-HT clearance mechanism during a vulnerable developmental period but can still be selectively reversed by pharmacological 5-HT$_{1A}$ receptor inhibition later in life, suggesting an enduring modulatory involvement of the 5-HT system.

In addition, 5-Htt deficient mice provide a practical tool to study the impact of genetic mechanisms on the development and plasticity of the brain, (Altamura et al. 2006; Di Pino et al. 2004; Persico et al. 2001, 2003; Salichon et al. 2001). The anxious-depressive phenotype in 5-Htt$^{-/-}$ mice is associated with increased dendritic branching in fear associated circuits, including the medial prefrontal cortex and the amygdala (Wellman et al. 2007; Nietzer et al., in press). However, despite growing evidence for a critical role of the 5-Htt in the integration of synaptic connections in the mouse brain during critical periods of development and adult life, knowledge of the machinery involved in these fine-tuning processes remain incomplete.

Several studies used heterozygous 5-Htt$^{+/-}$ mice (displaying a 50% gene dose-dependent reduction of 5-Htt expression, thus representing a practical model for individuals with the short 5-HTTLPR variant). These studies found that, although the mice do not show behavioural deficits at baseline, they developed increased anxiety and depression-like behaviour in adulthood when exposed to prenatal stressors (Heiming et al. 2009; van den Hove et al. manuscript submitted), to early life adverse experiences including maternal neglect (Wellman et al. 2007) or to psychosocial stress in adult life (Bartolomucci et al. 2010; Jansen et al. 2010; Lewejohann 2010) (Fig. 3). It is proposed that such animal G × E paradigms serve

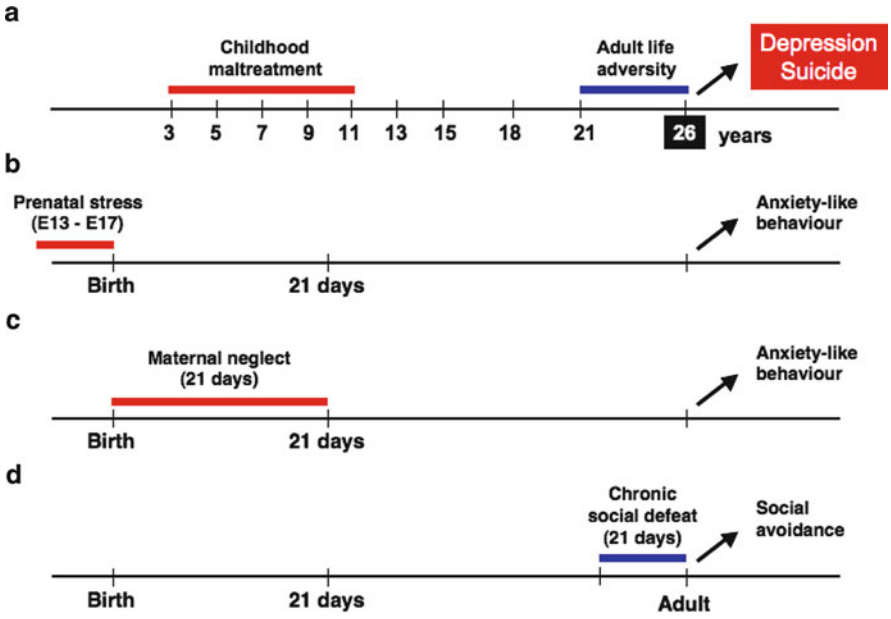

Fig. 3 Gene × enviromental adversity in the 5-HTT$^{+/-}$ mouse model based on the work by Caspi and coworkers (2003): (**a**), heterozygous 5-Htt$^{+/-}$ mice (displaying a 50% gene dose-dependent reduction of 5-Htt expression, thus representing a model for individuals with the short 5-HTTLPR variant) have used. These studies demonstrated that, although these mice do not show behavioural deficits at baseline, they developed increased anxiety and depression-like behaviour in adulthood when exposed to prenatal stressors (**b**), to early life adverse experiences including maternal neglect (**c**), or to psychosocial stress in adult life (**d**). These of modes of G × E serve as specific models for the increased vulnerability to environmental adversity across the lifespan in individuals with the low-expressing short 5-HTTLPR variant

as specific models for the increased vulnerability to environmental adversity across the lifespan in individuals with the low-expressing short 5-HTTLPR variant.

4.1 Prenatal Stress

Exposure to early life stress elicited by repeated mild electric footshock in the second postnatal week failed to further aggravate the increased anxiety and depression-like phenotype of 5-Htt deficient mice (Carroll 2007). Therefore, environmental adversity during early life may be more successfully modelled using ecologically relevant paradigms such as maternal neglect, simulation a threatening habitat for a female mouse and her pups or other species relevant adverse environmental challenges. Given the importance of early life stressors and their interaction with *5-HTT* genotype in the development of affective disorders in humans, it is important to model the interaction of 5-Htt deficiency with *prenatal* or *perinatal* stress in mice.

Heiming and co-workers (Heiming et al. 2009) exposed pregnant and lactating 5-Htt$^{+/-}$ females to the olfactory cues of unfamiliar adult males, signalling the risk of infanticide. When mothers had lived in a threatening environment, their offspring showed increased anxiety-like and reduced exploratory behaviour compared to controls and the effects were most pronounced in 5-Htt$^{-/-}$ mice. It was concluded that the behavioural profile of the offspring was shaped in an adaptive way, preparing the young for an adverse environment. When modulated by 5-Htt genotype, which alters 5-HT neurotransmission, offspring may develop potentially pathological levels of avoidance behaviour, which are determined by the G $\times$ E interaction.

Van den Hove and associates (2010, manuscript submitted) developed a prenatal stress paradigm by restraining pregnant 5-Htt$^{+/-}$ mice three times a day for 45 min in transparent glass cylinders filled with water up to a height of 5 mm, whilst being exposed to bright light. Prenatal maternal stress was performed daily during the last week of pregnancy (E13–E17). The results indicate that the long-term effects of prenatal stress on anxiety and depression-like behaviour in the offspring are partly dependent on the 5-Htt genotype. Genome-wide expression analysis of the hippocampus of these mice revealed G $\times$ E effects on apoptotic and psychoimmunological processes.

4.2 Maternal Neglect

Another species-relevant adverse environmental stressor in rodents is deficient maternal care in the first postnatal weeks. Previous investigations in rats indicated that maternal behaviour has long-lasting consequences on anxiety-like behaviour of the offspring. MS for several hours a day during the early postnatal period results

in increased anxiety-like behaviours as well as increased stress hormone reactivity in adult animals (Kalinichev et al. 2002). Similarly, pups that are raised by mothers that display low licking and grooming behaviour show higher levels of anxiety-like behaviour than pups raised by high licking and grooming mothers, and cross fostering studies show that these influences are primarily environmental (Caldji et al. 1998; Liu et al. 2000). Cross fostering offspring of low licking and grooming mothers to high licking and grooming mothers is able to impart low anxiety-like behaviour to the offspring, whereas the converse does not influence this behaviour. Offspring of high licking and high grooming mothers raised by low licking and grooming mothers do not show high anxiety-like behaviour, suggesting that specific genes inherited by the high licking and grooming offspring protect them from the effects of low licking and grooming mothering. Furthermore, Francis et al. (1999) have shown that the effect of high licking and grooming can be passed from one generation to the next. Females raised by high licking and grooming mothers themselves become high licking and grooming mothers and go on to produce low anxiety offspring regardless of whether their biological mother showed low or high licking and grooming. This epigenetic inheritance of anxiety-like behaviour underscores the power that environmental influences can exert to persistently remodel circuits in the brain during early development.

Studies using mice of defined genetic backgrounds have also begun to shed light on the molecular mechanisms of specific G × E interactions. Anisman et al. (1998) found that mice of the low licking and grooming Balb/c inbred strain cross-fostered at birth to the high licking and grooming C57BL/6 inbred strain display improvements in a hippocampus dependent memory task. Because the reverse cross fostering, where C57BL/6 pups are raised by Balb/c mothers, does not alter the behaviour of C57BL/6 mice, it appears that the C57BL/6 genetic background protects the pups from the effects of a Balb/c maternal environment. However, by transplanting C57BL/6 embryos into Balb/c foster mothers shortly after conception, Francis et al. (2003) were able to show that a combined *prenatal* and *postnatal* Balb/c maternal environment is sufficient to confer Balb/c behaviour on C57BL/6 offspring, demonstrating that intra- and extra-uterine maternal signals can synergistically induce long-term plastic changes in anxiety- and depression-related neural circuits.

To study the neural and molecular mechanisms underlying epigenetic programming by early adverse environment in an animal model amenable to genetic manipulation a G × E paradigm was developed in the mouse (Fig. 4a, b). It was shown that the effects of an adverse rearing environment on anxiety-related behaviour are modulated by mutations in *5-HTT* in a way that mimics the interaction between early stress and 5-HTT seen in humans (Carola et al. 2008). Mice experiencing low maternal care showed deficient gamma-aminobutyric acid-A (GABA-A) receptor binding in the amygdala and heterozygous 5-Htt$^{+/-}$ mice showed increased anxiety and depression-like behaviour and decreased serotonin turnover in hippocampus and striatum (Fig. 4c). Strikingly, levels of brain-derived neurotrophic factor (BDNF) mRNA in hippocampus were elevated exclusively in 5-Htt$^{+/-}$ mice experiencing poor maternal care, suggesting that developmental programming

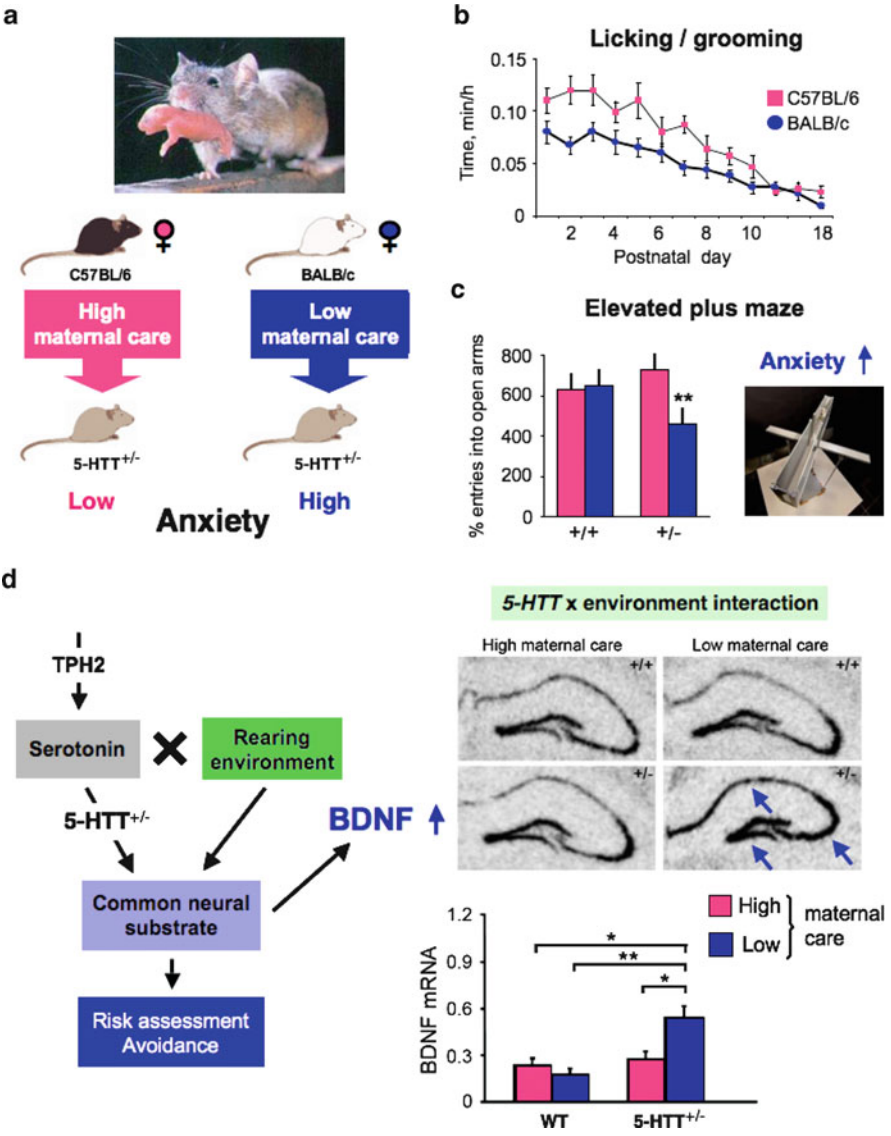

Fig. 4 Interaction between rearing environment and 5-Htt function in mice: BDNF as a molecular substrate of both *5-Htt* deficiency and maternal care (modified from Carola et al. 2008). (**a**) and (**b**), Epigenetic programming by early adverse environment in mice deficient for 5-Htt. The effects of maternal neglect on anxiety-like behaviour is modulated by 5-Htt inactivation in a way that mimics the interaction between early life stress and 5-HTTLPR genotype observed in rhesus macaques and humans. (**c**), heterozygous 5-Htt$^{+/-}$ mice experiencing maternal neglect showed increased anxiety-like behaviour. (**d**), Identification of neural and molecular substrates of G × E interaction: BDNF mRNA concentrations in hippocampus are elevated exclusively in heterozygous 5-Htt$^{+/-}$ mice experiencing poor maternal care, suggesting that developmental programming of hippocampal circuits may underlie the *5-HTT* × E adversity risk factor

of hippocampal circuits may underlie the *5-Htt* × E risk factor (Fig. 4d). These findings demonstrate that 5-HT plays a similar role in modifying the long-term behavioural effects of rearing environment in mice as it does in non-human primates and identifies BDNF as a possible molecular substrate of this risk factor. It is therefore predicted that some of the principle neural and molecular mechanisms which underlie G × E interactions in mouse and non-human primate models are relevant to the aetiology of disorders of emotional regulation in humans.

4.3 Chronic Psychosocial Stress in Adulthood

Although epidemiological evidence links exposure to stressful life events with increased risk for disorders of emotional regulation, there is significant individual variability in vulnerability to environmental cues and the penetrance of genetic variation is thought to play a major role in determining who will develop disorders. Identifying the molecular mechanisms underlying this G × E risk factor will facilitate an understanding of the individual differences in resilience to stress. Therefore, a mouse model of the *5-HTT* × adult life stress associated with winner or loser experience in a resident-intruder paradigm was recently generated.

Jansen et al. (2010) reported that male mice of all three 5-Htt genotypes experiencing social defeat on three consecutive days displayed increased anxiety-like behaviour and decreased exploration, irrespective of winning or losing. In losers, a distinct effect of genotype occurred. Homozygous 5-Htt$^{-/-}$ males showed more anxiety-like behaviour and less exploration than the other genotypes. In winners, no genotype-dependent variation was found. Genotypes did not differ in basal activation of the stress hormone system but there was a main effect of social experience with higher corticosterone levels in losers compared to winners. This effect was most pronounced in the heterozygous 5-Htt$^{+/-}$ mice, indicating that anxiety circuits and stress reactivity retain their plasticity throughout adulthood and can be shaped by genotype and social experiences during this phase of life.

Bartolomucci et al. (2010) subjected wild-type and heterozygous 5-Htt$^{+/-}$ male mice to 3 weeks of chronic psychosocial stress in a dominant/subordinate context of a resident-intruder paradigm (Fig. 5). The 5-Htt genotype did not affect the physiological consequences of stress as measured by changes in body temperature, body weight gain and plasma corticosterone. However, when compared with wild-type littermates, heterozygous 5-Htt$^{+/-}$ mice experiencing high levels of stressful life events, in the form of repetitive social defeat, showed significantly depressed locomotor activity and increased social avoidance toward an unfamiliar male in a novel environment. 5-Htt$^{+/-}$ mice exposed to high aggression stress also showed significantly lower levels of 5-HT turnover than wild-type littermates, selectively in the frontal cortex, which is a structure that is known to be involved in fear control and avoidance responses, and that is implicated in susceptibility to depression. These data point toward a useful animal model for better understanding the increased

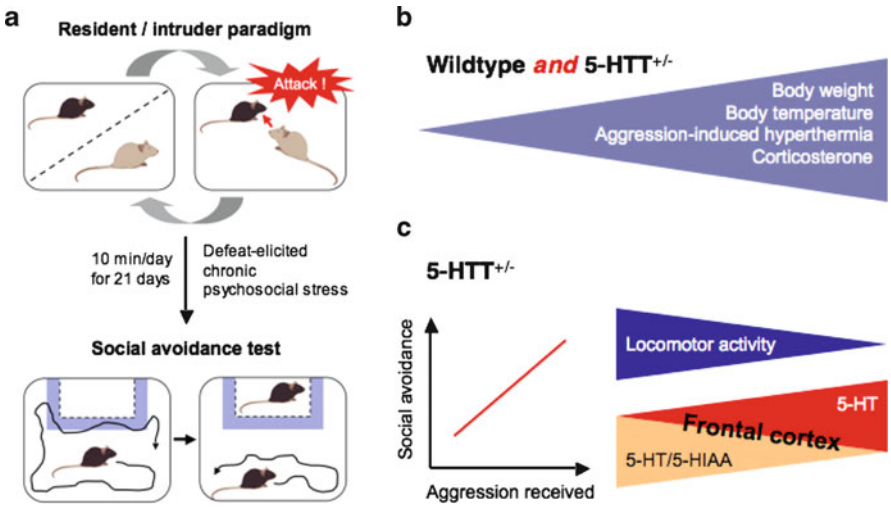

Fig. 5 The chronic social defeat mouse model (modified from Bartolomucci et al. 2010): Increased social avoidance (**a**), decreased locomotor activity, and increased serotonin concentrations (**c**) in the frontal cortex of mice in 5-Htt$^{+/-}$ mice receiving highest aggression in the resident-intruder paradigm, whereas both wildtype and 5-Htt$^{+/-}$ mice show increased body weight, body temperature, aggression-induced hyperthermia, and corticosterone plasma concentrations following social defeat (**b**)

vulnerability to stress which has been reported in individuals carrying the low-expressing short 5-HTTLPR variant, and suggest that social avoidance represents a behavioural endophenotype of the interaction between 5-HTT and psychosocial stress.

To identify changes in mRNA expression of novel candidate genes associated with altered prenatal, rearing and adult psychosocial environment and/or 5-HTT genotype, genome-wide microarray-based expression profiling of mRNA extracted from brain region-specific tissue and laser-dissected single neurons will have to be performed in mice from the various G × E paradigms (Ichikawa et al. 2008) (Fig. 7). By determining differential gene expression resulting from chromatin modification, future experiments promise to identify genes that are epigenetically regulated by environmental adversity and *5-Htt* in the mouse. These genes are likely to be candidate susceptibility genes for anxiety disorders and may serve as potential diagnostic and therapeutic targets. The evidence for epigenetic inheritance of anxiety and depression-like behaviour underscores the view that environmental influences can persistently remodel circuits in the brain during early development. 5-Htt deficient and other genetically modified mice will therefore be essential for the dissection of the molecular and neural mechanisms of epigenetic processes and of the neurodevelopmental–behavioural interface (Lesch and Mössner 2006).

5 Neural Mechanisms of Epigenetic Programming

As already outlined clinical evaluation and self-report of life events revealed that the effect of psychosocial stress on depression and suicidal behaviour is modified by allelic variation of 5-HTT function, which renders carriers of the short 5-HTTLPR variant more vulnerable to depression (see Sect. 2). As a first approximation toward identifying the neural circuits involved in controlling these epigenetic processes, individuals with self-reported life stress but no history of psychopathology were investigated with *multimodal* magnetic resonance imaging (MRI)-based imaging (functional, perfusion, structural). Based on functional MRI and perfusion data, support was found for a model by which life stress interacts with the effect of 5-HTTLPR genotype on amygdala and hippocampal resting activation, which may provoke a chronic state of vigilance, threat or rumination (Canli et al. 2006). Life events also differentially affected, as a function of 5-HTTLPR genotype, functional connectivity of the amygdala and hippocampus in response to emotional stimuli with a wide network of other regions, as well as grey matter structural features. These interactions may constitute a neural mechanism for epigenetic vulnerability or resilience against depressive illness and may have a morphological substrate in the increased dendritic branching and spine density within the fear circuit of 5-HTT deficient mice, including both the medial prefrontal cortex and the amygdala (Wellman et al. 2007; Nietzer et al. in press) (Fig. 6).

By the same token, the previously reported whole-brain analyses of activation, functional connectivity and grey matter density and volume also showed a moderation of the same prefrontal cortex-amygdala circuitry by 5-HTTLPR genotype × life stress interaction (Canli et al. 2006). In addition, the superior parietal lobule, superior temporal gyrus, inferior frontal gyrus, precentral gyrus and insula were affected by *5-HTT* × E. The remarkable fact about these regions is that they belong to circuits that integrate imitation-related behaviour, from which social cognition and behaviour in a social world has evolved (Iacoboni 2005). Social cognition is a construct comprising representations of internal somatic states, interpersonal knowledge and motivations as well as procedures used to decode and encode the self relative to other people. This complex set of processes, which are carefully orchestrated to support skilled social functioning and communication-facilitated networking, has recently been associated with activity in distinct neural circuits (Adolphs 2009; Kitayama and Park 2010).

Regions involved in imitation, imitative learning, social cognition and communication skills (Amodio and Frith 2006; Carr et al. 2003), and affected by *5-HTT* × life stress, include the superior parietal lobule, superior temporal gyrus, inferior frontal gyrus, precentral gyrus, insula, anterior cingulate and amygdala. Some of these regions contain mirror neurons (Rizzolatti and Craighero 2004; Uddin et al. 2005), which are activated during goal-directed behaviour or the observation of such behaviour in others, and Von Economo neurons, which are believed to play a role in social bonding. Dysfunction of both neural units is thought to cause social and communication disabilities associated with autistic syndromes (Allman et al. 2005; Dapretto et al. 2006). The morphological alterations in 5-HTT deficient mice

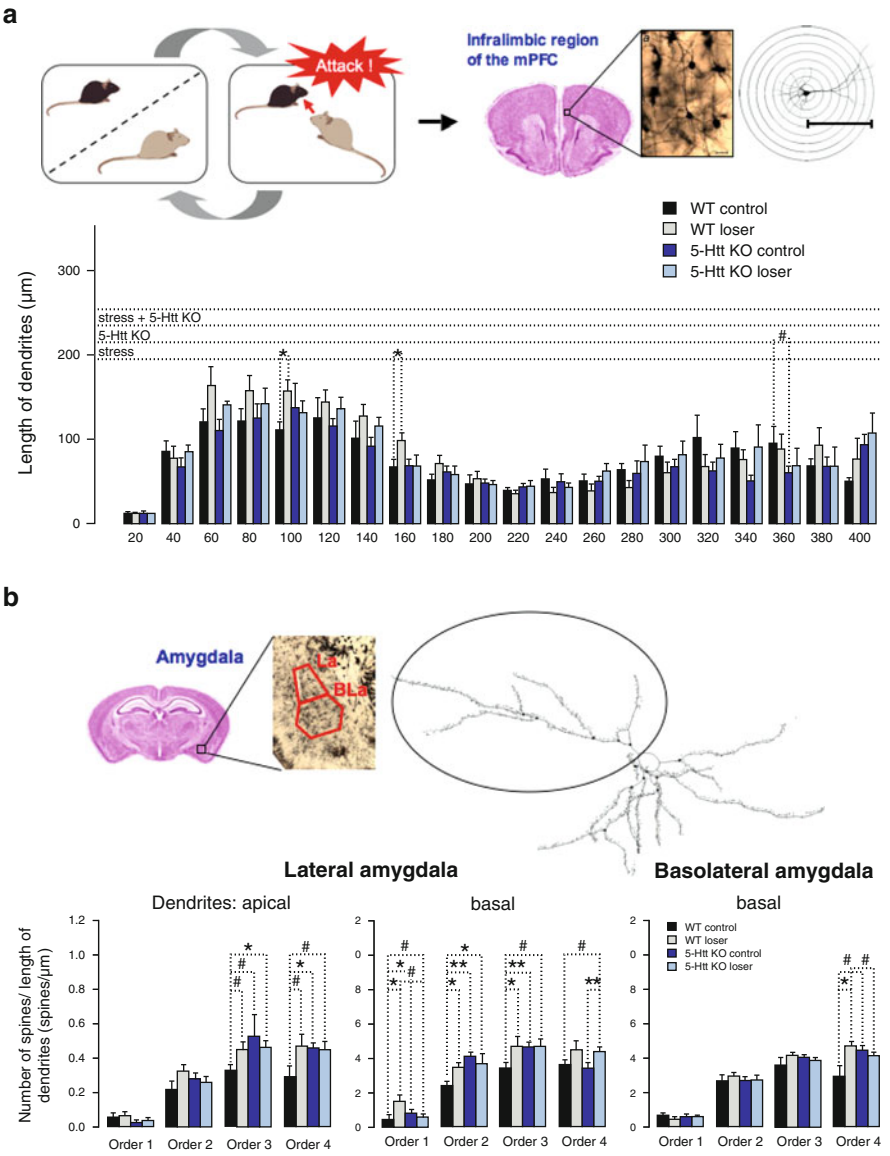

Fig. 6 Social defeat increases proximal length of apical dendrites in the medial prefrontal cortex (mPFC) (**a**), while both 5-Htt genotype and social defeat modify spine density in the amygdala (**b**) (modified from Nietzer et al., in press).

in conjunction with data G × E functional MRI data suggests that social competence and behaviour involving mirror or Von Economo neurons are neural targets of epigenetic moderation may be subject to an interaction between psychosocial adversity and 5-HTTLPR genotype (Canli and Lesch 2007).

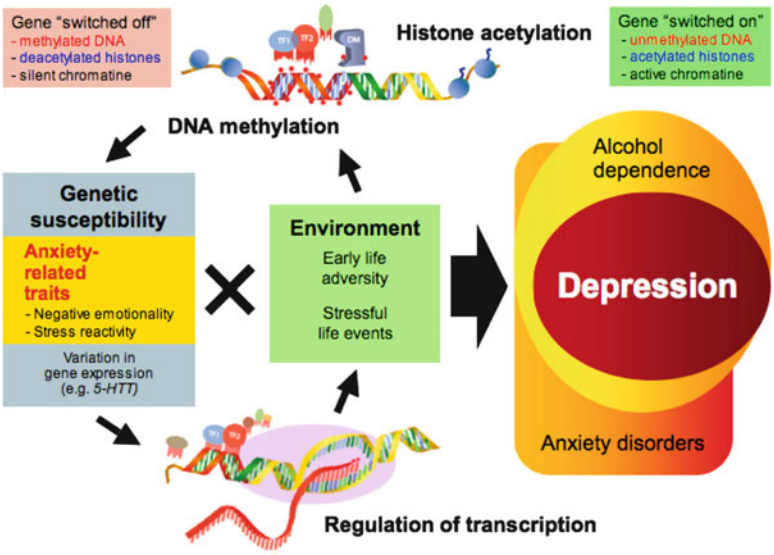

Fig. 7 The molecular cycle of epigenetics in the susceptibility of depression and related co-morbid disorders: Gene variation affecting transcriptional control is permissive for environmental adversity moderating epigenetic mechanisms that involve methylation of DNA and acetylation of histones which, in turn, impacts regulation of gene expression. Silencing chromatin by DNA methylation and histone deacetylation switches genes off, whereas activation of chromatin by DNA demethylation and histone acetylation switches genes on

Future psychophysiological and morphofunctional imaging studies in both the animal model and the humans will have to address whether an interaction of 5-HTTLPR and life stress moderates neural activation during imitation or social processing tasks. As investigations have begun to refine the methodologies for capturing epigenetic effects on the brain, we may better understand the mechanisms that render some individuals susceptible and others resilient to depression and other affective spectrum disorders.

6 "To Be or Not to Be": *5-HTT* × Psychosocial Adversity Interaction Is Subject to Evolutionary Pressure

Recently, a series of studies have emerged showing that the short 5-HTTLPR variant is associated with improved cognitive functions. Therefore, these factors may counteract or completely offset the negative consequences of the anxiety and depression-related traits (for review: Homberg and Lesch in press). For instance, it has been shown that carriers of the 5-HTTLPR short-allele exhibited increased emotionally cued startle responses (Brocke et al. 2006) and a stronger attentional

bias for anxious word stimuli (Beevers et al. 2007) along with greater difficulty disengaging their attention from stimuli with strong negative or positive valence (Beevers et al. 2009), and a bias to focus on positive images (Beevers et al. 2010). It has also been shown that in depression carriers of the short variant benefit most from social support (Brummett et al. 2008), indicating that sensitivity to positive stimuli can alleviate the negative consequences of sensitivity to adverse events. In a social context, short-allele carriers display more aggressive behaviours (Schwandt et al. 2010) and are characterized by an increased vulnerability to the adverse effects of psychosocial stress associated with subordinate status (Jarrell et al. 2008).

An analogous attentional bias in rhesus monkeys is particularly striking: The short variant of the rh5-HTTLPR has been associated with a reduction in time spent gazing at images of faces compared to non-face images, less time looking in the eye regions of faces and larger pupil diameters when gazing at photos of a high versus low status male macaques (Watson et al. 2009). The reluctance of rhesus macaques carrying the short allele to gaze directly at the eyes and faces of con-specifics, as well as their enhanced sympathetic response to the images of high-status males, suggests that these individuals experience greater anxiety than those homozygous for the long variant when viewing potential social threats. The greater pupil diameter indicates that the short-allele carrying monkeys found images of high-status male faces to be more arousing, which is in harmony with the observations of Beevers and associates (Beevers et al. 2010) that humans carrying the short-allele tend to exhibit a bias to focus on positive images and thus may experience more arousal when viewing rewarding pictures.

At the level of cognitive flexibility, it was found that the 5-HTTLPR short-allele is associated with improved performance in trials of attentional set shifting (Borg et al. 2009), which is in accord with increased attention towards task parameters (Roiser et al. 2007). The cognitive enhancement is also consistent with the observation that short-allele carriers show increased processing of negative feedback (Althaus et al. 2009). In rhesus monkeys, the short-allele of the rh5-HTTLPR was associated with superior performance on an array of cognitive tasks: the probability discounting task, the delay discounting task, the reversal learning task and the delayed match-to-sample task (Jedema et al. 2009). The better performance in the probability discounting task is consistent with the increased attention to high and low probabilities in the risky decision-making task in humans carrying the short 5-HTTLPR variant (Roiser et al. 2006) and may be explained by augmented cortico-limbic activation (Fallgatter et al. 1999, 2004) and superior ability to integrate feedback information over time to guide behaviour on subsequent choices (Althaus et al. 2009).

Connected to the medial prefrontal cortex-amygdala circuitry, other brain regions implicated in allelic 5-HTT function-mediated behavioural responses are the orbitofrontal cortex (OFC), the pulvinar nucleus of the thalamus and the bed nucleus stria terminalis. The OFC shows prominent morphological alterations and differential functional activation in response to environmental manipulation in rhesus monkeys carrying the short allele (Kalin et al. 2008). The OFC signals representations of expected outcomes and compares an expected with the actual

outcome of behaviour. When incongruent, it modulates the activity of downstream brain areas that are involved in response selection and action, such as the amygdala and striatum. In that way, increased OFC activity may allow short-allele carriers to flexibly adapt behaviour when a mismatch is detected between the expected and actual outcome of behaviour, for instance during reversal learning in rhesus macaques (Jedema et al. 2009).

Taken together, the studies in non-human primates complement the findings in human on emotionality in social settings and suggest that the 5-HTTLPR short-variant is associated with increased attention (Roiser et al. 2007) to task parameters and may contribute to improved decision making and improved cognitive flexibility (Borg et al. 2009; Jedema et al. 2009; Roiser et al. 2006, 2009). Moreover, it may be linked with heightened social vigilance and, generally, sensitivity to environmental cues per se, which could result in maladaptive responsiveness when danger or social threats are absent. However, it may also be highly adaptive in distinct contexts or circumstances and consequently subject to positive selective pressure. That is, seizing opportunities and the simultaneous avoidance of potential harmful antagonistic interactions may lead to social (and thus, reproductive) success.

Heightened vigilance, i.e. high attentional biases directed towards motivationally relevant stimuli, may be the common denominator in the high emotionality and cognitive enhancement associated with allelic variation of 5-HTT function (Homberg and Lesch in press). The challenge is now to understand how the brain determines when to respond either emotionally or cognitively depending on environmental stimuli. Integration of these findings will provide novel hypotheses for the understanding of the mechanisms underlying the co-occurrence of anxiety-related traits and enhanced cognitive function in association with *5-HTT* × E interaction.

7 Molecular Mechanisms of Epigenetic Programming

The molecular mechanisms by which early stress increases risk for disorders of emotional regulation in adulthood is not known, but is presumed to include epigenetic programming of gene expression (see Bountra et al. 2011; Weaver et al. 2004, 2006). Identifying the molecular mechanisms behind the long-term behavioural effects of altered rearing environments is a major goal of research in the field of developmental programming. Persistent changes in the expression of several genes have been documented in adult animals exposed to altered rearing environments. Decreased expression and function of the glucocorticoid receptor (GR) has been associated with a low maternal care environment and has been the subject of several studies (for review: Seckl and Meaney 2004). Decreased GABA-A receptor binding and subunit expression as well as increased corticotropin-releasing hormone (CRH) mRNA have been documented in the amygdala of offspring of low licking and grooming mothers and is proposed to be required for their increased anxiety-related and stress response behaviour, respectively (Calatayud et al. 2004; Caldji et al. 1998). Changes in hippocampal expression of NMDA receptor subunits, BDNF,

neural cell adhesion molecule (NCAM), synaptophysin and acetylcholine esterase have also been reported in offspring of low versus high licking and grooming mothers (Liu et al. 2000). Recent work in rats has suggested that changes in the activity of the GR promoter play a critical role in the epigenetic programming of adult stress response by rearing environment. Decreased methylation and increased histone 3 acetyl-K9 binding of the GR I7 promoter was found in hippocampus of adult rats receiving low licking and grooming (Weaver et al. 2004). Treatment of these rats in adulthood with the histone deacetylase inhibitor (HDAC), tricostatin A, reversed the effects of low licking and grooming on GR expression. Remarkably, tricostatin treatment also reversed the effects of rearing environment on stress hormone responses, demonstrating a role for chromatin remodelling in maintaining the long-term effects of rearing environment. In a parallel study by the same group, supplementing the diet of high licking and grooming mothers with methionine in adulthood increased GR promoter methylation and stress response, suggesting that epigenetic programming of GR can be pharmacologically adjusted either up or down with corresponding changes in stress response activity (Weaver et al. 2005).

8 Conclusions and Outlook

Modest advances in behavioural genetics are contrasted by giant leaps in an *epigenomic* era still in its infancy. The application of paradigms novel to neurogenetic approaches including generation of genetically modified mice, validation of G × E models in non-human primates and rodents, application of functional neuroimaging and next-generation sequencing technologies in the quest for rare genetic variants with high effect size but variable penetrance and inclusion of a more extensive phenotypic spectrum (e.g. higher cognitive functions, social competence, resilience) have strengthened the connection between 5-HTT, (social) cognition and emotionality and continue to enable a more profound understanding of how common and rare genetic variation modulates human behaviour.

In this overview I have attempted to integrate findings from studies in G × E animal models which underscore the central role of 5-HT and its fine-tuning by 5-HTT functionality in embryonic patterning events, brain development, and synaptic plasticity, particularly in neural circuits related to (social) cognitive and emotional processes. Increasing evidence suggests that epigenetic mechanisms may play a crucial role in neurodevelopmental programming of behavioural abnormalities. Among a number of environmental cues, social environment can act as a primary risk for the development of anxiety and depression-related phenotypes (Fig. 8). The best evidence derives from findings of developmental outcomes associated with institutional deprivation that consistently highlight the increased rates of disorders of emotional regulation. Further support comes from the risk-attenuating role of alternative social environments through resilience-focused therapeutic intervention. Moreover, the social environment has been shown to determine the extent to which patients with affective disorders develop co-morbidity. Through

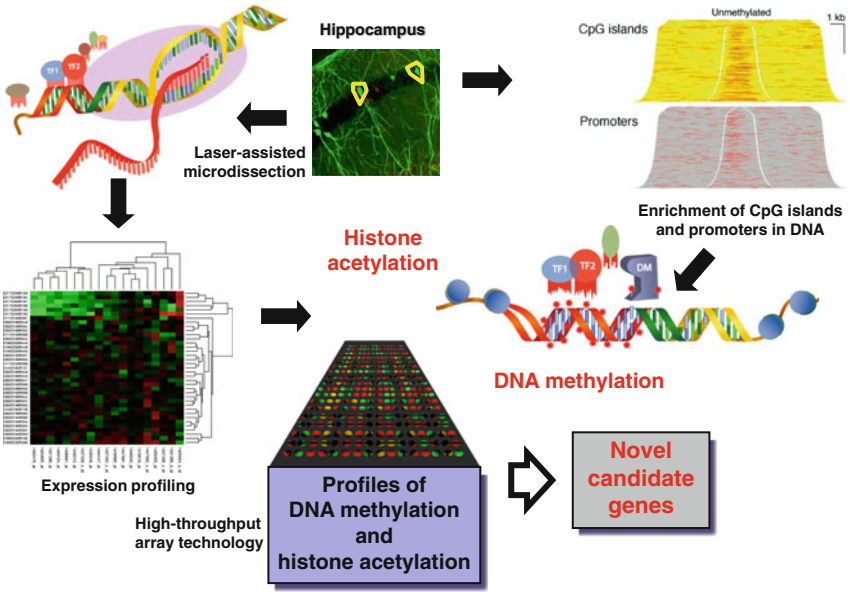

Fig. 8 Changes in mRNA expression of novel candidate genes associated with altered rearing environment and/or 5-Htt genotype may be identified by microarray-based expression profiling of mRNA extracted from brain region-specific tissue and laser-guided dissection of homogeneous neuron populations in mice from G × E paradigms. Whole-genome expression screening methods combined with high-throughput methylation and histone acetylation profiling microarray techniques both genome-wide and of selected genes in mice from stress-specific G × E paradigms promise to identify genes that are epigenetically regulated by *5-Htt* × E interaction in the mouse. These genes are likely novel candidate susceptibility genes for disorders of emotion regulation and will serve as potential diagnostic and therapeutic targets

modification of DNA and associated histones, epigenetic mechanisms translate environmental stimuli into changes in gene expression as neural correlates of epigenetic programming. Epigenetically influenced behavioural modifications are accompanied by changes in gene expression. Thus, these mechanisms are hypothesized to play an essential role in the interplay of genetic and environmental cues in determining anxiety and depression-related phenotypes. Epigenetic markers identified by genome-wide expression, DNA methylation, and histone modification profiles are dynamic and reversible and may also provide powerful targets for pharmacological intervention strategies. Therefore, deeper insight into the role of epigenetic regulation in the process of neurodevelopmental programmes, in particular in relation to depression and co-morbid disorders, will contribute to the establishment of early diagnosis and the development of innovative treatments targeting neural pathways that foster resilience.

Finally, anxiety disorders and depression are known to be influenced not only by environmental stressors but also by each individual's unique genetic background. The mouse models also permit analysis of synthetic mutant phenotypes or

polygenic characteristics based on epistatic interaction and pleiotropy. Epistasis with genes with strong evidence-based rationale, such as BDNF, has a potential to further refine the concept and indicate mechanisms underlying the observed G × E interaction (Ren-Patterson et al. 2005). However, the majority of neural substrates and circuitries that regulate emotional processes or cause affective disorders remain remarkably elusive. Among the reasons for the lack of progress are remaining conceptual deficiencies regarding the genetic and neural architecture of emotionality and behavioural despair, which make it difficult to develop and validate reliable models of depression. As analyses of epigenomes in non-human primates and rodents will likely contribute fundamentally to our understanding how humans have evolved, the next stage of complexity concerns the nature of genetic variation, either common or rare, and epigenetic programming among humans and its influence on inter-individual differences at the level of physiology and disease pathogenesis, as well as the relative impact of genetic and environmental determinants on cognition, emotion and, ultimately, behaviour.

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Animal Models of Epigenetic Regulation in Neuropsychiatric Disorders

Chas Bountra, Udo Oppermann, and Tom D. Heightman

Contents

Abstract Epigenetics describes the phenomenon of heritable changes in gene regulation that are governed by non-Mendelian processes, primarily through biochemical modifications to chromatin structure that occur during cell development and differentiation. Numerous lines of evidence link abnormal levels of chromatin modifications (either to DNA, histones, or both) in patients with a wide variety of

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diseases including cancer, psychiatry, neurodegeneration, metabolic and inflammatory disorders. Drugs that target the proteins controlling chromatin modifications can modulate the expression of clusters of genes, potentially offering higher therapeutic efficacy than classical agents with single target pharmacologies that are susceptible to biochemical pathway degeneracy. Here, we summarize recent research linking epigenetic dysregulation with diseases in neurosciences, the application of relevant animal models, and the potential for small molecule modulator development to facilitate target discovery, validation and translation into clinical treatments.

Keywords Chromatin · Epigenetics · Histone modifying enzymes · Inhibitors

Abbreviations

2-OG	2-Oxo-glutarate
AAV	Adeno-associated virus
BDNF	Brain-derived neurotrophic factor
BET	Bromodomain and extra C-terminal domain protein
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
CDK5	Cyclin-dependent kinase 5
ChIP	Chromatin immunoprecipitation
CoA	Coenzyme A
CpG	Short stretches of DNA in which the frequency of C and G base pairs are higher than other regions
CREB	cAMP response element binding protein
CRH	Corticotrophin releasing hormone
CTCL	Cutaneous T-cell lymphoma
DNMT	DNA methyltransferase
FAD	Flavin adenine dinucleotide
FPC	Frontopolar cortex
FRET	Forster resonance energy transfer
GABA	Gamma-aminobutyric acid
GR	Glucocorticoid receptor
H3K9Me2	Histone H3 dimethylated at lysine-9 ε-nitrogen
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HSV	Herpes simplex virus
IKB	IκB transcription factor
IKK	IκB kinase
JMJD	Jumonji (demethylase) domain
KDM	Lysine demethylase
KO	Knockout
LG	Licking and grooming
LSL	Lox P-stop-Lox P cassette

LTP	Long-term potentiation
MAOI	Monoamine oxidase inhibitor
MBD	Methyl-CpG-binding domain protein
MBT	Maligant brain tumour domain
MeCP2	Methyl CpG binding protein 2
miRNA	MicroRNA
NAc	Nucleus accumbens
NFκB	Nuclear factor-kappaB
NGFIA	Nerve growth factor inducible protein A
PHD	Plant homeodomain
PRMT	Arginine methyltransferase
PTGS	Post-transcriptional gene silencing
SAHA	Suberoyl-aniline hydroxamic acid
SirT	Sirtuin (NAD^+-dependent histone deacetylase)
SUMO	Small ubiquitin-like modifier
TSA	Trichostatin A
TSS	Transcription start site
WT	Wild-type

1 Introduction: Epigenetic Regulation of Gene Expression

The last decade has seen an impressive amount of data that has followed work by developmental biologist, Conrad Waddington, who created the concept of epigenetics. This basic model articulates how identical genotypes can result in a wide collection of phenotypes as development and differentiation proceeds in an organism (Waddington 1957). When Waddington coined the term, the physical nature of genes and their role in heredity was not known; he used it as a conceptual model of how genes might interact with their surroundings to produce a phenotype. Over time, this paradigm of phenotypic landscapes took an additional meaning by including "potentially heritable changes in gene expression that do not involve changes in DNA sequence" (Jaenisch and Bird 2003). It is now widely accepted that the eukaryotic chromatin structure is at the basis of all phenomena that are now collectively ascribed to "epigenetics" – that is gene regulation, gene imprinting or X chromosome inactivation. It is also obvious that now the original meaning of "epigenetics" is becoming more blurred, since many researchers use widely the phrase "epigenetics" also in the context of, for example genome integrity or cell-cycle regulation to describe in general chromatin modifications, architecture or gene regulation, thus spurring a semantic debate (Petronis 2010).

It is clear that chromatin states must be considered as highly dynamic and plastic, and hence any mechanism that changes a chromatin state is implicated as being potentially involved in epigenetic function. Chromatin modifications that have been related to epigenetic functions include methylation of DNA; post-translational modifications to chromatin proteins, especially histones; and

microRNAs, a recent addition to epigenetic mechanisms which add another layer of complexity to chromatin function and gene regulation. The chemical modification of chromatin components (often referred to as "marks") is not uniformly distributed in the genome, but is applied regionally where it serves to regulate "local" activity such as gene transcription or silencing. There are two important aspects to consider: first, several of the modifications appear "stable" and can be inherited from one cell or even organism to the next generation, thereby underpinning the concept of epigenetic landscape and heritability. Second, despite this apparent stability, it is now clear that most if not all modifications are potentially reversible, thus creating a high level of plasticity, allowing cells to respond dynamically to, for example, environmental cues. In this chapter, the different aspects of epigenetic mechanisms are briefly described, before outlining specific examples of the application of animal models to understand the role of epigenetics in neuropsychiatric system function. Finally, we outline progress in chemical biological approaches to investigate and dissect epigenetic phenomena in more detail.

1.1 DNA Methylation as Epigenetic Mark

In eukaryotes ranging from plants to humans, DNA methylation is found exclusively at the C5 position of the pyrimidine ring of cytosine residues. DNA methylation frequently occurs in repeated sequences, and helps to suppress the expression and mobility of transposable elements. The majority of all CpGs are methylated in mammals, whereas unmethylated CpGs are grouped in clusters called "CpG islands" (short stretches of DNA in which the frequency of C and G base pairs are higher than other regions) that are present in the 5′ regulatory regions of many genes (Klose and Bird 2006). Importantly, methylation patterns differ greatly between somatic and stem cell types as revealed by genome-wide methylcytosine sequence analysis (Lister et al. 2009). Furthermore, the discovery of hydroxymethylcytosine in mammalian cells (Penn et al. 1972; Kriaucionis and Heintz 2009), its implication in methyl DNA analysis, and its differential distribution in chromatin reveals that the "landscape" of DNA modification is not yet fully understood (Huang et al. 2010; Penn et al. 1972; Loenarz and Schofield 2009; Tahiliani et al. 2009). In many disease processes such as cancer, gene promoter CpG islands achieve abnormal hypermethylation (Smiraglia et al. 2001), which results in heritable transcriptional silencing, for example of tumour suppressor genes, which in turn provides the rationale for methyltransferase inhibitors such as azacytidine as epigenetic therapies. DNA methylation patterns are known to be established and modified in response to environmental factors by a complex interplay of at least three independent DNA methyltransferases, DNMT1, DNMT3A and DNMT3B, the loss of any of which is lethal in mice (Li et al. 1992). DNMT1 transfers patterns of methylation to a newly synthesized strand after DNA replication to hemimethylated DNA, and therefore is referred to as the "maintenance" methyltransferase. DNMT1 is essential for proper embryonic development, imprinting and X-inactivation (Goll and Bestor 2005).

DNA methylation may regulate the transcription of genes in two ways: directly, by impairment of binding of transcriptional regulators to the gene; and indirectly, by binding of methyl-CpG-binding domain (MBD) proteins to methylated DNA (Klose and Bird 2006). These MBD proteins recruit additional proteins to the target site, such as histone deacetylases, methyltransferases and other chromatin remodelling proteins that can modify histones, thereby forming inactive, silent chromatin (Fuks et al. 2003). Notably, loss of methyl-CpG-binding protein 2 (MeCP2) has been implicated in Rett syndrome (Kriaucionis and Bird 2003), and methyl-CpG-binding domain protein 2 (MBD2) mediates the transcriptional silencing of hypermethylated genes in cancer (Klose and Bird 2006).

1.2 Histone Modifications Dictate Chromatin Structure and Regulate Transcription

Eukaryotic DNA is packaged into a chromatin structure, consisting of repeating nucleosomes formed by wrapping 146 base pairs of DNA around an octamer of four core histones (H2A, H2B, H3, and H4). It has long been known that covalent modification of the proteinaceous component of chromatin, especially histones, plays an essential role in chromatin biology and epigenetics. The histones, particularly their N-terminal tails, are subject to a large number of post-translational modifications (Kouzarides 2007), which establish global chromatin environments, thereby regulating gene expression and genome function, and mediating DNA-based biological processes (see above). The H3 and H4 histones have long tails protruding from the nucleosome which can be covalently modified at multiple positions. Modifications of the tails include methylation, acetylation, phosphorylation, ubiquitination, sumoylation, citrullination and ADP-ribosylation (Kouzarides 2007) (Fig. 1 and Table 1).

Modification of specific residues results in distinct outcomes, for example acetylation of lysine residues in histones is associated with transcriptionally active genes, whereas the effect of lysine methylation on epigenetic functions is sequence and methylation-state specific (Kouzarides 2007). The cores of histones H2A and H3 can also be modified. The combinations of modifications are thought to form the so-called "histone code" (Strahl and Allis 2000; Jenuwein and Allis 2001) mediated by a system of so-called "readers, writers and erasers" (Table 2) allowing the chromatin to adopt a highly complex and dynamic nature.

1.3 MicroRNAs Emerge as a Novel Epigenetic Mechanism

MicroRNAs are short ~22 nucleotide RNA sequences that bind to complementary sequences in the 3′ untranslated region of multiple target mRNAs, usually resulting in their silencing (Bartel 2004; Bartel and Chen 2004). This post-transcriptional

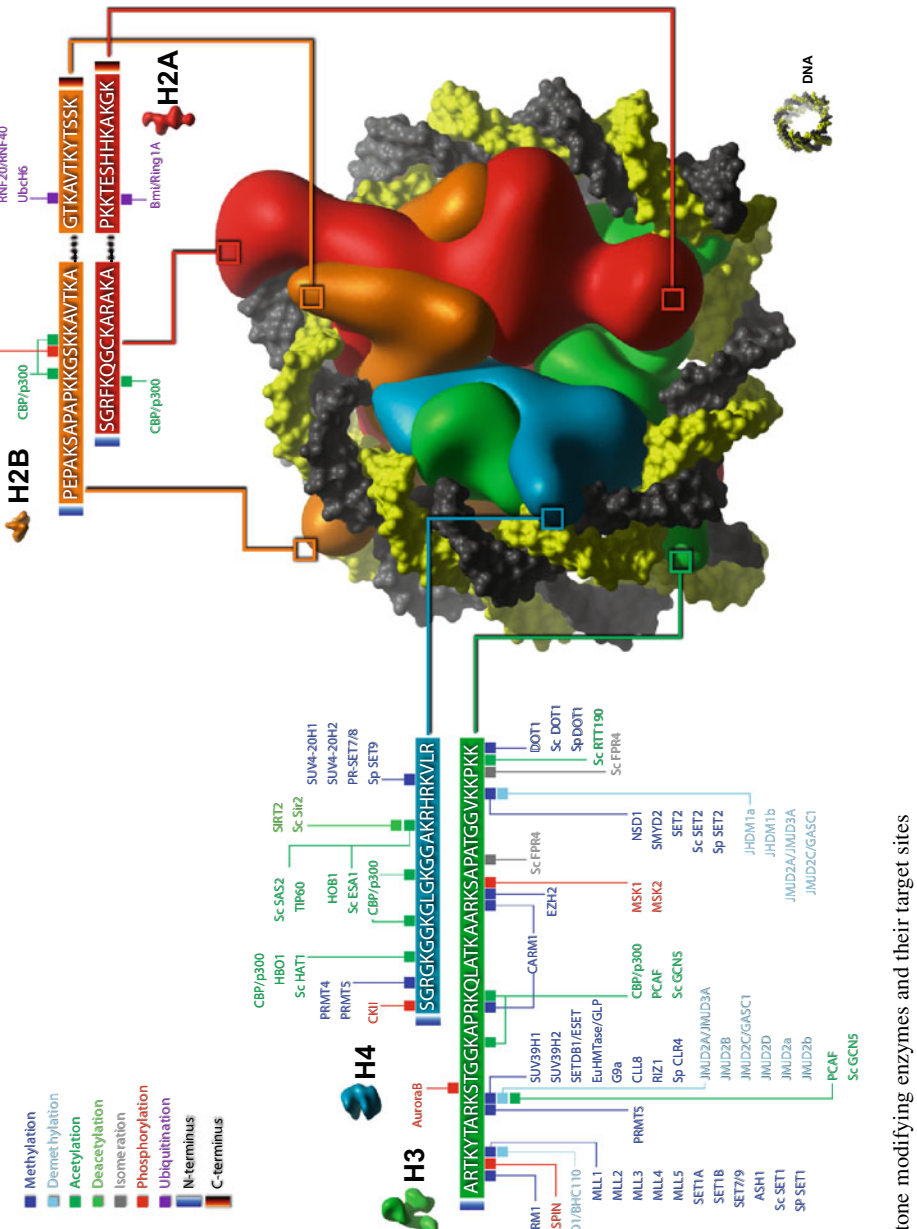


Fig. 1 Histone modifying enzymes and their target sites

Table 1 Histone modifications and their functions

Modification	Position	Function
KAc	H3K9, 14, 18	Activation
	H4K5, 8, 12, 16, 20	Activation
KMe	H3K4	Activation
	H3K9	Activation
KMe3	H3K4	Activation
	H3K9	Repression
	H3K27	Repression
	K3K36	Activation
	H3K79	Activation
	H4K20	Repression
Ser/Thr phosphorylation	H3T3	Mitosis
	H3S10	Mitosis
Ubiquitylation	H2AK119	Repression
	H2BK20	Activation
SUMOylation	H4, H2B	Repression
Biotinylation	H3K4, K9, K27	Repression
	H4K8, K12	Repression

Table 2 Readers, writers and erasers of histone modifications

Modification	Writer	Eraser	Reader
Lysine acetylation	Histone acetyl transferase (HAT)	Histone deacetylase (HDAC)	Bromodomain
Lysine methylation	Histone methyltransferase (HMT)	Histone lysine demethylase (KDM)	Tudor, chromo, MBT, PHD
Lysine phosphorylation	Kinases (Aurora, PRK1, Haspin)	Phosphatases	14-3-3
Ubiquitylation	Ubiquitin ligase	Isopeptidase	None known
SUMOylation	SUMO ligase	SUMO protease	None known
Biotinylation	Holocarboxylate synthetase	Biotinidase	None known

gene silencing (PTGS), which was initially considered a bizarre phenomenon limited to petunias and a few other plant species, has been shown to occur in both plants and animals and has roles in viral defense and transposon silencing mechanisms (Hammond et al. 2001). MicroRNAs possibly target ~60% of all genes, are abundantly present in all human cells and are able to repress hundreds of targets each. The recent discovery of non-coding RNAs (Sleutels et al. 2002) suggests that an RNA component may contribute to epigenetic gene regulation. Small interfering RNAs can modulate transcriptional gene expression via epigenetic modulation of targeted promoters. Importantly, one study implicates miRNA as a factor in the development of schizophrenia (Feng et al. 2009; Need et al. 2009).

2 Evidence for Epigenetic Processes in Neuropsychiatric Disorders

Drug discovery in epigenetics is in its infancy. Targeting the enzymes and recognition domains that regulate chromatin is an attractive approach to modulate the coordinated expression of multiple genes, potentially circumventing issues associated with traditional single-target pharmacology. Altered gene expression patterns linked with apoptosis and cell cycle arrest have prompted the development of DNA methyltransferase and histone deacetylase inhibitors, which have achieved success as chemotherapeutics. For example, vorinostat has been approved by the FDA for treating cutaneous T-cell lymphoma (CTCL). Other inhibitors FK228, PXD101, PCI-24781, ITF2357, MGCD0103, MS275, Valproic acid and LBH589 have also demonstrated therapeutic potential as monotherapy or in combination with other anti-tumour drugs in CTCL and other malignancies (Li and Chen 2009; Hrzenjak et al. 2010; Kim et al. 2010; Lee et al. 2010; Takai and Narahara 2010; Tan et al. 2010). Recently DNA methyltransferase inhibitors such as azacytidine have shown clinical efficacy in treatment of haematological malignancies and in neuroendocrine tumours (Brown et al. 2009; Voso et al. 2009; Alexander et al. 2010; Zhu et al. 2010). Clearly, these mechanisms also have the potential to modify regulatory cascades in post-mitotic cells, such as neurons. Epigenetic regulatory proteins play a leading role in neuronal development, but in addition, growing evidence suggests that adult neurons respond to various environmental signals via dynamic changes in DNA methylation and histone modifications. Because, in contrast to genetic alterations, changes in epigenetic marks are potentially reversible, these processes are important to mechanisms of memory formation and cognition via modulation of genes involved in synaptic plasticity, such as brain-derived neurotrophic factor (BDNF) and reelin (Levenson and Sweatt 2005; Szyf 2009). Epigenetic abnormalities, possibly introduced during either embryogenesis, puberty, or adulthood, have also been noted in several psychiatric disorders, including drug addiction, depression and schizophrenia (Tsankova et al. 2007). In the following sections, we survey the evidence for involvement of epigenetic processes in the aetiology of neuropsychiatric disorders.

2.1 Addiction

Drug addiction involves long-lasting adaptations in the brain's rewards circuitry, mediated in part by alterations in gene expression. Models of addiction in which the molecular, cellular and behavioural responses in rodents to psychostimulant drugs of abuse, for example cocaine are described by Heidbreder (2011).

2.1.1 Role of Methyltransferases

Repeated cocaine exposure leads to persistent changes in gene expression and altered neuronal morphology within rodent nucleus accumbens (NAc), a key component of the brain's reward circuitry (Robinson and Kolb 2004; Hyman et al. 2006). Chromatin remodelling underlies these changes (Kumar et al. 2005; Renthal et al. 2007, 2008, 2009; Borrelli et al. 2008; Stipanovich et al. 2008; Brami-Cherrier et al. 2009). Maze et al. (2010) have shown that repeated cocaine administration in mice produces repression of the methyltransferase G9a in NAc, leading to decreased levels of the repressive mark H3K9me2, selective gene induction and thereby increased preference for cocaine in the place preference paradigm (Fig. 2). Furthermore, the authors demonstrate an increase in the density of dendritic spines in this region of the brain.

These changes were mimicked by pharmacological inhibition of G9a, using BIX01294 or attenuated/inhibited by overexpression of G9a using herpes simplex vectors (HSV-G9a). The repression of G9a is also shown to be mediated via an increased expression of ΔFosB (a stable splice product of the immediate early gene fosB) in NAc. The latter was selectively induced in NAc using bi-transgenic NSE–tTA × tet OP ΔFosB mice (Transgenic ΔFosB). Such induction produces the same sequelae as repeated cocaine administration. Bilateral intra NAc injections of adeno-associated virus (AAV) vectors expressing ΔFosB (AAV–ΔFosB) also produced a repression of G9a in NAc. Conversely, overexpression in the NAc of ΔJunD, a dominant negative mutant protein that antagonizes ΔFosB transcriptional activity, blocked the ability of repeated cocaine to increase dendritic spine formation in the NAc (Fig. 2).

Together, these experiments elegantly demonstrate that chronic drug abuse produces selective gene induction, via repression of a methyltransferase, correlating with a behavioural outcome (increased preference for the drug) and a structural change (increased spine density). These effects are mimicked by a pharmacological

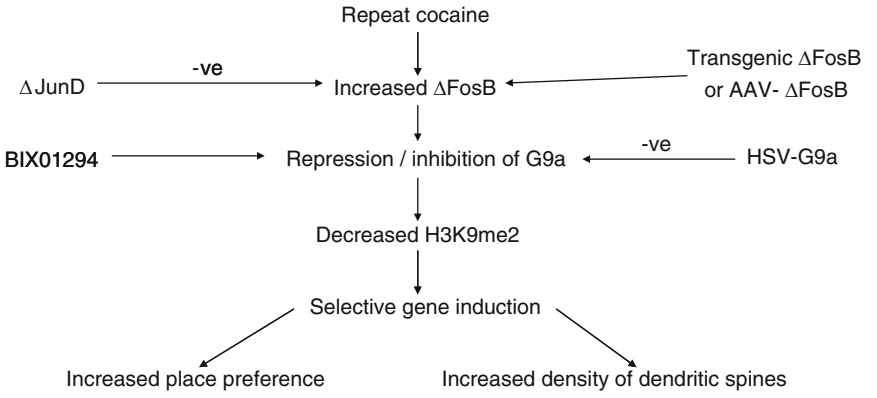


Fig. 2 Repeat cocaine produces transcriptional, anatomical and behavioural changes via repression of a methyl-transferase (G9a)

inhibitor of the methyltransferase, or attenuated by upregulation of the same enzyme (Maze et al. 2010)

2.1.2 Role of Histone Deacetylases

Renthal and colleagues (Renthal et al. 2009) using the same paradigm (repeat cocaine administration), reported the effects on acetylation and methylation patterns of gene promoters in NAc. They demonstrated increased acetylation of Histone 3 (H3) at 1,004 gene promoters and decreased acetylation at 83 gene promoters. The former included FosB, Cdk5 (cyclin-dependent kinase 5) and BDNF. Similarly there was hyperacetylation at Histone 4 (H4) in 692 and hypoacetylation at 123 gene promoters. There was little overlap in hyper (15%) and hypo (1%) acetylation patterns at H3 and H4 proteins. Levels of acetylation of H3 and H4 were maximal between −500 and +200 base pairs (relative to transcription start site, TSS) and showed a bimodal peak for H3 and unimodal for H4. Chronic cocaine did not alter the spatial distribution of acetylation, only the magnitude.

These workers also demonstrated that chronic cocaine produced a decrease in dimethylation of H3K9 and H3K27 (repressive marks) at 209 gene promoters and an increase at 898. This methylation was broader, occurring at −1,600 to +500 base pairs relative to TSS. Similar to the acetylation patterns, cocaine did not alter the spatial distribution.

There was only a 2% overlap in genes showing a decrease in histone acetylation and an increase in histone methylation, indicating that activation or repression of cocaine regulated promoters in NAc occurs via independent alterations in acetylation and methylation patterns. The authors conclude that the most common mechanisms for activation are acetylation (rather than demethylation) and for repression are methylation (as opposed to deacetylation).

Using chromatin immunoprecipitation (ChIP), Renthal et al. (2009) showed that cocaine treatment led to 4% more (1,553) promoters being bound to the transcription factor ΔFosB, compared to saline treated animals. About 13% of these ΔFosB gene targets showed coincident increases in histone acetylation, indicating that ΔFosB may be acting as a transcription factor at these genes. However, ΔFosB may also act as a repressor since 8% of ΔFosB target genes showed increased H3 K9/K27 methylation.

The group also demonstrated increased ΔFosB binding to Sirt1 and 2 promoters (silent information regulator of transcription, NAD-dependent histone deacetylase, also called sirtuins), and an associated increase in H3 acetylation. In order to demonstrate a functional consequence of this binding (ΔFosB) and the chemical modification (histone acetylation), they incubated NAc lysates with the fluorescent substrate for Sirt1 or Sirt2 and thereby concluded that chronic cocaine did indeed increase Sirt1 and 2 activity in NAc. Furthermore the non-selective inhibitor of Sirts (sirtinol) produced a silencing of NAc neurons, assessed using whole cell current clamp of spiny neurons. The non-specific Sirt activator (resveratrol) produced the opposite effect i.e. increased excitability in these neurons. These pharmacological

agents also produced opposite effects on cocaine reward. Resveratrol given systemically (since it penetrates the blood brain barrier) produced an increase in cocaine induced place conditioning and sirtinol (given directly into NAc) produced a decrease in this behaviour. It will be informative to re-assess these conclusions using more specific reagents, ideally administrated via an identical route.

Since cAMP response element binding protein (CREB, another transcription factor) is activated by phosphorylation at Ser 133, the same group performed ChIP for phospho CREB and showed that it binds nearly 38% (1,743) more genes after chronic cocaine compared to saline. In 12% of these, there was an increase in histone acetylation (promoting transcription) and in 1% an increase in H3 dimethyl K9/K27 (promoting repression) marks. Examples of genes binding phospho CREB included Pdyn, cFos, neurogranin and Grin2A.

This extensive study by Renthal and colleagues (Renthal et al. 2009) demonstrates the large number of promoters that are acetylated or methylated by chronic cocaine, the little overlap in the activating or repressing marks, lack of correlation between activating marks and the binding of two transcription factors (ΔFosB and phospho CREB), increased activity of the deacetylases Sirt1/2 and the key roles of these two enzymes in modulating activity of spiny neurons in NAc and the behavioural effects of cocaine (Fig. 3).

Kumar et al. (2005) showed hyperacetylation of histones in NAc following both acute and chronic cocaine administration. Furthermore, the behavioural responses to cocaine were increased following systemic administration of the non-selective HDAC inhibitor – suberoyl-anilide hydroxamic acid (SAHA – inhibits class I and II HDACs). Renthal et al. (2007) have shown that mice receiving intra NAc injections of SAHA also produced an increase in the rewarding responses to cocaine (illustrating the pivotal role of NAc in these behaviours). The same workers found that HDAC 3 and 5 were most highly expressed in NAc, but their respective mRNA levels were unaffected by cocaine.

Since Class II HDACs (4, 5, 7 and 9) are phosphorylated and hence exported out of the nucleus, the group explored the effects of cocaine on phosphorylation of these enzymes. They showed that chronic cocaine administration caused a significant induction of phosphorylated HDAC5 (Ser 259) at 30 min post, but this alteration

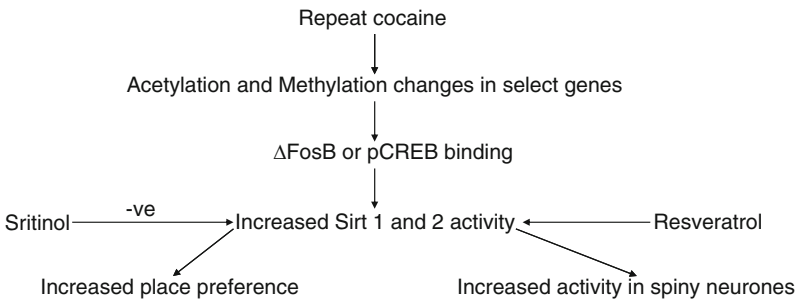


Fig. 3 Repeat cocaine produces chromatin modifications, transcription factor binding, increased HDAC activity and thereby electrophysiological and behavioural changes

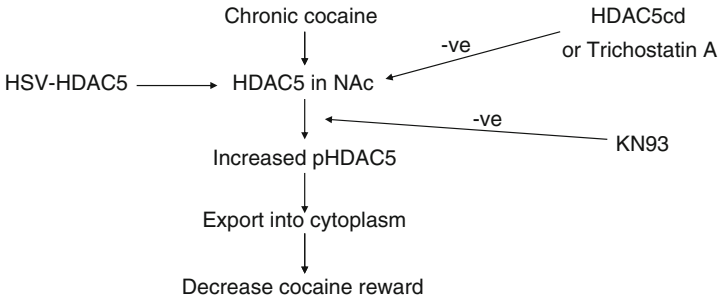


Fig. 4 HDAC5 produces a decrease in cocaine reward through phosphorylation and subsequent transport into cytoplasm

had resumed basal levels 24 h later. These effects were not observed after an acute dose. Renthal et al. (2007) investigated the sub-cellular localization of HDAC5 by infecting NAc with HSV vector expressing flag-tagged HDAC5 (HSV-HDAC5). Nuclear export after chronic cocaine in vivo was confirmed using anti-flag antiserum. This translocation of phospho HDAC5 (pHDAC5) from nucleus to cytoplasm was inhibited by the CaMK inhibitor, KN93. The authors conclude that phosphorylation and subsequent nuclear export of HDAC5 is a mechanism for regulating the promoter acetylation of cocaine-induced genes (Fig. 4).

Since HSV-mediated transgene expression is transient (max at 1–4 days, normalized by day 7; Barrot et al. 2002, Carlezon et al. 1998; Green et al. 2006), Renthal and colleagues used this method for overexpressing HDAC5 during training of place conditioning as opposed to during expression of place preference. Bilateral overexpression of HDAC5 in NAc using this approach was shown to decrease the rewarding effects of cocaine. Viral vectors expressing HDAC5 lacking the catalytic domain (HDAC5cd) and hence function, attenuated these changes. Treating mice with systemic Trichostatin A (non-selective HDAC inhibitor), produced similar behavioural effects (Fig. 4).

HDAC5 KO mice and littermate controls were given daily cocaine injections for 7 days, and then tested for cocaine CPP after 4 days withdrawal. Whilst the cocaine naïve HDAC5 KO mice displayed similar preference to cocaine as their littermate controls, in the animals which had previously been exposed to cocaine, the HDAC5 KO displayed hypersensitization to cocaine reward. This reward hypersensitivity was normalized in HDAC5 KO mice, which received intra-NAc injections of HSV-HDAC5 during the chronic cocaine course, prior to conditioned place preference training.

Renthal et al. (2007) also explored the HDAC5 target genes mediating this reward hypersensitivity. They found 1,616 genes differentially regulated in NAc in HDAC5 KO treated with chronic cocaine, versus wild-type (WT) animals treated with chronic cocaine. One hundred and seventy two of these were regulated by cocaine in KOs (KO cocaine versus KO saline). Nearly all of this subset were regulated in the same direction in cocaine treated – KO vs WT and in KO cocaine vs vehicle. Since HDAC5 functions as a transcriptional repressor, it was surprising to

see a subset of genes which were downregulated in NAc of HDAC5 KO. One possible explanation for this is that the upregulated genes can act as transcriptional repressors, for example the histone methyltransferase SUV39H1 (Bannister et al. 2001; Lachner et al. 2001). Many of these 172 genes are implicated in dopamine transmission in NAc, neuronal excitability in NAc or in cocaine responses.

The important feature of this study is that it illustrates how HDAC5 function is modulated by chronic cocaine, but not acute. Following chronic injections, nuclear HDAC5 function is decreased by a CaMK-mediated phophorylation, followed by transport into cytoplasm.

2.1.3 Role of NFκB

Although chronic cocaine-induced changes in dendritic spines on NAc neurones have been correlated with behavioural sensitization, the molecular pathways underlying these changes are poorly understood. The transcription factor NFκB is rapidly activated by diverse stimuli and regulates expression of many genes known to maintain cell structure.

Russo et al. (2009) have studied the role of NFκB signalling on neuronal morphology (spine density) and the rewarding effects of cocaine. They initially demonstrated that chronic cocaine treatment produced an increase in expression of NFκB subunits p65/Rel A and p105/p50, a trend towards an increase in IKBβ and no change in IKBα. These changes were associated with an increase in the activating mark, acetylation at H3 (H3ac), at p105/p50, p65/RelA and IKBβ genes. Not surprisingly, there was no change in this mark at IKBα genes. Chronic cocaine did, however, produce a decrease in the repressive mark H3K9me3 in all four of the above sub-populations of genes. This would suggest that increased activation and decreased repression are acting synergistically for this subset of genes – not a conclusion arrived at by Renthal and colleagues (Renthal et al. 2009) when studying a broader set of genes.

Russo et al. (2009) used virally mediated gene transfer to either express a constitutively active (HSV-IKKca) or dominant negative (HSV-IKKdn) mutant of inhibitor of Kβ kinase. They showed that overexpression of IKKdn (inhibition of NFκB) produced a decrease in the density of basal dendritic spines on NAc neurons, and over-expression of IKKca produced an increase. Importantly, HSV-IKKdn blocked the chronic cocaine induced increase in dendritic spines (Fig. 5).

When explored in vivo, HSV-IKKdn injected bilaterally into NAc led to a decrease in cocaine-induced place preference and blocked the development of reward sensitization (prior exposure to cocaine produces a sensitization to the rewarding effects; see Heidbreder 2011). However, activation of NFκB via bilateral injection of HSV-IKKca produced no significant effect. This study highlights the importance of a transcription factor NFκB, which is well documented in inflammatory pathology (Biswas and Lewis 2010; Dong et al. 2010; Langereis et al. 2010) to have a profound effect on neuronal structural and behavioural plasticity.

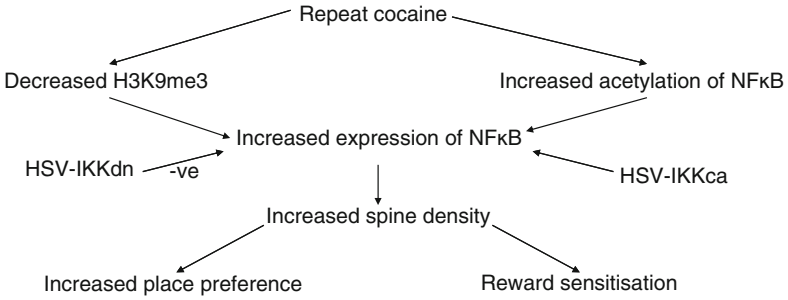


Fig. 5 A well-documented inflammatory mediator, NFκB plays a key role in cocaine induced neuro-anatomical and neuro-behavioural changes

2.2 Suicide and Depression

2.2.1 Role of Methyltransferases

McGowan et al. (2008) investigated methylation patterns of ribosomal RNA (rRNA) genes in human hippocampi of suicide victims (history of childhood abuse or neglect) and control subjects (sudden death without history of childhood abuse or neglect). They showed that the rRNA promoter and 5′ regulatory region showed increased methylation in suicide subjects compared to controls. Most CpG sites (21 out of 26) showed greater methylation in suicide victims compared to control, with no site specificity in methylation. No CpG site showed greater methylation in controls. There were no significant differences in methylation in cerebella (a region not associated with psychopathology), between suicide and control subjects. Furthermore, there was no relationship between methylation status and diagnosis, for example substance abuse victims and mood disorder victims (non substance abuse). The increased methylation in suicide victims was associated with a decrease in expression of rRNA in suicide victims, relative to controls.

This study is certainly consistent with the hypothesis advocated by this lab that early life experiences/events alter epigenetic status of genes mediating neuronal functions. One limitation of the study design is the absence of samples from control subjects with a history of child abuse. The sample size is understandably small ($n = 12$ for abuse and $n = 12$ for non-abuse), but the results are consistent with others suggesting that suicide has a developmental origin (using psychological autopsy methods and epidemiological longitudinal designs).

In a parallel study, Poulter et al. (2008) investigated the expression of DNA methyltransferase (DNMT) in brain regions in depressed suicide victims compared with control subjects who had died suddenly of causes other than suicide. Since the group had previously reported (Merali et al. 2004) a region-specific decrease in mRNA for GABAA receptor α1 subunit in suicide brain, they also investigated methylation status of this promoter. They report a decreased expression of DNMT1 and an increased expression of DNMT3B in frontopolar cortex (FPC) in suicide

victims. There were no significant differences in DNMT3A between suicide and control groups. The increase in DNMT3B mRNA correlated with an increase in protein. Interestingly, there was no correlation between age and DNMT expression, in either of the two groups. In hippocampi, DNMT levels did not vary between suicide and control cohorts, but in amygdala there was a decrease in DNMT1 and DNMT3B in the former group.

Of the 16 CG sites investigated in GABAA receptor α1 subunit gene, three were hypermethylated in the suicide group compared to control (fragment 1, 200–300 bp, about 500 bases from TSS – site 2 suicide = 16%, control = 4%; site 4 suicide = 20%, control = 10%). The hypermethylation was found to be gene specific, since GABAA receptor α5 subunit was equally methylated (8% or more) in both cohorts. The study is important in that it highlights region-specific alteration in DNMTs in human "diseased" vs normal tissue, and gene and site-specific effects of these enzymes.

2.2.2 Role of Histone Deacetylases

There is a large literature suggesting overlaps or similarities in responses to cocaine and stress (Erb et al. 1996; Ahmed and Koob 1997; Koob and Kreek 2007). Furthermore, there are many reports documenting that anti-depressant drugs alter histone acetylation and methylation in specific brain regions (Lee et al. 2006; Tsankova et al. 2006) in animals. Indeed, sodium butyrate, a weak and non-selective HDAC inhibitor exerts some anti-depressant-like effects in animals (Tsankova et al. 2006; Schroeder et al. 2007). Renthal and colleagues (Renthal et al. 2007) have used a model of social defeat stress to mimic the behavioural aspects of human depression (Berton et al. 2006). Social avoidance induced by chronic defeat stress is reversed by chronic treatment with anti-depressants (Berton et al. 2006; Tsankova et al. 2006). Renthal and co-workers showed that chronic social defeat stress leads to decreased levels of HDAC5 mRNA in NAc without altering phosphorylation or the subcellular distribution. Acute social defeat did not alter HDAC5 levels. Furthermore, chronic treatment with the anti-depressant imipramine produced a reversal of the decrease in HDAC5 mRNA. Other HDACs tested (1, 2, 3, 4 and 9) did not demonstrate such a reciprocal regulation by stress and imipramine.

The HDAC5 KOs developed greater social avoidance after chronic social defeat stress compared to the wild-type animals. The HDAC5 KO did not differ in response to acute defeat nor in models of acute stress or anxiety (forced swim test, elevated plus maze and open field). These experiments further highlight the important role of HDAC5 in modulating stress responses.

Covington et al. (2009) analysed brains from stress and control mice in social defeat assay. They showed that acetylation levels of H3K14ac were decreased by about 50% 1 h after stress; but are significantly increased 1 and 10 days after the test. A similar finding was reported in human post-mortem brain (depressed vs matched control individuals), from patients who were or were not taking anti-depressants.

In the same study, the authors report that the anti-depressant fluoxetine was without effect on H3K14ac in the mouse NAc. The laboratory also showed decreased levels of HDAC2 mRNA (no change in HDAC 1 and 3) and reduced enzyme levels up to 15 days after the defeat episode. A parallel effect on HDAC2 protein levels in clinical samples was also noted. Once again fluoxetine produced no effect on HDAC2 enzyme activity in the mouse assay. Furthermore, Covington et al. (2009) showed that chronic infusion (5 days continuous infusion via bilateral cannulae into NAc) of the HDAC inhibitors MS275 (100 μm) or SAHA (100 μm) reversed the reduction in social interaction observed in defeated mice. The HDAC inhibitors also reversed the reduced preference for sucrose and the reduction in mobility (forced swim test) in defeated animals.

MS275 (intra NAc infusion, 10 days) and fluoxetine (systemic pellet implantation) reduced many of the gene expression changes induced in NAc by social defeat stress. Overall MS275 affected a larger number of genes than fluoxetine, but similarly regulated about 37% of fluoxetine affected genes. Other fluoxetine regulated genes were either not affected (15%) or regulated in the opposite direction (48%) by MS275. Of the HDAC inhibitor affected genes fluoxetine had similar effects, no effects or opposite effects on 48, 9 and 43%, respectively. The data indicate some overlap in mode of action of MS-275 with fluoxetine and hence potential clinical efficacy. The differences may be indicative of superior or inferior profiles in efficacy or adverse events.

Many of the genes regulated by MS275 are involved in affecting dendritic/synaptic plasticity and gene transcription. There are a number of important findings in this study: the effects on acetylation and levels of the likely enzyme mediator (HDAC2) are prolonged, both effects were similar to those observed in clinical samples and in social defeat paradigm the effect of the HDAC inhibitor was behaviourally comparable to a widely used clinical anti-depressant fluoxetine, but at the transcription level the effects were on a broader number of genes. The latter may possibly translate into therapeutic superiority, either as greater or as broader efficacy.

2.3 Psychotic Behaviour and Schizophrenia

2.3.1 Role of Methyltransferases

There are no clinical data on efficacy for drugs which target epigenetic mechanisms in schizophrenia. However, there is a literature on similar hypermethylation patterns of specific promoters in post-mortem brain from schizophrenics and in a mouse model (Reeler heterozygous mice). An excellent review on reelin, glutamic acid decarboxylase 67 (GAD67), GABAergic neurons and psychoses has been written by Tueting and colleagues (Tueting et al. 2006). Briefly, they report that, in GABA-ergic neurones in post-mortem brain tissue from schizophrenic patients (Fig. 6), Reelin levels are decreased by about 50%, DNMT1 levels are increased

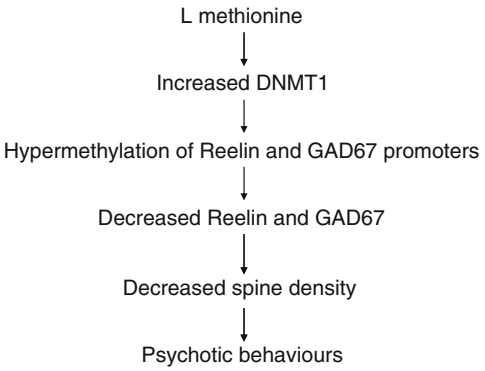


Fig. 6 Increased methionine intake in mice produces protein and behavioural changes, also observed in post-mortem psychotic brain

and Reelin and GAD_{67} promoters are hypermethylated. Reeler heterozygous mice (+/rl) exhibit pathologies similar to those found in post-mortem brain tissue taken from schizophrenic patients and that levels of GAD_{67}, dendritic arborization and spine density (in cortex and hippocampus) are all decreased. Treating normal mice with methionine (a methyl group precursor for the methyl donor in transfer reactions, S-adenosyl-methionine) produces increases in DNMT1 levels, brain and behavioural abnormalities, similar to +/rl animals (Fig. 6).

2.3.2 Role of Histone Deacetylases

Prolonged treatment of schizophrenia patients with L-methionine leads to psychotic symptoms in 79% of patients (Wyatt et al. 1971). Furthermore, cytosine hypermethylation down-regulates gene expression by affecting binding of transcription factors or by attracting methyl-CpG-binding domain (MBD) proteins, for example, histone deacetylases MeCP2 and MBD2, both of which are highly expressed in the brain (Ng and Bird 1999; Fan and Hutnick 2005). Both these protein families bind to selective gene promoters with a high affinity for symmetrically hypermethylated CpG dinucleotides. It has been reported (Dong et al. 2005) that L-methionine-induced hypermethylation is associated with increased binding of MeCP2 and MBD2 and that hypermethylation and decreased protein expression are attenuated with valproate (an HDAC inhibitor, Tremolizzo et al. 2002, 2005). Dong et al. (2005) have also shown that L-methionine administered to mice (5.2 mmol/kg sc, twice daily, up to 15 days) down-regulates reelin and GAD67 mRNA and protein expression in frontal cortices of mice (Fig. 7). This was associated with increased binding of MeCP2 and MBD2 to the respective promoters, as assessed using ChIP assays. This effect of L-methionine is gene specific since it does not affect mRNA or protein expression of GAD65 and its binding of MeCP2 antibody.

Valproic acid (2 mmol/kg) increases acetylated H3 in brain (Tremolizzo et al. 2002, 2005); but importantly increases this in reelin and GAD67 promoter sites. Furthermore, valproic acid decreases L-methionine induced MeCP2 binding to

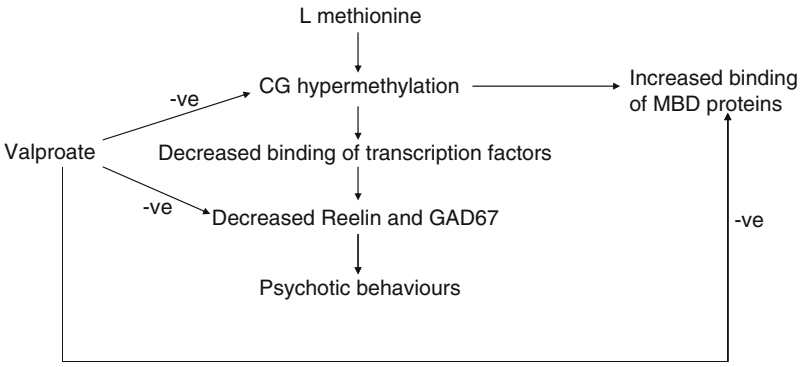


Fig. 7 HDAC inhibitors reduce methionine-induced hypermethylation, binding of MBD proteins and protein expression changes in GABAergic neurones

reelin and GAD67 promoters (to levels seen in vehicle treated controls). The effect of valproic acid cannot be attributed to its anti-convulsant activity because imidazenil, an anti-convulsant, potentiates GABAA receptors containing α5 subunits, but has no effect on receptors containing α1 subunits and does not increase acetylated H3 in the brain. Imidazenil also had no effect on L-methionine induced MeCP2 binding to reelin promoters. This study highlights the potential for HDAC inhibitors in normalising the decrease in reelin and GAD67 expression in cortical GABAergic neurones and thereby offering a potential new treatment modality for schizophrenia.

2.4 Rett Syndrome

Rett syndrome (RS) is a post-natal progressive neuro-development disorder observed in girls in early childhood. In the second year of life, there is a deceleration of head growth (microcephaly), developmental stagnation and muscle hypotonia. These are followed by loss of hand skills, speech and social interaction. Patients even as early as the third year of life have respiratory abnormalities, seizures, autistic features, weight loss and severe motor deterioration. Most girls with RS are confined to a wheelchair by their teenage years. As patients get older they often develop parkinsonian features. Some patients survive until the sixth or seventh decades of life. The disease is mediated by mutations in MeCP2 (Amir et al. 1999). MeCP2 mutations have now been noted in males and in other neuropsychiatric diseases (Jan et al. 1999; Lam et al. 2000; Watson et al. 2001; Klauck et al. 2002; Maiwald et al. 2002; Carney et al. 2003; Budden et al. 2005; Masuyama et al. 2005; Milani et al. 2005; Dayer et al. 2007).

There are at least three mouse models with different MeCP2 (a methyl-CpG-binding protein) mutations. The conditional knockout (Chen et al. 2001; Guy et al. 2001) undergoes a period of normal development followed by severe progressive

neurological dysfunction and then death at 8–10 weeks. Another model truncates MeCP2 at amino acid 308 (Shahbazian et al. 2002). These mice appear normal until 6 weeks and then develop progressive neurological phenotypes. The truncated protein maintains normal chromatin localization but H3 is hyperacetylated in the brain (Shahbazian et al. 2002; Moretti et al. 2005; McGill et al. 2006). Finally, in the third model, MeCP2Tg, there is a twofold increase in expression of human MeCP2 resulting in abnormalities starting at about 10 weeks of age (Collins et al. 2004).

Transcriptional profiling studies in brain from MeCP2 null mice have not identified major gene expression changes, indicating that MeCP2 is not a global repressor. Several studies have therefore probed candidate genes. BDNF was identified as a target of MeCP2 through a candidate gene approach (Chen et al. 2003a, b) who have shown that MeCP2 binds the promoter of BNDF in silent wild-type cultured neurons. Membrane depolarization leads to CaMKII-mediated phosphorylation of MeCP2 at serine 421, and hence unbinding from the promoter, thus allowing transcription (Chen et al. 2003a, b; Zhou et al. 2006). Neurons expressing the S421A mutant were not able to be phosphorylated and hence are not released from the BDNF promoter by depolarization (Zhou et al. 2006). Although these cortical culture studies indicate MeCP2 binding to BDNF promoter and hence affecting its transcription in an activity-dependent manner, the in vivo studies are less clear.

MeCP2 null mice brains show increased expression of the glucocorticoid regulated genes, serum glucocorticoid inducible kinase 1 (Sgk1) and FK506 binding protein (Fkbp5) in pre-, early- and late symptomatic animals. These changes are not mediated by high glucocorticoid levels since these were unaltered in the basal state. Nuber et al. (2005) have also demonstrated MeCP2 binding to Sgk1 and Fkbp5 genes in wild-type brain. It is certainly possible therefore that RS may at least partly be influenced by MeCP2 modulation of these and potentially other stress responsive genes.

McGill et al. (2006) have shown that MeCp2 308/y mice exhibit increased anxiety-like behaviours, an exaggerated stress response (increased corticosterone levels), increased levels of corticotrophin-releasing factor (CRF) and increased binding of MeCP2 to the CRF promoter. The increased expression of CRF, however, appeared to occur only in neurons normally expressing CRF and not in non-expressing tissues, suggesting that MeCP2 is a not a "silencer" of gene expression, but rather is a "modulator".

Guy et al. (2007) have explored the reversibility of RS symptoms using a mouse in which a Lox-stop cassette (MeCP2lox-stop), which behaves as a null mutation, is conditionally activated through a tamoxifen inducible Cre transgene. Small, repeated doses of tamoxifen reversed the late onset neurological phenotype observed in adult MeCP2lox-stop Cre heterozygotes. As activation of MeCP2 produces an attenuation of the symptoms, the study shows that MeCP2 deficient neurones are not permanently damaged.

Giacometti et al. (2007) have further demonstrated partial rescue by post-natal reactivation of MeCP2 in mutant mice. This group targeted a transgene carrying the

MeCP2 cDNA downstream of a Lox P-stop-Lox P (LSL) cassette (in colla1 locus). The LSL MeCP2 transgene was then rescued by excision of the stop signal. Four different transgenes were used to activate MECP2; (1) in neurones and glia (Nestin-Cre), (2) in post-mitotic neurons during embryogenesis (Tau-Cre), (3) in forebrain, hippocampus, mid-brain and brainstem at P0–P15 (CamKII-Cre-93) and finally in forebrain at P15–P30 (CamKII-Cre-159). Activation of the LSL MECP2 transgene prolonged lifespan and delayed motor neuron deterioration in MeCP2-/y mice. The magnitude of these effects was time specific and regionally specific. Greatest rescue, which extended lifespan to 8 months, was obtained in lines providing earlier and widest Cre expression in neurons, that is in Tau and Nestin transgenes. Post-natal rescue (Cre 93 and Cre 159) in comparison only extended lifespan by 4 weeks with effects in Cre 93 being better than Cre I59. The authors concluded that it is desirable to normalize MeCP2 levels in a broad neuronal population as early as possible. These elegant studies demonstrate the potential reversibility of symptoms in adult animals.

2.5 Effects of Early Life Experience and Diet

Several lines of evidence suggest that the intra-uterine and post-natal environment can have major effects on the course of chronic disease in later life. Maternal care in early life is one such determinant. Increased licking and grooming (LG) of pups by maternal rats has been shown to increase expression of glucocorticoid receptors (GRs) in hippocampus, decrease hypothalamic CRF, and provoke an attenuated hypothalamic–pituitary–adrenal (HPA) stress response (Liu et al. 1997; Francis et al. 1999). Cross fostering can reverse these effects. McGowan et al. (McGowan and Kato 2008) have demonstrated that offspring of high LG mothers exhibit increased histone acetylation, decreased DNA methylation, and increased GR promoter binding of transcription factor nerve growth factor inducible protein A (NGFIA). Maternal care not only affected expression of GR but also modulated levels of several hundred genes (Weaver et al. 2005). These effects were shown to be reversible. Weaver et al. (2004) injected Trichostatin A (TSA), a non-selective inhibitor of HDACs (Yoshida et al. 1990) into brains of pups with low LG mothers. This promoted GR acetylation, reduced DNA methylation (by facilitating interaction of demethylases with DNA), increased GR levels and hence reduced stress responsivity. All parameters approached those of pups with high LG mothers. The converse effect could also be produced by injecting L-methionine into lateral ventricles (100 μg/ml, 2 ml daily for 7 days) of offspring with high LG mothers. This promoted DNA methylation, reduced GR expression and increased stress responsiveness. The animals also exhibited reduced anxiety as assessed by time spent in the centre of an open field (Weaver et al. 2005, 2006). Methionine affected expression of 300 genes. This effect is specific since this represented only 1% of genes evaluated on the array.

Champagne et al. (2008) have demonstrated that offspring with low LG mothers exhibit shorter dendritic branch length and lower spine density in CA1 cells, impaired long-term potentiation (LTP) under basal conditions but enhanced LTP in response to high corticosterone in vitro, enhanced memory in hippocampal-dependent contextual fear conditioning paradigm in vivo, and reduced hippocampal levels of glucocorticoid and mineralocorticoid receptors compared to offspring of high LG mothers. These studies demonstrate that early life experiences can affect anatomical substrates (dendrite length), but the outcome of these experiences may be modulated in later life by environmental influences such as stress, corticosterone levels and memory or LTP.

The findings on the effects of maternal care now have some quite remarkable parallels with clinical findings (McGowan et al. 2008). More recently, McGowan et al. (2009) have demonstrated that suicide victims with a history of childhood abuse have decreased levels of GR mRNA associated with increased levels of promoter methylation and decreased binding of NGFIA, compared to suicide victims with no history of childhood abuse. Since cytosine methylation is largely considered stable, the differences in DNA methylation are unlikely to be attributable to events immediately preceding death or during the post-mortem period. This study reinforces the hypothesis that social environment in childhood affects hippocampal gene expression through altered epigenetic processes. Stable epigenetic marks such as DNA methylation may then persist into adulthood to influence vulnerability to psychopathology and susceptibility to chronic disease.

There are numerous preclinical reports documenting effects of dietary methionine on brain development, aging and neurodegenerative pathologies (Slyshenkov et al. 2002; Van den Veyver 2002). Developmental choline deficiency alters SAM levels (Niculescu et al. 2006; Kovacheva et al. 2007) and dietary factors including zinc and alcohol can influence SAM formation (Ross 2003; Davis and Uthus 2004; Pogribny et al. 2006; Ross and Milner 2007). Other dietary components may act as HDAC inhibitors, for example broccoli contains sulforaphane which has been reported to increase H3 and H4 acetylation in PBMCs in mice a few hours after consumption (Dashwood and Ho 2007).

3 Chemical Probes for Epigenetic Target Validation

As described in the introduction, the epigenetic code is comprised of modifications to DNA bases (methylation and hydroxymethylation), and post-translational modifications to histones. Modulation of the writers, readers and erasers of these histone modifications holds the potential to treat chronic, multifactorial diseases in a more holistic way than traditional single-target pharmacology. In order to understand the potential therapeutic consequences of pharmacological intervention at these targets, a systematic study of their roles is required, ideally using a combination of genetic, biochemical and small molecule intervention in vitro and in vivo.

In the following sections, we briefly review progress towards the generation of chemical probes for epigenetic histone-modifying enzymes and recognition domains.

3.1 Modulators of Histone Acetylation

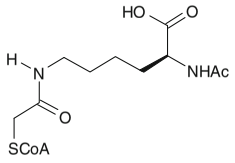
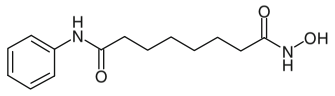
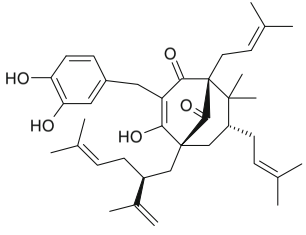
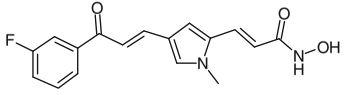
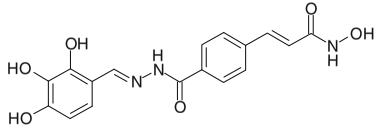
Histone acetylation occurs at the ε-nitrogen of lysine residues within histone core and tails. The writers and erasers of histone acetylation are, respectively, histone acetyltransferases (HATs) and histone deacetylases (HDACs). The simplest consequence of histone lysine acetylation is a decrease in electrostatic charge on histones leading to reduced affinity for DNA, which correlates with transcriptional activation: hence, many transcription factors contain a HAT domain or form complexes with HATs. Conversely, histone deacetylation results in an increase in the electrostatic attraction between DNA and histones, resulting in tighter binding and thereby repression of transcription. However, histone acetylation also influences the recruitment of transcriptional regulators (including other histone modifying enzymes), likely through acetyllysine recognition by bromodomains, which constitute the "readers" of this modification.

3.1.1 Histone Acetyltransferase Inhibitors

Histone acetyl transferases use acetyl CoA as co-factor, and can be classified into three broad families depending on their catalytic domains: the GNAT (GCN5-related *N*-acetyltransferase) family, including SAGA and PCAF; the MYST (MOZ, Ybf2/Sas3, Sas2, Tip60) family, including HBO1; and "orphan" HATs with greater sequence diversity from classical HAT domains, including HAT1, p300 and CBP. The potential clinical impact of HAT modulation covers a wide range of indications including multiple cancers, HIV, diabetes mellitus, cardiac disease and neurodegeneration (Manzo et al. 2009).

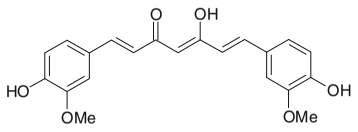
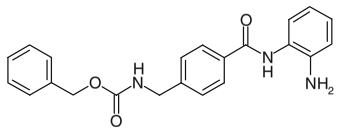
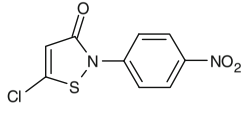
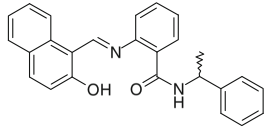
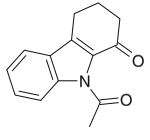
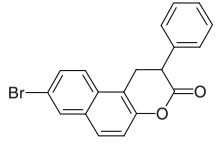
There is a clear need for drug-like HAT inhibitors for use in target validation experiments. A bisubstrate class of inhibitors link CoA and substrate peptide to mimic the Ac-CoA-lysine intermediate. The simplest analogue, LysCoA, showed a K_i of 16 nM against p300 (Table 3). This and a number of LysCoA-containing histone peptides have been used as in vitro probes for a range of HATs (Lau et al. 2000). A number of natural products, including curcumin and garcinol, show non-selective inhibition of HATs. Modified garcinol analogues show varying selectivity profiles across HATs, with some compounds showing selective inhibition of p300 in the micromolar range (Mantelingu et al. 2007). A series of isothiazolones that inhibit p300 and PCAF was discovered by high-throughput screening. One analogue, CCT077791, reduced total acetylation of histones H3 and H4, levels of specific acetylated lysine marks and acetylation of A-tubulin. These compounds were shown to be covalent modifiers of HATs (Stimson et al. 2005).

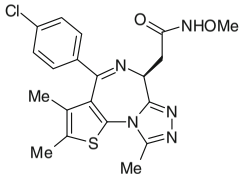
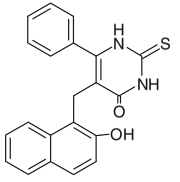
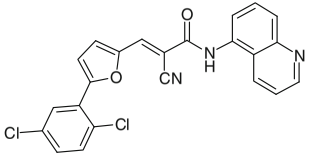
Table 3 Small molecule modulators of readers, writers and erasers of histone acetylation marks

Modulator	Structure	Activity/selectivity	Modulator	Structure	Activity/selectivity
Histone acetyl transferases			*Histone deacetylases*		
LysCoA		p300: K_i 16 nM Selective vs PCAF	SAHA (Vorinostat)		HDAC 1,2,3,6: K_i 1–5 nM HDAC 8: K_i 480 nM
Garcinol		p300: IC_{50} 7 μM PCAF: IC_{50} 5 μM	MC1568		HDAC1: 0% inh. at 5 μM HDAC1: 55% inh. at 5 μM
			Pandacostat		HDAC 1,2,3,6,8: K_i 0.05–0.12 μM HDAC 4,5,7,9: K_i 0.18–1.1 μM

(*continued*)

Table 3 (continued)

Modulator	Structure	Activity/selectivity	Modulator	Structure	Activity/selectivity
Curcumin		p300: IC_{50} 25 μM PCAF: no inhibition at 100 μM	MS-275		HDAC 1,2,3: K_i 22–360 nM
CCT077791		PCAF: IC_{50} 2 μM p300: 95% inhibition at 35 μM	Sirtinol		SIRT2: IC_{50} 38 μM
Bromodomains					
MS7972		CREBBP: K_D 20 μM	HR73		SIRT1: IC_{50} <5 μM

Example 17		BRD4: IC_{50} 13 nM	Cambinol		SIRT1: IC_{50} 56 μM SIRT2: IC_{50} 59 μM SIRT3: no inh. SIRT5: 42% inh. at 300 μM
			AGK2		SIRT1: IC_{50} >40 μM SIRT2: IC_{50} 3.5 μM SIRT3: IC_{50} >40 μM

3.1.2 Histone Deacetylase Inhibitors and Activators

HDACs are grouped into four classes based on sequence homology and mechanism. The first two classes, referred to as "classical" HDACs, are zinc dependent and their activity is inhibited by the hydroxamic acid TSA. The third class, referred to as sirtuins (see above), is NAD^+-dependent proteins and is not affected by TSA. The fourth class is also zinc dependent, but is considered an atypical category based on low sequence homology to the classes I and II.

Unlike HATs, the development of HDAC inhibitors is well advanced and two HDAC inhibitors, vorinostat (Table 3) and romidepsin, are already marketed for the treatment of CTCL. At the time of writing, these and at least eleven other inhibitors are in clinical trials for a variety of other cancers, including Hodgkin's lymphoma, melanoma, pancreatic, nasopharyngeal and prostate (Ma et al. 2009; Tan et al. 2010).

The potential of HDAC inhibitors to activate the expression of mRNAs that are down-regulated in schizophrenia, depression, drug addiction and other conditions has led to interest in embarking on clinical trials with HDACis such as MS-275, which show some evidence of limited CNS-penetration (Grayson et al. 2008). Histone acetylation homeostasis has recently been shown to be perturbed in the neurodegenerative mechanisms of Huntington's disease, Parkinson's disease, Kennedy disease, amyotropic lateral sclerosis and Rubinstein–Taybi syndrome as well as stroke. Clinical trials have been initiated to evaluate the safety and efficacy of HDAC inhibitors for the treatment of Huntington's disease, amyotropic lateral sclerosis and spinal muscular atrophy (Hahnen et al. 2008). A recent discovery showing that inhibition of HDAC2 negatively regulates memory formation and synaptic plasticity has created excitement that the development of specific inhibitors of this isoform could be used as cognitive enhancers, particularly in neurodegenerative patients (Guan et al. 2009). In addition, HDAC inhibitors have effects on the acetylation of key factors that regulate immune cell function via modulation of inflammatory transcriptions: for example HDAC3 and 6 have been shown to control acetylation of the NFκB subunit p65, suggesting that these agents could be used to modulate neuroinflammatory conditions (Adcock 2007).

The success of vorinostat has encouraged the development of many HDAC inhibitors with diverse selectivity profiles and there are numerous reviews on the subject, for example (Grayson et al. 2008; Hahnen et al. 2008; Paris et al. 2008; Tan et al. 2010). The majority of HDAC inhibitors conform to a "cap-linker-chelator" pharmacophore. Those that have entered clinical trials are variants of one of two zinc-chelating groups: the hydroxamic acid, present in vorinostat and TSA, or the aminobenzamide moiety present in MS-275 (Table 3). Other zinc-chelating groups under investigation include mercaptoacetamides, alpha-keto oxazoles, trifluoromethyl ketones, and silane-diols (Wang and Dymock 2009).

In general, the hydroxamic acids in clinical trials show broad activity across HDAC isoforms, while the aminobenzamides show some selectivity for class I over class II isoforms. The nature of the linker and cap is also important in determining isoform selectivity: within the cinnamic hydroxamic acids, the pyrrole-linked MC-1568 shows unusual selectivity in favour of class II isoforms, whilst the

phenyl-hydrazine linked pandacostat is almost completely pan-active (Bradner et al. 2009) (Table 3). It should be noted that to date HDAC inhibitors in the most-characterized hydroamic acid (vorinostat) and benzamide (MS-275) classes show poor brain penetration, limiting their usefulness as tools and potential therapeutics for CNS diseases (Dhalluin et al. 1999) (Palmieri et al. 2009; Hooker et al. 2010).

Sirtuin inhibitors include sirtinol Table 3, splitomycins including the derivative HR73 which shows activity on human SirTs, and cambinol, which inhibited growth of Burkitt lymphoma xenografts in mice (Grozinger et al. 2001; Pagans et al. 2005; Heltweg et al. 2006). Selective SIRT2 inhibition by AGK2 has been shown to effect neuroprotection in models of Parkinson's and Huntington's diseases by decreasing sterol biosynthesis (Outeiro et al. 2007; Luthi-Carter et al. 2010). Several compounds including resveratrol have been characterized as activators of SIRT1 and used to propose a mechanistic link between SIRT1 activation and delay in the onset of age-related diseases such as type 2 diabetes, Parkinson's and Alzheimer's, although controversy has ensued after it was demonstrated that these compounds do not act directly on SIRT1, serving as a reminder of the importance of using high quality, well-characterized chemical probes in drug target validation studies (Pacholec et al. 2010).

3.1.3 Bromodomain Inhibitors

The bromodomain is a conserved ~110 amino acid module which recognizes acetylated lysines in proteins including histones (Dhalluin et al. 1999), thereby participating in deciphering the histone code (Strahl and Allis 2000). The human genome encodes for 57 distinct bromodomains contained in 42 proteins, with some proteins containing two or more distinct bromodomains.

Bromodomain-containing proteins have been classified into several distinct subgroups based on their function: (1) HATs, including GCN5, PCAF and TAFII250; (2) ATP-dependent chromatin-remodelling complexes, including Brahma, Swi2, Snf2 and Brg1; (3) the BET (bromodomain and extra C-terminal domain) family, a class of transcriptional regulators carrying two tandem bromodomains and an extra terminal (ET) domain (Florence and Faller 2001). Although the function of a number of bromodomain-containing proteins has been studied in depth, allowing some association with specific diseases, the roles of many bromodomains remain to be elucidated. Within the BET family, BRD2 is genetically associated with juvenile myoclonic epilepsy (Cavalleri et al. 2007), while knockdown experiments have implicated BRD4 in a range of transcriptional co-activation targets including NFκB (Huang et al. 2009). Bromodomain-containing HATs including CREBBP, PCAF and GCN5, have been extensively studied but conclusions drawn from gene knockdown studies do not allow the individual contributions of the different domains to be elucidated.

At the time of writing, very few small molecule ligands for bromodomains have been described. The carbazole MS7972 (Table 3), discovered by NMR screening of commercially available acetamide-containing small molecules against CREBBP,

showed a K_D of ~20 uM as determined by titration in tryptophan fluorescence experiments. In U2OS cells, MS7972 at 200 uM modulated p53 stability and function in response to DNA damage (Sachchidanand et al. 2006).

The triazolothienodiazepine (Example 17, Table 3) is claimed to displace acetylated histone H4 peptides from BRD4 with an IC_{50} of 13 nM using a FRET assay with FLAG-tagged BRD4 and biotinylated H4 peptide. This compound showed antiproliferative effects at submicromolar concentrations against a panel of cancer cell lines, in particular a GI_{50} of 14 nM against MV4-11 leukemic cells (Mitsubishi/Tanabe Int. Pat. Appl. WO09/084693).

3.2 Modulators of Histone Methylation

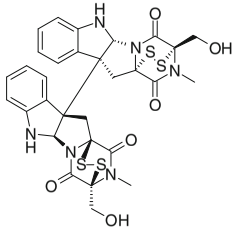
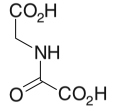
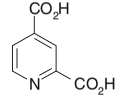
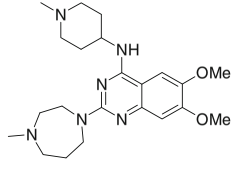
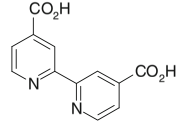
Like acetylation, histone methylation occurs within histone core and tails at the ε-nitrogen of lysine residues but also on arginine guanidyl groups. The writers and erasers of histone lysine methylation are, respectively, histone lysine methyltransferases (HMTs) and histone demethylases (KDMs). Arginine is methylated by protein arginine methyltransferases (PRMTs) and demethylated by histone arginine demethylase (HAD). Methylated lysines and arginines are recognized by a range of different binding modules including chromodomains, tudordomains, MBT domains and PHD domains, which are often constituents of catalytic enzyme complexes, enhancing specificity and/or activity of a catalytic domain towards specific histone marks.

3.2.1 Histone Lysine Methyltransferases

Histone lysine methyltransferases (HMTs) catalyse mono-, di-, or tri-methylation of lysine residues of histones and other proteins. More than 20 human HMTs have been identified (Blackledge and Klose 2010), and with the exception of DOT1L, all contain the evolutionarily conserved SET domain, which uses S-adenosyl methionine as co-factor (Fog et al. 2007). The SET domain containing HMTs are classified into five subfamilies based on SET domain homology and are named after their founding members: SUV39, SET1, SET2, RIZ and SMYD3.

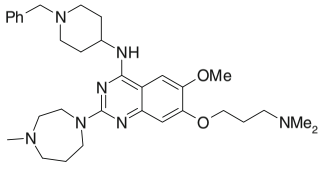
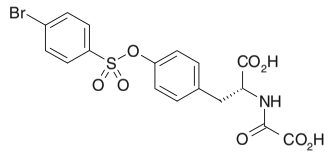
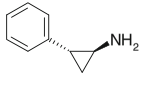
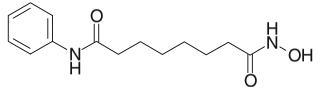
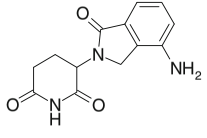
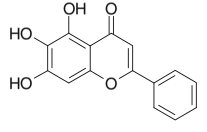
To date, three selective small molecule HMT inhibitors have been reported (Greiner et al. 2005; Kubicek et al. 2007; Liu et al. 2009). Chaetocin (Table 4), a fungal mycotoxin that belongs to the class of 3–6 epidithio-diketopiperazines (Greiner et al. 2005) inhibits the human SUV39H1 ($IC_{50} = 0.8$ μM), an H3K9 HMT, and is selective for H3K9 HMTs over HMTs that do not target H3K9. Although cytotoxic under some conditions, 0.5 μM chaetocin shows marked cellular reduction of di-methylation and tri-methylation of H3K9 and no changes in the methylation state of H3K27, H3K36 and H3K79. BIX01294 has been characterized as a selective small molecule inhibitor of G9a ($IC_{50} = 1.9$ μM) and GLP ($IC_{50} = 0.7$ μM) with excellent selectivity over several H3K9 HMTs including SUV39H1 ($IC_{50} > 45$ μM) and ESET ($IC_{50} > 45$ μM), other HMTs such as SET7/9

Table 4 Small molecule modulators of readers and writers of histone methylation marks

Modulator	Structure	Activity/selectivity	Modulator	Structure	Activity/selectivity
Histone lysine methyl transferases			*Histone demethylases (2-OG dependent)*		
Chaetocin		SUV39H1: IC_{50} 0.8 μM Selective for H3K9 methylating enzymes	*N*-Oxalyl glycine		JMJD2E: IC_{50} 78 μM
			2,4-Pyridine dicarboxylic acid		JMJD2E: IC_{50} 1.4 μM
BIX01294		G9a: IC_{50} 1.9 μM GLP: IC_{50} 0.7 μM SUV39H1, ESET, SET7/9, PRMT1: IC_{50} >45 μM	Bipyridyl-*bis*-carboxylic acid		JMJD2E: IC_{50} 6.6 μM

(*continued*)

Table 4 (continued)

Modulator	Structure	Activity/selectivity	Modulator	Structure	Activity/selectivity
UNC0224		G9a: IC_{50} 16 nM GLP: IC_{50} 20 nM SET7/9: IC_{50} 16 nM SET8/PreSET7: IC_{50} 16 nM	*N*-Oxalyl tyrosine derivative		JMJD2E: IC_{50} 5.4 μM
Histone demethylases (FAD dependent)					
Tranylcypromine		LSD1: K_i 357 μM	SAHA (Vorinostat)		JMJD2E: IC_{50} 14 μM
Pomalidomide		Uncharacterized in vitro	Baicalein		JMJD2E: IC_{50} 4.0 μM

($IC_{50} > 45$ μM), and the arginine methyltransferase PRTM1 ($IC_{50} > 45$ μM – Table 4). In cellular assays, BIX01294 at 4.1 μM reduced the H3K9Me2 level of bulk histones; however, it was toxic at higher concentrations (Kubicek et al. 2007). A more potent analogue, UNC224, has recently been obtained using structure-based design (Liu et al. 2009).

3.2.2 Histone Lysine Demethylase Inhibitors

Lysine demethylases fall into two major classes defined by their oxidative mechanism and cofactor. The LSD family, homologues of the flavin-containing monoamine oxidases, use the cofactor FAD (Yang et al. 2007), while the family of Jumonji-domain containing demethylases use Fe(II) together with the cofactor 2-oxoglutarate (Bannister and Kouzarides 2005).

Several small molecule LSD1 modulators have been reported: these include known non-specific monoamine oxidase inhibitors (MAOIs), including tranylcypromine (Culhane et al. 2010) and pargyline (Metzger et al. 2005), and thalidomide derivatives pomalidomide and lenalidomide (Escoubet-Lozach et al. 2009) (Table 4). The latter two compounds show effects on histone H3K9 and H3K4 methylation levels in cells, apparently mediated by modulation of LSD1-catalysed demethylation, although direct action on the enzyme has not been demonstrated in vitro. The inhibition by tranylcypromine has been shown to occur by formation of a covalent adduct with the FAD cofactor.

JMJD2 demethylases are inhibited by analogues of the co-factor 2-oxoglutarate (2-OG), including *N*-oxalylamino acids, pyridine dicarboxylates and related bipyridyl derivatives (Table 4). Other chemotypes that are also presumed to bind to the active site Fe(II) include catechols, hydroxamic acids (including the clinically used HDAC inhibitor SAHA/Vorinostat), and cofactor byproducts/analogues, such as succinate and fumarate (Rose et al. 2008; Sakurai et al. 2010). Targeting the catalytic Fe(II) brings with it the challenge of achieving selectivity, since the co-factor and Fe(II)-binding sites of 2-OG oxygenases are generally very similar. Recently, the protein crystal structure of JMJD2A has been combined with a dynamic combinatorial chemistry approach to derive a series of substituted oxalyl tyrosines that exploit a subpocket of this enzyme to achieve selectivity over PHD2 (Rose et al. 2010).

3.2.3 Histone Methyl Lysine Binding Domain Inhibitors

Several families of structurally distinct protein modules have been shown to bind to methylated lysines in histones and other proteins: the so-called “royal family” – made up of Tudor, Agenet, chromo, PWWP and MBT domains; the plant homeodomain – PHD; the WD40 repeat protein – WDR5, and ankyrin repeats (Taverna et al. 2007; Collins et al. 2008).

In general, the binding of these domains to methyl-lysine containing peptides is fairly weak, typically in the range 10–100 uM (Kd). A recent review of the available high-resolution X-ray crystal structures of these domains summarizes the key recognition features of various KMe states as an electron-rich aromatic cage interacting with the lysine cation with additional charge neutralization and H-bonding by from 0 to 2 acidic functionalities – depending upon the methylation state of lysine (Adams-Cioaba and Min 2009). Although no small molecule methyl-lysine binding domain inhibitor has been described to date, high-throughput assays have recently been described allowing detection of compounds that disrupt the interaction between MBT or Tudor domains and histone peptides, so non-peptide antagonists of methyl-lysine binding should appear from diversity screening and structure-based design approaches in the near future (Quinn et al. 2009; Wigle et al. 2010).

3.3 Modulators of Other Histone Modifications

3.3.1 Histone Phosphorylation

Serine, threonine and tyrosine residues in histones are subject to phosphorylation by a variety of kinases. As for non-histone proteins, phosphorylation result in specific recognition events, recruiting other proteins into complex formation. For example, phosphorylation of H3S10 by the kinase PIM1 results in binding of a 14-3-3 protein, which recruits HATs to nucleosomes, leading to a cascade of histone modification and protein binding events, with the overall effect of activating transcriptional elongation (Zippo et al. 2009). AuroraB-catalysed phosphorylation of H3S10 (and other proteins) is required for chromosome condensation, and its inhibition or down-regulation leads to a variety of errors in mitosis: a large number of inhibitors are under study as anticancer agents (Perez Fidalgo et al. 2009). Phosphorylation of H3T11 by the kinase PRK1 apparently accelerates demethylation of H3K9me3 by the demethylase JMJD2Cs (Metzger et al. 2008); in contrast, phosphorylation of H3S10 prevents demethylation of H3K9 by the JMJD2 demethylase (Ng et al. 2007). Phosphorylation levels on histones are controlled by interplay between kinases and phosphatases, which include Protein Phosphatase Type 1 (PP1).

3.3.2 Histone Ubiquitylation, SUMOylation, Biotinylation and Poly(ADP) ribosylation

Ubiquitination of lysine residues is classically associated with protein degradation in the cytosol and has only recently been shown to occur on histones (Suganuma and Workman 2008). Ubiquitination of H2A and H2B histones is believed to act as a master switch for other post-translational modifications that ultimately regulate gene expression (Minsky et al. 2008). Small molecule ubiquitination inhibitors

have been identified, such as Ro106-9920 which blocks NFκB-dependent cytokine expression in cells and rats (Swinney et al. 2002).

Small ubiquitin-like modifier (SUMO)-specific activating, conjugating and ligating enzymes catalyse the ligation of SUMO proteins to lysines in histones, particularly in histone H4, where SUMOylation is associated with transcriptional repression, through the recruitment of HDACs and HP1 (Shiio and Eisenman 2003).

Histone biotinylation is catalysed by holocarboxylase synthetase, although later studies have shown that the activated biotinyl-5′-AMP is capable of biotinylating histones in the absence of enzyme (Hassan and Zempleni 2008; Healy et al. 2009). Eleven biotinylation sites have been identified in histones H2A, H3, and H4. K12-biotinylated histone H4 is enriched in heterochromatin, repeat regions, and plays a role in gene repression. No inhibitors of histone biotinylation have been reported.

ADP-ribosylation of histones can be can be mono- or poly-, mediated by MARTs (Mono-ADP-ribosyltransferases) or PARPs (poly-ADP-ribose polymerases), respectively (Hassa et al. 2006). Although this modification has been known for over two decades, only one site, H2BE2ar1, has been definitively mapped, and so the direct effect on chromatin regulation is poorly understood. Despite this, numerous PARP inhibitors have been developed, including several in clinical trials for oncology (Ferraris 2010).

3.4 Freely Available Chemical Probes for Epigenetics: A Novel Paradigm

As will have become clear in the preceding sections, although broad spectrum HDAC inhibitors have been successfully developed as anticancer therapies, there remains a great need for well characterized, potent, selective and cell-permeable chemical probes for all of the protein families involved in epigenetic regulation of gene expression.

In order to address this need, we have recently initiated an international consortium in which industry and academia are collaborating to generate chemical probes for histone modifying enzymes and recognition domains (Edwards et al. 2009). The partnership comprises the Structural Genomics Consortium (SGC), the Universities of Oxford and Toronto, the US National Institutes of Health (NIH) Chemical Genomics Center, GlaxoSmithKline, Pfizer and a network of academic collaborators, combining capabilities in purified protein production, structural biology, biochemical and biophysical assay development, high-throughput screening, medicinal chemistry and cellular biology. The research builds on the output of the SGC, which has produced most of these human proteins in purified form and has determined the three-dimensional structures of many.

We are taking a systematic approach to generating the chemical probes. Rather than selecting specific "therapeutically relevant" proteins at the outset, the major

protein families involved in modifying and recognizing histone marks are being studied as protein family systems. For each protein family, ligand mimetic chemical libraries are designed and synthesized using structure-based approaches. The libraries are screened at each of the collaborating groups, using newly developed biochemical and biophysical assays; this structure-driven knowledge-based approach is complemented by diversity screens that are identifying otherwise unpredicted ligand chemotypes. Iterative improvement of potency and selectivity is accelerated by the family-wide availability of the purified proteins at the SGC and the growing level of structural understanding of the protein families and their interactions with ligands. The overall progress is being overseen by a committee of scientists having expertise in epigenetics and chromatin biology.

To ensure that the chemical probes will be used immediately and with maximal benefit to the biomedical community, they are treated as precompetitive reagents and made available to all researchers without restriction on use (http://www.thesgc.org/chemical_probes/epigenetics/). The success of the program and the continued involvement of industry both rely on the scientific community's willingness to use the chemical probes properly and share their data without restriction on use.

Taken together, we expect that the chemical probes will enable a scale and depth of experiments on epigenetic signalling by both academic and industrial investigators that could not otherwise be achieved. We look forward to the many exciting experiments that will guide the medical research community's understanding of which epigenetic targets can be modulated to elicit a therapeutic benefit, and thereby trigger the development of novel treatments for unmet medical need.

4 Animal Models and Reagents to Probe Biology

This area of science is relatively new, the animal models are established and the reagents are invariably limited in number and quality. We aim to help address the latter, by generating high quality, well characterized (potent, selective, cell penetrant) small molecules "probes". These may then be used to dissect biological pathways, and disease mechanisms, and hence drive new targets into drug discovery. It is of course right that we cannot model a "system" in a cellular assay and hence we have to use in vivo animal models. The data we have given in this chapter is without exception compelling and exciting. It has allowed the labs to explore hypotheses and establish correlations between molecular, cellular and behavioural parameters. The question regarding the predictive utility or translatability of these in vivo animal models remains. It is with this in mind that we have emphasized wherever possible any clinical data for neuro-psychiatric disease.

The challenges of drug discovery are compounded for neuropsychiatric diseases by the need in most cases for penetration across the blood–brain barrier and often a lack of access to relevant clinical disease tissue. Furthermore, it is conceivable that in many of these diseases we have "overestimated" the role of genetics and underestimated the role of environmental factors or epigenetics in their aetiology and

plasticity. Herein lies our optimism – by exploring this area of biology we will identify novel targets which will deliver highly effective therapeutics for these chronic diseases.

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Functional and Pharmacological MRI in Understanding Brain Function at a Systems Level

Angelo Bifone and Alessandro Gozzi

Contents

Abstract Functional magnetic resonance imaging (fMRI) methods have been extensively applied to study the human brain and its functional organization in healthy and disease states. A strong rationale exists for the extension of this approach to animal models as a translational tool to bridge clinical and preclinical research. Specifically, the development of pharmacological MRI (phMRI), i.e., the use of fMRI to map spatiotemporal patterns of brain activity induced by pharmacological agents, has provided a robust and flexible tool to resolve brain circuits and mechanism-specific functional changes produced by selective intervention in different neurotransmitter systems in vivo. This chapter describes the methodological aspects of fMRI and phMRI in preclinical species, and some of the key findings,

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with a special emphasis on the translational potential of these methods in neuropharmacological research.

Keywords Brain circuits · Complex networks · fMRI · Functional connectivity · Pharmacological MRI · phMRI

Abbreviations

5-HT	5-Hydroxytryptamine
Acb	Nucleus accumbens
BOLD	Blood oxygen level dependent
CBF	Cerebral blood flow
CBV	Cerebral blood volume
CRF	Corticotropin-releasing factor
EEG	Electro-encephalogram
FDG	^{18}F-fluoro-deoxy-glucose
fMRI	Functional magnetic resonance imaging
GABA	γ-Aminobutyric acid
GlyT-1	Glycine transporter type-I
mCPP	1-(*m*-Chlorophenyl) piperazine
MEG	Magneto-encephalography
mPFC	Medial prefrontal cortex
MRI	Magnetic resonance imaging
nAChR	Nicotinic acetylcholine receptor
NK1	Neurokinin 1 (NK1)
NMDAR	*N*-methyl-D-aspartate receptor
PCP	Phencyclidine
PET	Positron emitting tomography
phMRI	Pharmacological magnetic resonance imaging
rCBV	Relative cerebral blood volume
VTA	Ventral tegmental area

1 Introduction

Since its inception in the 1970s, magnetic resonance imaging (MRI) has rapidly become a widely applied radiological technique owing to its superior soft-tissue contrast, absence of ionizing radiation exposure and versatility. Multiple combinations of pulse sequences, acquisition parameters and exogenous contrast agents can be used to sensitize image contrast to different tissue characteristics and

physiological parameters, thus enabling a variety of clinical applications to musculoskeletal, oncological, cardiovascular, and neurological imaging.

The recent discovery (Ogawa et al. 1990) that brain MR images are sensitive to changes in tissue perfusion and blood oxygenation levels paved the way for the development of noninvasive MRI of brain function, dubbed functional magnetic resonance imaging (fMRI). The close interrelation between regional blood flow, metabolism, and neuronal activity has been known for a long time. The Italian physiologist Angelo Mosso was the first to record regional changes in vascular pulsation in the human cortex during mental activity (Mosso 1881; Ogawa et al. 1990), an observation that was confirmed and expanded by Roy and Sherrington in experimental models (Roy and Sherrington 1890). It was not until the early 1990s, however, that Ogawa and other authors conceived the application of MRI methods to map regional hemodynamic changes as a surrogate for neuronal activation (Ogawa et al. 1990; Kwong et al. 1992; Bandettini et al. 1992; Frahm et al. 1992). Since then, the inherent advantages of fMRI over other functional imaging methods (e.g., water PET, EEG, MEG etc) in terms of spatiotemporal resolution and noninvasiveness have determined the prevalence of this technique in functional neuroimaging.

Following the first pioneering studies, fMRI has been extensively applied to demonstrate and study *functional segregation* in the human brain. In this context, the regional localization of fMRI responses elicited by motor and sensory stimuli, or by cognitive and emotional tasks, is interpreted as evidence of the functional specialization of the underlying neuronal substrate. Complex paradigms, involving comparisons between probe and reference tasks, have been developed to isolate the functional role of specific brain areas. By way of example, the subject may be asked to perform a word retrieval task, and the activation recorded during the task performance is then contrasted against passive viewing of words, thus enabling the researcher to disentangle retrieval-specific activity from the complex pattern of fMRI responses elicited by the task. This framework, grounded on the principle of cognitive subtraction and variations thereof (Posner et al. 1988), provides the conceptual basis for the increasingly sophisticated neuropsychological paradigms used to investigate the functional organization of the human brain.

More recently, considerable attention has been devoted to the functional interactions between brain regions (Horwitz 2003), a viewpoint that is complementary to the traditional focus on functional specialization. The idea underlying this approach is that complex brain processes do not merely arise from "switching on" or "switching off" individual brain structures, but rather from the concerted modulation of activity in a set of spatially and anatomically distinct regions. From this angle, correlations between the fMRI signals in remote brain regions engaged by a task or even at rest may be interpreted in terms of mutual interactions, or *functional connectivity* (Friston 1996; Horwitz 2003). These two frameworks for the analysis of fMRI data, i.e., functional localization versus connectivity, reflect the concept that higher brain function emerges from the interplay between the seemingly conflicting principles of functional segregation and integration. Consistent with this picture, the spatiotemporal patterns of brain activity measured by

fMRI methods are often thought of as *circuits* of lumped elements (functionally specialized brain regions) and their links (structural or functional connections among different regions).

Functional MRI has been widely applied to study the neuronal circuits engaged by neuropsychological paradigms in the healthy human brain, and has brought about considerable progress in our understanding of the brain functional architecture. Likewise, functional imaging methods have provided novel tools to investigate the neurobiological substrates of psychiatric and neurological illnesses. Psychiatric diseases are heterogeneous disorders, often described as clusters of symptoms, and are inherently complex, with polygenic and multifactorial origins (Gottesman and Gould 2003). This complexity makes it difficult to unambiguously identify specific genes or neuropathological features that may underlie their onset and progression. Hence, the quest for "endophenotypes," i.e., more objective and measurable markers that might help deconstruct these complex syndromes into more tractable components, whose genetic or environmental underpinnings may be more readily identified. Functional imaging methods have contributed a new powerful means to this end by enabling the association of symptoms and phenotypes with specific brain circuits. For example, a number of recent studies have demonstrated abnormal BOLD fMRI activation of the amygdala and related circuits in depressed subjects engaged in emotion-recognition paradigms, thus pointing to emotion-processing circuitry as a potential pathophysiological substrate for depression (Sheline et al. 2001; Fu et al. 2004; Pezawas et al. 2005; Chen et al. 2007). While these intriguing results have yet to be replicated a larger scale in the general patient population, the impact of functional neuroimaging methods on the understanding of psychiatric conditions is potentially huge.

The development of functional MRI methods has been driven primarily by human investigations, but there exists significant scope for their application in preclinical species. Provided that a degree of homology exists between brain circuits in humans and animals, it is conceivable to apply fMRI methods to understand and demonstrate the construct-validity of disease models, thus improving their relevance to the human condition and their predictivity. Moreover, the combination of functional MRI with more invasive techniques in preclinical species may be useful to understand the physiological basis of the fMRI responses, and to validate the imaging endpoint for clinical investigation. Last but not least, animal models may provide useful tools to test the effects of putative medicines on the activity of specific brain circuits thought to be implicated in aspects of the human disease prior to proceeding to more complex and expensive clinical trials, thus expediting the drug-discovery process. Hence, the translational potential of a noninvasive imaging technique such as fMRI is attractive for both basic and applied brain research.

However, several constraints and limitations have made the application of fMRI methods to animal models, and particularly to rodents, less than straightforward. First, imaging experiments in rodents require the use of anesthesia to prevent motion artifacts and to reduce animal stress (Flecknell 1987). Moreover, the vast repertoire of paradigms used to probe cognitive or emotional aspects of brain

function in freely moving and behaving animals cannot be applied under the constrained experimental conditions of an fMRI experiment. An interesting approach to overcome some of these limitations relies on pharmacological manipulation, and has been dubbed "pharmacological MRI" (phMRI) (Leslie and James 2000), i.e., fMRI as applied to map spatiotemporal patterns of brain activity induced by pharmacological challenges. The main merits of phMRI lie in its ability to elicit robust and reliable fMRI signals even under anesthesia, and to enable selective stimulation of different neurotransmitter systems, thus providing a means to study the neurochemical basis of fMRI responses and the corresponding circuitry engaged. A number of recent studies have demonstrated mechanism-specific patterns of activation corresponding to key circuits in the rat brain, and phMRI is gaining momentum as a translational neuroimaging tool.

In this chapter, we review the application of phMRI in rodents, with the aim to emphasize the ability of this method to resolve brain circuits and to provide a systems level view of brain function in preclinical species. In Sect. 2, we focus on the anatomical localization of phMRI signals and their dependence on the mechanism of action of the probe agent. Selected examples are presented to illustrate the neurofunctional and anatomical specificity of phMRI activation patterns, and their correspondence with key circuits involved, e.g., in reward processing, motivated behavior or fear processing. In Sect. 3, we focus on functional connectivity in the rodent brain as measured by phMRI. The principles of this approach are reviewed, and demonstrated with examples that emphasize the close correspondence between functional connectivity networks and neurotransmitter pathways engaged by the stimulation paradigm. Finally, we discuss the translational potential of this approach in the light of recent applications of phMRI in humans.

2 Pharmacological Magnetic Resonance Imaging

The term phMRI was introduced by Leslie and James (2000) to indicate the application of functional MRI methods to map the spatiotemporal patterns of brain activity elicited by acute pharmacological challenge. In this context, acute drug administration serves as a probe to stimulate or inhibit activity in neuronal circuits, or to study the modulatory effects of behavioral preconditioning, pharmacological pretreatment or genetic background on drug-induced patterns of activation. While the effects of drugs on neuronal responses, vascular reactivity and neurovascular coupling can be complex, multimodal studies have corroborated the idea that in many instances the phMRI responses can be interpreted in terms of neuronal (de)activations induced by the rapid change in receptor or transporter occupancy following drug challenge. Hence, we find it natural to organize the review of phMRI studies by the specific neurotransmitter system stimulated by the primary drug challenge. In the following subsections, we review recent phMRI studies involving some of the major excitatory, inhibitory, and modulatory

neurotransmitter systems, with a special focus on the different brain circuits involved, and on the neurofunctional specificity of the phMRI responses.

2.1 Glutamate

Glutamate is the most important excitatory neurotransmitter in the brain. In conjunction with its inhibitory counterpart γ-aminobutyric acid (GABA), glutamate exerts a tight control of both tonic and phasic activity of wide neuronal populations and circuits (Kandel et al. 2000). Moreover, glutamate plays a key role in the neurometabolic cascade that determines the hemodynamic response to changes in neuronal activity (Zonta et al. 2003).

Several phMRI studies have described the functional effects of pharmacological manipulation of glutamatergic neurotransmission. Of particular interest is a recent series of studies with antagonists of the *N*-methyl-D-aspartate receptor (NMDAR) such as ketamine and phencyclidine (PCP) (Gozzi et al. 2008a, b, c, 2010a). These drugs are the subject of extensive investigation due to their ability to induce symptoms reminiscent of those of schizophrenia in healthy subjects, a finding that has led to the hypothesis that a decreased NMDAR function may be a predisposing or even causative factor for this disabling disease (Farber 2003; Kristiansen et al. 2007). The similarity between NMDAR antagonist-induced psychosis and schizophrenia is also widely exploited preclinically to model schizophrenia symptoms and to provide experimental models that may prove useful in the development of novel treatments for the human disorder. Specifically, the ability of drugs to inhibit behaviors induced by NMDAR antagonists may be interpreted as a pharmacodynamic signal of pharmacological activity, or as a predictor of the efficacy of novel pharmacological treatments for schizophrenia (Large 2007). Within this framework, noninvasive neuroimaging techniques such as phMRI can be applied to spatially resolve the neuronal circuitry engaged by glutamate NMDAR antagonism in humans and preclinical species, thus providing a valuable translational tool for schizophrenia and psychosis research.

The use of NMDAR antagonists to model schizophrenia symptoms in preclinical models is however complicated by the strong dose-dependence of the effects they exert. Indeed, at sufficiently high doses PCP and ketamine act as anesthetics and their psychotogenic effects arise only at lower, subanesthetic doses (Krystal et al. 1994; Morris et al. 2005). As rodent neuroimaging studies are typically performed under anesthesia, the interaction of NMDAR antagonists with the anesthetic agents needs to be carefully assessed to identify workable doses and anesthetic regimens.

This aspect was specifically investigated in a recent experiment where the phMRI response to PCP in halothane-anesthetized rats was mapped for varying levels of anesthesia and different PCP challenge doses (Gozzi et al. 2008c). Not surprisingly, both anatomical distribution and sign of the response depended strongly on anesthetic level and challenge dose, with sustained and widespread deactivation at higher PCP doses or anesthesia levels, a signal of positive

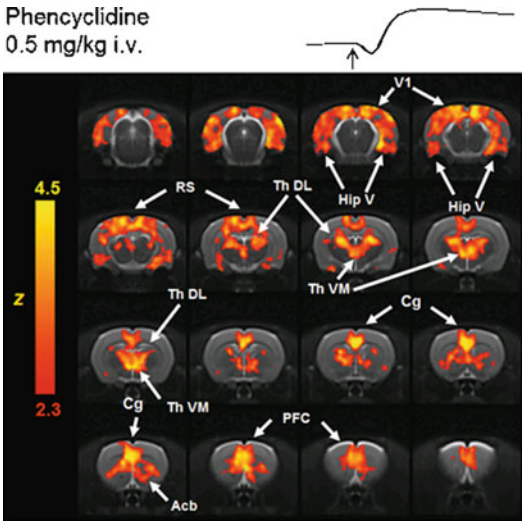

Fig. 1 Maps of rCBV response following acute PCP challenge (0.5 mg/kg i.v.; $n = 24$) relative to vehicle ($n = 6$), showing significant activation of the cortico–limbo–thalamic circuit. Adapted from Gozzi et al. (2008b, 2010b) with permission. Abbreviations: *V1* primary visual cortex; *VHip* ventral hippocampus; *RS* retrosplenial cortex; *DLth* dorso-lateral thalamus; *VMth* ventro-medial thalamus; *Cg* cingulate cortex; *Acb* nucleus accumbens; *mPFC* medial prefrontal cortex

interaction between the drug and the anesthetic agent. However, at appropriate combinations of PCP and anesthetic doses a focal and robust pattern of activation was observed in cortico–limbo–thalamic areas, including visual, orbitofrontal, cingulate cortices, the amygdala, dorsolateral, and ventromedial thalamus, ventral and posterior hippocampus and basal ganglia (Gozzi et al. 2008b, c) (Fig. 1).

Analogous patterns of activation have been shown using various NMDA receptor antagonists (dizolcipine/MK801 and ketamine) and functional readouts (i.e., BOLD) under similar anesthetic conditions (Littlewood et al. 2006; Roberts et al. 2008). Importantly, the activation pattern highlighted in these studies is consistent with that observed in conscious rats with 2-deoxyglucose autoradiography (Duncan et al. 1998a, b, 1999; Miyamoto et al. 2000), single unit electrophysiological recording (Homayoun et al. 2005), [^{14}C]-iodoantipyrine CBF measurements (Cavazzuti et al. 1987) and immediate-early gene expression (Nakki et al. 1996). Thus, a judicious choice of anesthetic regimen and drug dose appears to preserve the neuroanatomical substrates stimulated by NMDAR antagonists in conscious subjects. A good correspondence was also found between these animal findings and the patterns of brain activity measured in humans under ketamine infusion using either metabolic (FDG-PET) or hemodynamic (BOLD) neuroimaging techniques (Langsjo et al. 2003; Deakin et al. 2008; Gozzi et al. 2008b). Hence, the circuitry recruited by acute NMDAR antagonism appears to be consistent across species and imaging modalities, thus making the use of NMDAR antagonists an attractive translational paradigm.

The focal nature and anatomical localization of the pattern of activation produced by NMDAR antagonism may provide important information regarding the circuitry mediating psychosis. A number of clinical neuroimaging studies show evidence of a strong correlation between fronto–thalamo–hippocampal hyperactivity and cognitive and perceptual alterations observed in unmedicated schizophrenia

patients (Parellada et al. 1994; Silbersweig et al. 1995; Liddle et al. 2000; Ngan et al. 2002; Soyka et al. 2005). Moreover, functional impairment of limbic cortical areas such as cingulate and retrosplenial cortices has been associated with the development of thought disorder, disturbance of consciousness, and overall cognitive decline (Mitelman et al. 2005; Kircher and Thienel 2005). Likewise, the identification of robust foci of activation in the thalamus is in agreement with recent evidence supporting a critical role of thalamic gating disturbance in the pathophysiology of schizophrenia (Clinton and Meador-Woodruff 2004). Finally, PCP-induced activation of mesolimbic and nigrostriatal structures is in good agreement with the classical dopamine hypothesis of schizophrenia, where dysregulation of dopamine transmission is implicated at the onset of positive symptoms (Carlsson et al. 1999). These observations demonstrate a significant degree of correspondence between the brain areas activated by PCP challenge, and some of the key brain circuits that are thought to be dysfunctional in schizophrenia. This suggests that phMRI with NMDAR antagonists may provide a useful paradigm to study the neuroanatomical substrate of psychosis, and to test the effects of existing and putative antipsychotics on these circuits.

Recently, Gozzi et al. (2008b) investigated the modulatory effects of several antipsychotic agents with distinct pharmacological mechanisms on the patterns of activation induced by PCP in the rat. Dopaminergic agents such as the dopamine D_2 receptor antagonists raclopride did not significantly affect the response to PCP, consistent with a downstream implication of the dopamine system with respect to the mechanism that elicits the phMRI response to the PCP challenge. On the other hand, agents known to inhibit aberrant glutamatergic activity, such as the metabotropic glutamate receptors ($mGluR_{2/3}$) agonists LY354740, or the sodium channel blocker lamotrigine, suppressed entirely the activation induced by PCP, thus indicating a primary role of glutamatergic neurotransmission in the functional response to PCP. Consistent with this notion, a recent study showed that stimulation of the glycine coagonist site of the NMDAR either by direct agonism with D-serine, or by blockade of glycine reuptake with the glycine transporter type 1 (GlyT-1) inhibitor SSR504734 completely prevented PCP-induced phMRI activation in anesthetized rats (Gozzi et al. 2008a). Some of these findings in animal models have been confirmed in psychobiological (Krystal et al. 1999; Anand et al. 2000) or neuroimaging (Deakin et al. 2008) studies with the NMDAR antagonist ketamine in humans. Interestingly, the $mGluR_{2/3}$ receptor agonist LY2140023 was shown to provide significant therapeutic benefit to schizophrenia patients in a randomized phase-II clinical trial (Patil et al. 2007), a finding that has not been replicated in a recent second study, where however a larger than anticipated placebo response was observed (Kinon et al. 2010). Although further research is needed to ascertain the exact therapeutic contribution of this mechanism in schizophrenia, the limited clinical data produced so far support a putative pathophysiological role of NMDAR dysfunction and the translational use of neuroimaging assays exploiting the psychotogenic effects of NMDAR antagonists.

The ability of neuroimaging methods to spatially resolve patterns of activation is critical to identify the neuronal substrate of drugs with multireceptor targets. In a

recent study, pretreatment with the prototypical second-generation antipsychotic clozapine, a drug characterized by a complex receptor profile (Meltzer 1996), resulted in a region-dependent modulation of the phMRI response to PCP, with complete suppression of the relative cerebral blood volume (rCBV) response in the thalamus (Gozzi et al. 2008b). In the light of the key role of altered thalamic gating to the etiopathology of schizophrenia, it is tempting to interpret the region-dependent effect of clozapine as a mechanistic marker of its superior antipsychotic efficacy, particularly in refractory patients. Individual components of the complex profiles of many antipsychotics can be studied using the PCP/phMRI paradigm in combination with selective tool compounds. By way of example, selective serotonin $5HT_{2A}$ receptor antagonism, an important component of clozapine and other second generation antipsychotics, was shown to regionally inhibit the pattern of activation produced by PCP in the septo–fronto–hippocampal circuit (Fig. 2) (Gozzi et al. 2010a). This observation is of particular interest given the ample clinical evidence suggesting a correlation between fronto–hippocampal hyperactivity and cognitive and perceptual alterations in unmedicated schizophrenia patients (Parellada et al. 1994; Silbersweig et al. 1995; Liddle et al. 2000; Medoff et al. 2001; Ngan et al. 2002; Soyka et al. 2005). Notably, glucose metabolism studies using positron emission tomography (PET) demonstrated a tight correlation between depression of cortico–hippocampal activity and antipsychotic action elicited by a single-dose of the atypical antipsychotic risperidone (Liddle et al. 2000), thus corroborating the clinical significance of this circuit in schizophrenia.

Collectively, all these preclinical and clinical findings support the use of NMDAR antagonists in combination with functional neuroimaging as a valuable translational paradigm to study the neuropathological processes that might contribute to the symptoms of schizophrenia, and to investigate how these processes are modulated by antipsychotic agents.

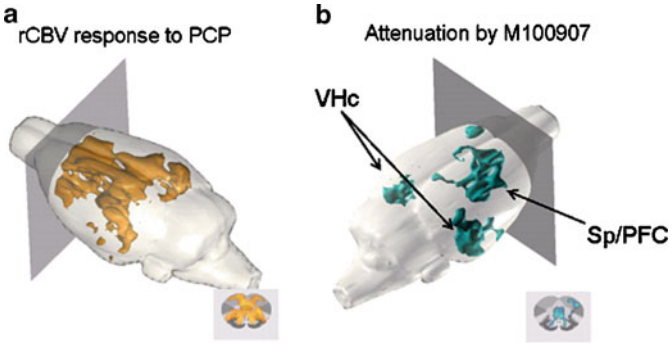

Fig. 2 (**a**) Volumetric reconstruction of the pattern of rCBV activation produced by acute challenge with PCP with respect to vehicle, and (**b**) attenuating effect of pretreatment with the selective 5-HT_{2A} antagonist M100907 (1.5 mg/kg i.p., group 1 vs. group 2) in fronto–septo–hippocampal regions. *PFC* medial prefrontal cortex; *VHc* ventral hippocampus; *Sp* septum. Adapted from Gozzi et al. (2010a) with permission

2.2 γ-Aminobutyric Acid

Consistent with the fundamental role of the inhibitory neurotransmitter GABA in controlling local and general excitatory tones, pharmacological modulation of GABA elicits robust phMRI responses. Early studies showed sustained and regional brain activation upon infusion of the GABAA antagonist and epileptogenic drug bicuculline in the rat (Reese et al. 2000). More recently, Kalisch et al. (2004) demonstrated differential BOLD-contrast responses to the anxiolytic and GABA-modulator diazepam as a function of the trait-anxiety phenotype in different rat strains. The same authors reported a striking anatomical correspondence between phMRI and Fos immunoreactivty measurements under similar experimental conditions (Kalisch et al. 2004). These findings suggest that insufficient activation of anxiety-inhibiting structures (i.e., medial prefrontal cortex) may play a key role in determining high trait anxiety. The involvement of GABA-mediated signal changes has also been suggested for the BOLD-response to the opioid and addictive drug heroin (Xu et al. 2000; Xi et al. 2004).

On the methodological side, GABA-releasing agents can be used as tools to unravel the individual neurometabolic components underlying cerebral deactivation and the elusive nature of negative fMRI responses. In a recent study, Gozzi and colleagues applied a multimodal approach to investigate the negative phMRI signal changes produced by the GABA-transporter-inhibitor and anticonvulsant drug tiagabine (Gozzi et al. 2006a). The drug-induced dose-dependent increases in extracellular GABA-concentration that were correlated with decreases in hemodynamic parameters. Interestingly, reduced tissue perfusion was accompanied by increased tissue oxygen tension, thus demonstrating an overall reduction of oxidative metabolic activity. Similar approaches have been exploited to qualify the specific hemodynamic and metabolic processes involved in negative BOLD-response to different stimuli, or to describe their dependence on resting GABAergic tone (Chen et al. 2005b).

2.3 Dopamine

Dopamine is involved in many brain functions, including reward processing, control of motivated and goal-oriented behavior, and cognition (Kandel et al. 2000). Moreover, dopamine is thought to mediate the reinforcing properties of drugs of abuse, and the long-term plastic changes responsible for addictive behaviors, craving and ultimately relapse (Koob and Le Moal 2001; Volkow et al. 2007). Hence, the dopaminergic system has been extensively studied in the preclinical imaging arena.

A number of studies with dopamine-mimetic drugs or dopamine-releasing agents including apomorphine (Schwarz et al. 2006b) amphetamine (Chen et al. 1997; Nguyen et al. 2000; Schwarz et al. 2004a, c; Dixon et al. 2005; Chen et al. 2005a;

Preece et al. 2007) and cocaine (Marota et al. 2000; Febo et al. 2004; Schmidt et al. 2006) have demonstrated consistent patterns of phMRI responses including mesolimbic and mesocortical dopamine pathways, as well as cortical regions. However, dopamine may induce vascular responses independent of changes in neuronal activity by interacting with dopamine D_1 and D_5 receptors expressed in the microvasculature (Choi et al. 2003; Jenkins et al. 2003). Moreover, many psychostimulants also induce strong changes in arterial blood pressure, a potential confound that might affect central hemodynamic responses.

Several studies have addressed these issues, and the role of different neurochemical and neurometabolic contributions to the functional MRI response to dopaminergic agents, with the aim of demonstrating the specificity of signal changes. Concurrent phMRI and microdialysis measurements showed significant correlation between dopamine release and hemodynamic response in the rat striatum (Schwarz et al. 2004b, c). However, no correlation was found between cerebral blood volume (CBV) changes and local dopamine levels in the cortex, thus suggesting that direct interactions of dopamine with cortical vasculature cannot account for the strong phMRI response to cocaine (Schwarz et al. 2004c). Consistent with this finding, multimodal CBF and tissue oxygen level measurements in situ demonstrated a strong coupling between cocaine-induced hemodynamic responses and local metabolic changes, thus confirming that a purely vascular effect is unlikely to be the primary determinant of the phMRI response (Ceolin et al. 2004).

Several studies have investigated the role of blood-pressure changes as a potential confound in phMRI experiments. In an elegant study, Luo et al. (2003) used a nonbrain penetrant salt form of cocaine (cocaine-methyliodide) to rule out potential peripheral contributions to the pattern of fMRI activation elicited by cocaine. Nonbrain penetrant vasopressors were also used to demonstrate that autoregulation in the rat brain is functional over a wide range of arterial blood pressure values under the anesthetic conditions used for the phMRI experiments, and that abrupt changes in blood pressure within that window do not result in significant central phMRI confounds (Gozzi et al. 2007). Febo et al. (2004) observed mesolimbic increases in BOLD signal in response to intracranial injections of cocaine in awake rats undergoing phMRI, thus suggesting that stimulant-induced fMRI responses generalize to the waking state. These studies provide converging evidence that the peripheral effects of stimulants (e.g., increased heart rate and blood pressure) cannot account for the central hemodynamic changes measured with phMRI.

The neurochemical specificity of the functional responses produced by amphetamine and other dopamine-releasing agents has been investigated by several research groups. A recent paper (Knutson and Gibbs 2007) reviewed the experimental evidence suggesting that the hemodynamic response to dopamine-mimetic agents predominantly depends on agonism at dopamine D_1 receptors on postsynaptic neural membranes, with D_2 receptors playing an indirect, modulatory role. These mechanistic studies in animals have greatly advanced our understanding of neurovascular transduction of dopaminergic stimuli, and provide a robust interpretative framework for clinical fMRI paradigms that probe dopamine

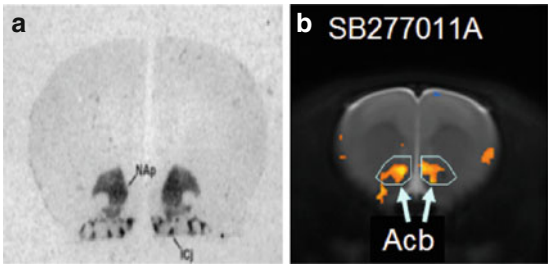

Fig. 3 (**a**) Distribution of dopamine D3 receptors in the rat brain and (**b**) pattern of potentiation of the phMRI response to amphetamine challenge by dopamine D3 receptor antagonist SB277011A. SB277011A modulates phMRI response in the Nucleus Accumbens (Nac), a key structure in the mesolimbic dopamine system, consistent with the focal expression of DA D3 receptros in this region

neurotransmission and its modulation by genetic, pharmacological, or psychodynamic conditioning (Knutson and Gibbs 2007).

Dopamine-releasing agents can also be used as probe challenge to study the modulatory effects of pharmacological pretreatment on the evoked pattern of phMRI response. This approach has been recently applied to the characterization of the functional effects of a dopamine D_3 receptor antagonist (SB277011), a class of compounds that reduce drug-seeking behavior in animal models, and have potential as pharmacological agents to treat addiction (Micheli et al. 2007). A single dose of dopamine D_3 receptor antagonist administered prior to an amphetamine or a cocaine challenge increased evoked-dopamine outflow (Congestri et al. 2009) and phMRI response in the cingulate cortex and multiple areas of the mesolimbic dopamine system, including the nucleus accumbens (Acb), a dopaminergic afferent region rich in dopamine D_3 receptors and involved in the control of motivation and goal-oriented behavior (Fig. 3) (Schwarz et al. 2004a; Micheli et al. 2007). To correlate these findings with the behavioral evidence showing a robust effect of these compounds as inhibitors of compulsive drug-seeking and relapse (Heidbreder et al. 2005), it has been suggested that potentiation of dopaminergic responses by D_3 receptor antagonism may rescue hyporeactivity of the dopamine system produced by long-term drug-abuse and restore dopaminergic tone in states of addiction (reviewed by Volkow et al. 2007).

2.4 Acetylcholine

Most functional neuroimaging studies of cholinergic neurotransmission in rodents involve the prototypical nicotinic acetylcholine receptor (nAChRs) agonist nicotine.

Acute exposure to nicotine enhances cognitive functions such as attention, learning, and memory in humans and preclinical species (Levin and Simon 1998; Rezvani and Levin 2001; Levin and Rezvani 2002). The drug also has also

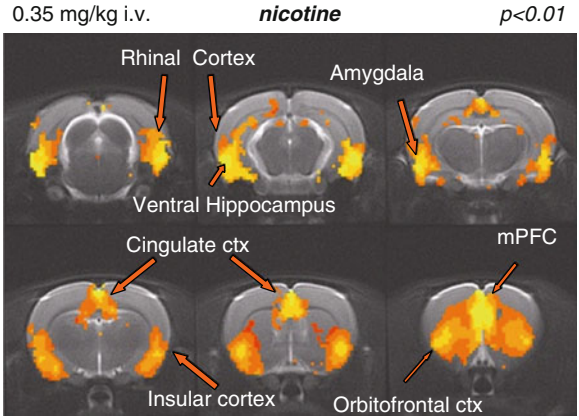

Fig. 4 Maps of rCBV response to acute nicotine challenge (0.35 mg/kg i.v.) in the rat brain, showing significant activation of key cortical areas involved in cognitive processing, including prefrontal, orbitofrontal, insular and cingulate cortices. Adapted from Gozzi et al. (2005) with permission

addictive properties, an effect that probably involves the recruitment of the mesolimbic dopamine system and its cortical terminals (Mathieu-Kia et al. 1998). Consistent with its pharmacological profile, acute administration of nicotine to drug-naïve rats produced focal activation of corticolimbic regions including the amygdala, ventral hippocampus, medial prefrontal, orbital, insular, and anterior cingulate cortex (Fig. 4) (Gozzi et al. 2006b). Analogous studies were performed by Stein and colleagues in human smokers using BOLD fMRI (Stein et al. 1998). Interestingly, the pattern of activation observed by Stein in humans is remarkably similar to that activated by nicotine in the rat. The cingulate and several frontal lobe divisions, including the dorsolateral, orbital, and medial frontal, were among the most prominently activated regions in both studies. The frontal lobes with their rich dopaminergic innervations and the cingulate cortex, through its connections with many neocortical association, motor, and sensory regions, are thought to be involved in cognitive functions known to be affected by nicotine intake (Warburton 1990). In addition, nicotinic receptors are present on both the somatodendritic and the axon terminals of locus ceruleus noradrenergic neurons, which are known to project to much of the forebrain and hippocampus.

These neurons and their projections are thought to regulate or modulate behavioral arousal and vigilance (Aston-Jones et al. 1994). This converging evidence suggests that the regions identified by human and rat phMRI studies represent a plausible neuronal substrate of the psychopharmacological action of nicotine. Consistent findings have also been reported by Nagata et al. (1995), who measured increased blood flow in the frontal lobes after cigarette smoking using ^{15}O positron emission tomography (PET).

Gozzi et al. (2006b) also investigated the relative contribution of α7 and α4β2* nAChR subtypes to the observed activation pattern of nicotine by using selective nAChR subtype agonists, and found that the selective α4β2* nAChR selective agonists 5-iodo-A-85380 elicits a pattern of activation identical to that of nicotine, whereas no signal changes were observed with the α7 agonist cpdA. A later study of

the effect of a selective α4β2 nAChR agonist using a CBV-weighted protocol in awake rats produced consistent results (Skoubis et al. 2006). These studies suggest that, of the many nAChR subtypes at which nicotine show significant activity, the α4β2* nACh receptors predominantly mediate the psychopharmacological and functional effects of nicotine, a finding corroborated by genetic and behavioral studies (Levin 2002; Picciotto 2003; Kauer 2005).

2.5 Serotonin

Serotonin (5-HT) is a modulatory neurotransmitter involved in key brain functions such as mood control, cognition, sleep and pain processing, through interactions with a heterogeneous family of receptor subtypes (Kandel et al. 2000). Several phMRI studies have addressed aspects of the complex role of serotonergic neurotransmission by pharmacologically targeting specific receptors subtypes.

In a recent study, the effects of the 5-HT_{1B} and 5-HT_{2C} agonist mCPP on the regulation of feeding behavior in rats were examined using phMRI (Stark et al. 2006, 2008). The authors identified several structures with increased activity and, more importantly, structures exhibiting decreased activation not evident from standard immunochemistry markers of neuronal activity such as Fos. The study also allowed the identification of a plausible mechanism by which mCPP induces hypophagia in terms of both a specific receptor subtype (i.e., 5-HT_{2C}) and the neuroanatomical circuitry (i.e., hypothalamus and medulla oblongata).

An early demonstration of the exquisite sensitivity of phMRI measures to serotonergic stimulation was provided by Scanley et al. (2001), who used CBV fMRI to investigate the effects of the 5-HT_{1A} receptor agonist 8-OH-DPAT in the rat. Acute challenge with 8-OH-DPAT resulted in a reduction of CBV (negative response) in brain areas rich in 5-HT_{1A} receptors, including hippocampus, septum, caudate, cingulate cortex, and thalamus. The pharmacological specificity of the effect was corroborated by the ability of the selective 5-HT_{1A} antagonist WAY-100635 to block the response to 8-OH-DPAT. The presence of functional deactivation is consistent with the molecular role of the 5-HT_{1A} receptor, a G-protein coupled inward rectifying potassium channel that produces rapid membrane hyperpolarization and suppresses neural firing upon endogenous or exogenous stimulation (Tsetsenis et al. 2007).

The effects of acute selective inhibition of the reuptake of serotonin in drug naïve rats was recently investigated using the antidepressant fluoxetine (Schwarz et al. 2007b). The pattern of activation induced by acute fluoxetine administration was widespread, and involved both cortical and subcortical regions, consistent with the ubiquitous presence of serotonin in the brain. Importantly, a good delineation of the ascending serotonergic pathways originating in the raphe nuclei was observed using correlation analysis (Schwarz et al. 2007b) (see next section), thus corroborating the serotonergic origin of the phMRI responses. The implications of these findings are discussed more in detail in a later section of this chapter (Sect. 3.1).

2.6 *Neuropeptides*

The ability of phMRI to resolve circuits recruited by specific neurotransmitter systems can be exploited to map the effects of substances that do not effectively cross the blood–brain barrier, such as neuromodulatory peptides, in preclinical species, where more invasive methods of administration can be applied. The potential of this approach was demonstrated in a study of the rCBV changes induced by intracerebroventricular (ICV) infusion of synthetic psychoactive peptides through MR compatible indwelling cannulae. ICV administration of the metabolically stable neurokinin 1 (NK_1) receptor agonist GR73632 elicited sustained CBV increase in several brain structures, including the amygdala, the caudate putamen and the cortex (Gozzi et al. 2005). Interestingly, the onset of the response was rapid, within minutes, thus suggesting that the response was initiated by interactions of the peptide with periventricular receptors and propagated downstream to deeper structure through functional connections. The same technique has also been applied to study the effect of corticotropin-releasing factor (CRF) (Gozzi et al. 2004). This method may represent a valuable tool for studying the functional correlates of the central activity induced by agents characterized by poor brain penetration, or to separate peripheral and central effects of psychoactive agents targeting widely distributed receptor pools. A recent example of this technique has been provided by Febo et al. (2005), who demonstrated the feasibility to separate central and peripheral contributions produced by psychostimulants.

3 Functional Connectivity Analysis of Brain Circuits

FMRI has been instrumental to study brain functional segregation, i.e., the functional specialization of discrete brain regions engaged by specific stimuli (Posner et al. 1988), including pharmacological stimuli, as extensively discussed in the previous section. To this end, fMRI time series are typically analyzed with massively univariate methods, testing the hypothesis of a greater (or lower) activity during execution of a task, or under drug challenge, than at baseline on a pixel-by-pixel basis (spatial smoothing notwithstanding) (Friston 1997). Functional specialization is then inferred from the segregation of activated pixels in anatomically or functionally defined brain regions, further emphasized by the application of statistical thresholds for the significance of fMRI activation.

While the evidence of functional specialization of brain cortical regions appears compelling, even the simplest sensorimotor task involves the integrated activity of multiple brain areas (Luria 1973), a notion consistent with the dicothomic principles of functional segregation and integration underlying the brain's functional organization. Multivariate analyses of fMRI time series can be applied to assess the distributed nature of brain neurophysiological response and to address *interactivity* among different structures (Friston et al. 1994; Rogers et al. 2007).

These approaches rely on the evaluation of some definition of correlation or covariance between spatially remote neurofunctional events. In this context, statistical dependencies among signals originating in different brain regions are interpreted in terms of *functional connectivity*, as opposed to *structural connectivity*, which denotes the presence of physical neuronal connections between remote brain structures (Ramnani et al. 2004). While the concept of functional connectivity is thought to capture the flow of information between brain regions, and the parallel nature of brain functional processes, it should be noted that it necessarily implies neither the presence of actual physical links between the regions involved, nor a causal relationship between spatially remote activations.

Functional connectivity has been investigated in both humans and laboratory animals with a number of techniques, and using different definitions of the notion of covarying activity. At the cellular level, functional connectivity has been measured by assessing the temporal coherence in the spiking activity of individual neurons (Gerstein and Perkel 1969). On a larger spatial scale, electroencephalography (EEG) has been applied to evaluate cross-correlation between signals from pairs of scalp electrodes and their coherence in specific frequency bands (Adey et al. 1961; Pfurtscheller and Andrew 1999) under evoked response or resting conditions. Interregional correlations in the metabolic responses across subjects have been used to investigate functional connectivity from PET studies with FDG in humans (Horwitz et al. 1984; Bartlett et al. 1987), or from autoradiographic data in laboratory animals (Soncrant et al. 1986).

The inception of fMRI has significantly expanded the repertoire of methods to study resting-state or task-evoked functional interactivity in the human brain. Increased flexibility has also enabled the exploration of different measures of interregional covariance, including temporal correlation, and correlations in the fMRI response amplitude across conditions and subjects [for a review of these methods see Horwitz (2003) and references therein]. It should be noted that while all these approaches represent legitimate definitions of functional connectivity, they interrogate different aspects of the regional interdependence and are not necessarily equivalent.

The recent discovery that spontaneous, low frequency fluctuations in the fMRI signals from the human brain at rest exhibit coherent patterns within defined networks has opened an interesting avenue of investigation, often referred to as "resting-state fMRI" (Beckmann et al. 2005). By way of example, a network of functional connectivity corresponding to brain regions whose activity is higher at rest than during an experimental task has been identified, and interpreted as evidence in support of the existence of a "default mode" of baseline brain function (Greicius et al. 2003). Interestingly, alterations in resting state functional connectivity have been observed under a number of pathological conditions, including Alzheimer's disease (Li et al. 2002), multiple sclerosis (Lowe et al. 2002), and schizophrenia (Zhou et al. 2008).

While there is a large body of work assessing functional connectivity in the human brain, the extension of these methods to preclinical species, and particularly to rodents, is very recent. The study of functional connectivity in laboratory

animals, where imaging can be combined with more invasive methods, may provide a better understanding of the physiological basis of the interregional correlations in fMRI signals observed in humans, and of the origin of the alterations in functional connectivity observed in pathological states. Several studies in laboratory animals have followed the path of human resting state fMRI. Synchronous low-frequency fluctuations were detected in bilateral primary somatosensory cortex in the rat brain at rest under alpha-chloralose anesthesia (Lu et al. 2007). Interestingly, significant correlation between fMRI responses and delta oscillations measured with epidural EEG were observed, thus suggesting a link between spontaneous fMRI fluctuations and the underlying neuronal electrical activity. Similarly correlated fluctuations were also detected in the rat under isoflurane (Kannurpatti et al. 2008), halothane (Majeed et al. 2009) or medetomidine anesthesia (Pawela et al. 2008, 2009; Zhao et al. 2008). All these studies used a seed-correlation region approach (i.e., signal time-course correlations were computed with respect to a specific region chosen by the experimenter a priori) and demonstrated encouraging correlations between bilaterally symmetrical cortical regions. However, coherent networks of connectivity akin to those observed in humans (e.g., the default mode network) have not been observed in the rat using this method to date. Whether this reflects experimental impediments (e.g., the use of anesthesia, or high stress-levels due to restraint) or rather a different functional architecture of the rodent brain with respect to that of higher species such as primates is the subject for further investigation. However, the presence of multiple reports describing the identification of coherent functional-connectivity default mode networks in deeply anesthetized monkeys (Vincent et al. 2007) or humans (Greicius et al. 2003; Kiviniemi et al. 2005; Horovitz et al. 2008) as well as in comatose patients (Boly et al. 2009) intriguingly favors the latter hypothesis.

A conceptually different approach to the study of functional connectivity in rodents has been explored using phMRI, a strategy that has proven very effective in determining the circuital and neurochemical basis of pharmacologically evoked fMRI responses. Several recent studies using this approach have shown exquisite delineation of focal patterns of correlated responses corresponding to key neurotransmitter pathways (Schwarz et al. 2007a, b, c, 2008, 2009). Moreover, network analysis of functional connectivity patterns obtained with phMRI has provided the first evidence of organized networks of functional connectivity in the rat brain, thus demonstrating the potential of this method to explore the brain functional architecture, an aspect discussed in greater detail in sections (Sect. 3.3).

Functional connectivity analysis of phMRI data requires image analysis approaches that are substantially different from those applied to study low-frequency spontaneous fluctuations. In this section, we summarize the key principles and methodological aspects of correlation analysis of phMRI data, and review recent studies exploring pharmacologically induced or modulated functional connectivity in the rat brain. Moreover, we discuss novel statistical methods based on a network representation of functional connectivity data, and their application to resolve the functional organization of the rat brain.

3.1 Correlation Analysis in phMRI

In a typical phMRI experiment, a drug challenge is administered systemically (e.g., intravenously or intraperitoneally) during the acquisition of the fMRI time series (Schwarz et al. 2004c; Gozzi et al. 2006b, 2008b). The time resolution of fMRI is determined by the timescale of the hemodynamic response following neuronal excitation, and is of the order of seconds. This temporal resolution is sufficient to capture the time-course of the slow spontaneous fluctuations measured by resting state fMRI (typically below 0.15 Hz). However, the phMRI signal changes following a drug challenge are much slower (typically lasting tens of minutes or more) than the hemodynamic response to a single neural "event" (Schwarz et al. 2003). Moreover, the signal time-course reflects drug kinetics and pharmacodynamic effects, and is similar in all brain regions affected (Schwarz et al. 2004c, 2005). Hence, intrasubject temporal correlations of the phMRI responses tend to be anatomically nonspecific; variability across subjects further complicates the generation of a suitably representative and meaningful group image.

To circumvent this problem, an alternative approach has been proposed that calculates interregional correlations in the response amplitude *across subjects* (Schwarz et al. 2007b), in the fashion of the procedures applied in metabolic PET or autoradiography studies (Horwitz et al. 1984; Soncrant et al. 1986). Schematically, the correlation analysis procedure used in phMRI studies can be summarized in terms of the following steps. First, individual subject time series are coregistered to a common stereotaxic space (Schwarz et al. 2006a). Time courses from individual image voxels are then extracted for each subject, and suitable regressors are fitted to the data to determine response amplitudes (Schwarz et al. 2005). In this context, we intend the response amplitude to represent the magnitude of the postinjection signal change elicited by the acute drug challenge. These values provide voxel-specific vectors of response amplitude across subjects. Intersubject correlations are then calculated for each voxel in reference to a selected "seed" region, using the vector of response amplitudes from step B. Finally, statistical correlation maps, i.e., maps of voxels whose response amplitude correlates significantly (in a statistical sense) with those in the reference region are generated.

This approach leverages variations in *the spatial profile* of the response observed across subjects following drug challenge. Figure 5 provides an outline of this process, and an exaggerated visual impression of the intersubject variability in the phMRI response profile.

A recent study (Gozzi et al. 2010b) has significantly expanded the area of application of these methods by combining phMRI, intersubject functional connectivity and advanced mouse genetics to provide a direct link between cells, circuits, and behavior. Specifically, the authors combined phMRI and focal pharmacogenetic silencing to spatially resolve behavior-specific circuits controlled by a discrete neuronal population expressed in the central nucleus of the amygdala (CeA), a brain region involved in emotional processing and behavioral response to fear (LeDoux 2003). Reversible suppression of neural activity in a subset ("type-I") of CeA

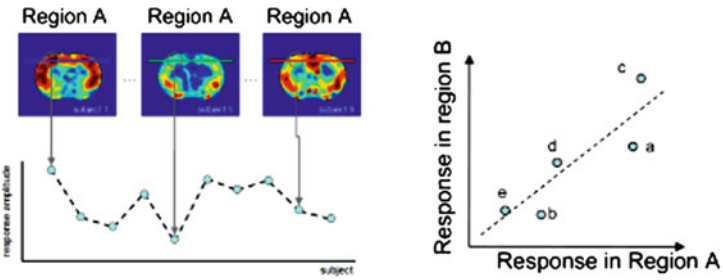

Fig. 5 Schematic overview of the principle underlying across-subject functional correlation. Different subjects show different spatial profiles of responses to the pharmacological challenge. This variability is leveraged to extract response vectors for each image voxel (*left-hand panel*). Intersubject correlations are then calculated for each pair of image voxels, or for a voxel in reference to a seed region. Adapted from Schwarz et al. (2009) with permission

neurons was achieved by inducing cell-specific expression of the serotonin 1A receptor (Htr1aR) in mice devoid of the endogenous receptor (the resulting mice called $Htr1a^{CeA}$), a strategy recently described by Gross and coworkers (Tsetsenis et al. 2007). The Htr1aR is coupled to GIRK potassium channels, and its activation induces rapid and reversible membrane hyperpolarization of the cells expressing the receptor. Hence, the use of systemically administered Htr1aR agonists (like the selective compound 8-OH-DPAT) can be exploited to obtain cell-specific inhibition of spontaneous neuronal firing in behaving animals (Tsetsenis et al. 2007). By combining fMRI with this pharmacogenetic inhibition strategy, the authors were able to identify a novel pathway in the mammalian brain apt to regulate passive and more active components of fear responses. Specifcally, selective inhibition of type-I CeA cells led to widespread increased cortical arousal as visualized by phMRI upon acute administration of the selective Htr1AR agonist 8-OH-DPAT. Intersubject functional connectivity analyses of phMRI time-courses was critical in resolving the neuronal circuitry underlying the increased cortical activity. Significant correlation was found between the CeA and several ventral forebrain cholinergic nuclei such as the substantia innominata and the diagonal band, and the same cholinergic nuclei exhibited tight correlations with the cortical areas activated upon inhibition of type-I CeA neurons. The involvement of the cholinergic system was confirmed in additional phMRI studies showing that the cortical arousal was blocked by central (but not peripheral) cholinergic antagonists. Remarkably, an analysis of the behavioral correlates of cortical activation in $Htr1a^{CeA}$ mice highlighted a pivotal role of type-I CeA cells as suppressors of cholinergic-activity and exploratory behavior and promoters of freezing (passive) fear responses, thus leading to the identification of a novel neural pathway that biases fear responses toward either passive or active coping strategies. Methodologically, this work expands the applicability of functional connectivity analyses to genetically modified mouse models, and provides at the same time a compelling demonstration of the combined use neuroimaging methods and advanced pharmacogenetic systems as a new powerful paradigm to identify and resolve behaviorally relevant neural circuits in the living brain.

3.2 *Functional Connectivity in Neurotransmitter Systems*

The first demonstration of this approach applied correlation analysis of rCBV changes in the rat induced by acute challenge with D-amphetamine and fluoxetine, two widely used (and abused) drugs that target key monoaminergic systems (Schwarz et al. 2007b).

One of the main effects of D-amphetamine in the brain is the presynaptic stimulation of dopamine release resulting in increased extracellular levels of dopamine at the terminals of dopaminergic neurons (Everitt and Robbins 2005). The psychoactive and reinforcing properties of D-amphetamine are thought to be mediated by stimulation of the mesolimbic dopamine pathway, a small bundle of neurons originating from the ventral tegmental area (VTA) in the midbrain and projecting to the ventral part of the striatum, the Acb (Everitt and Robbins 2005). Consistent with this hypothesis, correlation maps of phMRI responses referenced to the VTA clearly delineated the parallel major axes of this pathway forward through the ventromedial thalamus to the ventral striatum (Fig. 6). Other downstream patterns of functional connectivity could be identified by moving the reference region along the functional connectivity pathway. Using the shell of the Acb as the seed region, a functional connectivity pattern largely coincident with that of the

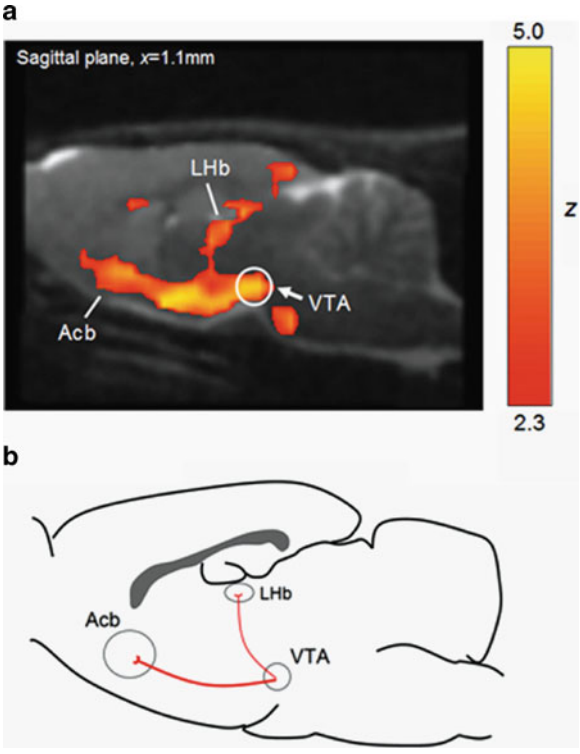

Fig. 6 Map of the responses to D-amphetamine covarying (across-subjects) with those in the ventral tagmental area (VTA). The pattern of connectivity delineates the mesolimbic dopamine pathway extending forward to the striatum. Adapted from Schwarz et al. (2007b) with permission. *LHb* lateral habenula; *Acb* nucleus accumbens

VTA, but extending to the medial prefrontal cortex (mPFC), was identified, consistent with the known reciprocal projections of this region to the accumbens. Moving further down the dopamine pathway, broader cortical involvement, including somatosensory and motor cortices was observed. In this way, it was possible to discriminate between connectivity in the primary dopaminergic projections, and connectivity with downstream structures.

A second remarkable example of the use of functional connectivity to delineate circuits engaged by specific neurotransmitters was obtained by the same authors with fluoxetine, a selective serotonin reuptake inhibitor (SSRI) (Stahl 1998) and a clinically effective antidepressant. The main source of serotonergic projections to the forebrain originates in the raphe nuclei of the brain stem. Correlation analysis using the raphe as a reference region showed a focal delineation of major ascending serotonergic projections to the forebrain and cortex, and a network of subcortical structures including hippocampus and amygdala, a pattern of connectivity consistent with the drug's mechanism of action (Fig. 7).

This study represented the first in vivo demonstration of functional connectivity in discrete neurotransmitter systems. The correlation analysis was guided by a judicious choice of seed region that was informed by previous knowledge of the systems under investigation. While the confirmatory evidence produced in this study represented convincing validation of the method, approaches that are independent of the specific choice of reference region would be desirable for more exploratory studies where the mechanism of action of the drug is unknown.

A more general approach to study patterns of connectivity from phMRI data has been proposed (Schwarz et al. 2007a), whereby a hypothesis-free cluster analysis was applied to resolve networks of correlated brain activity stimulated by D-amphetamine without prior definition of a seed correlation region. Forty-eight volumes of interest (VOIs) providing a reasonably complete breakdown of the image volume acquired were delineated automatically using a three-dimensional

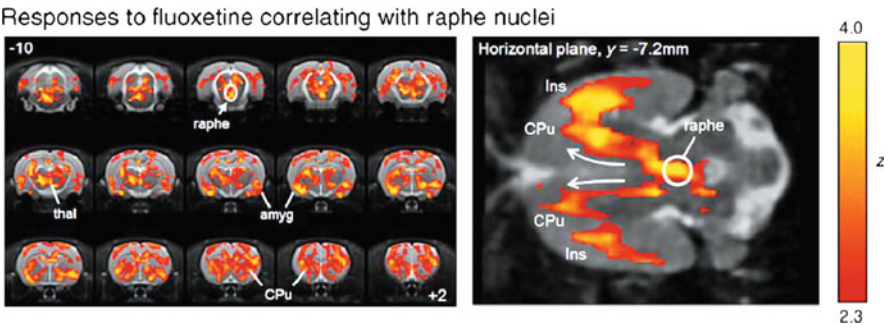

Fig. 7 Map of the responses to fluoxetine covarying (across-subjects) with those in the Raphe nucleus. The responses were predominantly subcortical and included regions in mesencephalic areas, the thalamus, amygdala and caudate putamen. Ascending projections to forebrain regions are visible in the horizontal slice shown at *right*. Adapted from Schwarz et al. (2007b) with permission. *Ins* insular cortex; *thal* thalamus; *CPu* caudate putamen; *amyg* amygdala

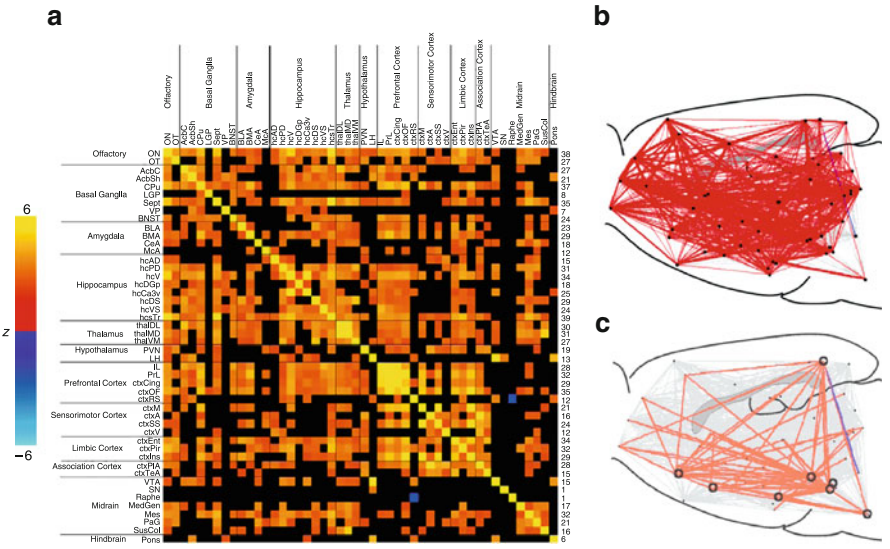

Fig. 8 Interregional correlations in response to D-amphetamine. (**a**) Cross-correlation matrix for the 48 brain structures examined; (**b**) Connections between the 48 structures in sagittal view. Each region is indicated by a *black dot*, and the connections by *red lines*. (**c**) A cluster of structures identified by the *k*-clustering algorithm, corresponding to the primary projection of the mesolimbic dopamine system. Adapted from Schwarz et al. (2007a) with permission

digital reconstruction of the Paxinos and Watson rat brain atlas coregistered with the MRI rat brain template (Schwarz et al. 2006a). Pairwise correlations within the set of selected structures were calculated by computing the Pearson linear correlation coefficient r between the response vectors in each pair of structures, yielding a 48×48 correlation matrix summarizing the similarities in response profile among the selected VOIs (Fig. 8). To identify groups of brain structures within the full correlation matrix that were closely coupled in their response to D-amphetamine, these authors applied a *k*-means clustering algorithm (Everitt et al. 2001), an optimization-based method that minimizes a distance measure between the N_v response vectors. When applied to a D-amphetamine phMRI data set, the algorithm indicated that the 48 response vectors should be optimally clustered into three groups of VOIs to maximize intercluster over intra-cluster dispersion in the normalized cross-subject response profiles. Interestingly, one of the clusters predominantly consisted of brain structures involved in the mesolimbic and nigrostriatal dopamine pathways, including the VTA and substantia nigra (SN), together with ventral structures along the major axes of these pathways (Fig. 8c). It is important to emphasize that the clustering results did not identify merely a trivial solution in which strongly responding regions were grouped together while those responding more weakly were clustered apart. Instead, key brain areas involved in primary dopamine pathways emerged naturally from the data in the form of a group of tightly interconnected structures, thus overcoming the limitations imposed by the need of introducing a priori knowledge in the analysis.

In the phMRI approach, a drug challenge serves the purpose of eliciting a brain response whose intersubject variability provides a basis to calculate interregional correlations. From a pharmacological point of view, it is also important to ascertain whether drug treatment can *modulate* brain functional connectivity, since this might shed light on the drug mechanism of action at a systems level. One interesting approach to addressing this problem has been proposed by Schwarz et al. (2007c), whereby the effects of a compound of interest were assessed by examining how it modulated the correlated responses to a different probe compound. Specifically, this study investigated the effects of a selective dopamine D_3 receptor antagonist, a putative treatment of addiction, on the pattern of functional connectivity stimulated by D-amphetamine. The DA D_3 receptor has a particularly focal distribution in the brain, with high levels of expression in mesolimbic brain structures involved in reward-related processes, including the VTA and the Acb (Schwarz et al. 2004a; Heidbreder et al. 2005). The phMRI responses to amphetamine were acquired for the two groups, one treated with the selective DA D_3 receptor antagonist SB277011A, and one with vehicle. The effects of pretreatment with SB277011A were calculated by comparing the correlation matrices describing functional connectivity in the two treatment groups. Interestingly, main changes in interregion correlations were associated with the VTA, the origin of dopaminergic projections to the forebrain. Specifically, these involved connectivity of the VTA with the ventral subiculum and ventral CA3 fields in the hippocampus, dorsolateral and midline dorsal thalamus, piriform cortex and superior colliculi. In the midline dorsal thalamus and hippocampus, the relationship was altered from a significantly positive correlation with the VTA to a significantly negative one, with a dramatic inversion of the sign of the connectivity.

The thalamus and hippocampus are important components of a limbic brain circuit, and thought to be involved in drug addiction, providing modulatory inputs to the prefrontal cortex, an important brain region involved in goal-directed behavior. Pharmacological modulation of functional connectivity within this circuit may explain the inhibition of drug-seeking behavior consistently observed with SB277011A and other selective dopamine D_3 receptor antagonists (Cervo et al. 2007). Methodologically, this study extended the applicability of intersubject functional connectivity analyses of phMRI data to antagonist–agonist experiments, where modulations in the correlation structure underlying the response to a probe signal may be detected.

3.3 Complex Network Analysis and Community Structure of Functional Connectivity in the Rat Brain

Functional connectivity analyses of neuroimaging data aim to elucidate relationships between signals originating in spatially distinct brain regions. This emphasis on interaction between different brain structures is a good conceptual match for

representing the data as a graph, or network, of nodes and links. In this representation, image voxels or parcellated brain regions represent the nodes, and a measure of similarity or correlation in their responses defines the edges linking the nodes (Salvador et al. 2005; Eguiluz et al. 2005; Achard et al. 2006; Achard and Bullmore 2007). In recent years, networks from a wide variety of fields, describing connection patterns as diverse as social collaborations, metabolic pathways or air traffic, have been characterized and found to exhibit rich behaviors beyond those of simple random networks; accordingly, they are referred to as "complex networks" (Strogatz 2001). Network analysis of functional connectivity data to date has concentrated on the characterization of global properties of the network, which can reveal much about the properties of the system. For example, networks derived from human brain imaging data have been shown to possess a scale-free degree distribution and exhibit "small world" behavior – a finding that has implications for information transfer in the brain (Bullmore and Sporns 2009).

Complex networks of functional connectivity derived from phMRI data in the rat have been recently investigated (Schwarz et al. 2008, 2009). In these papers, the nodes were defined as individual image voxels (0.128 mm^3) in the 3D image volume, and the strength of the *edge* between each pair of nodes was based on the Pearson correlation coefficient between the intersubject response amplitude vectors in the two voxels. This value was converted into an equivalent z-statistic using Fisher's r-to-z transformation. The magnitudes of these normalized correlation values were used to describe the strength of the correlation between each pair of nodes and used to construct an edge weight matrix. Finally, the edge weight matrix was thresholded and binarized to define a binary adjacency matrix. Analysis of the structural properties of the resulting networks outlined interesting topological features, including a small-world structure, and a long tailed distribution of node degrees (i.e., number of connections for certain node), indicative of the presence of hubs, namely a subset of highly connected nodes in cortical regions.

Beyond global characterization, the coexistence of functional segregation and integration in brain activity (Tononi et al. 1994) suggest that some degree of modularity i.e., subnetworks or clusters of more tightly linked nodes, might exist within functional connectivity networks. The issue of the identification of such groups of nodes characterized by denser connections between their constituents than to other nodes outside the group has received considerable recent attention in the field of complex networks. This concept originated in the study of social relationships and has been consequently dubbed "community" structure. The first application of this concept to the analysis of brain functional connectivity networks has been recently demonstrated using phMRI data in rodents challenged with fluoxetine (Schwarz et al. 2008). In this paper, a network-theoretic community structure algorithm, based on the maximization of a mathematical formalism of "modularity," was applied to resolve functionally and anatomically segregated communities within a functional connectivity network derived from phMRI responses. Specifically, an algorithm based upon maximizing the value of a mathematical definition of *modularity* Q (Newman and Girvan 2004; Newman 2006) was applied to identify number and composition of subnetworks within a widely

distributed network of functional connectivity obtained under pharmacological challenge with fluoxetine. For a certain partition of the network, Q measures the difference between the fraction of the edges connecting nodes within communities and the same fraction in the case of a randomly connected network with the same partition. The closer the value of Q is to its theoretical maximum 1, the stronger the community structure, i.e., the more modular the network. Algorithms seeking a network partition that maximizes modularity yield an estimate of the number of communities into which the network should be optimally split, the composition of each community and an associated value of max(Q). In the case of functional connectivity networks, an optimal partition can reveal the system-level functional organization of the brain under the particular experimental conditions studied, as well as providing a measure of the emergent modularity. The application of this method to the functional connectivity network in the rat challenged with fluoxetine revealed three communities of nodes (Fig. 9). The pixels in the two largest communities were symmetrically distributed between the left and right hemispheres and their distributions corresponded closely to known anatomical and functional subdivisions of the rat brain. It is important to emphasize that division was obtained from a network representation based on image pixels with neither imposition of symmetry nor any prior anatomical constraints. Interestingly, one of the communities comprised nodes corresponding primarily to subcortical structures – striatum, thalamus, and amygdala – but also regions of the hippocampus and entorhinal, medial prefrontal and cingulate cortices (Fig. 9a). The finding of pixels in the cingulate and prefrontal areas grouped with those in striatal and thalamic structures is consistent with the fact that these cortical regions are the main cortical target of input from the basal ganglia via extensive reciprocal connections with the thalamus (Uylings et al. 2003). These cortical regions are anatomically similar to other regions of the cortex yet *functionally* distinct. Similarly, pixels in the entorhinal cortex and in the hippocampus were assigned to the same community, reflecting the dense connections between these structures. Most other cortical nodes, including those located in the motor, somatosensory, and visual cortices, were assigned to a second community (Fig. 9b), while a third community (Fig. 9c) mainly comprised pixels near the brain edge, the ventricles and in white matter and the cerebellum.

The community structure approach is somewhat akin to other multivariate methods, such as Principal Component Analysis and Independent Component Analysis, which have been extensively applied to seek structure within imaging data in a model-independent way (Friston 1997; McKeown et al. 1998; Beckmann and Smith 2004). Unlike these, however, this community structure approach explicitly takes into account the topology of the functional connections, with communities defined on the basis of link density and distribution. This concept may be more readily interpretable in biological terms than measures such as the orthogonality (Friston et al. 1993; Friston 1997) or statistical independence (McKeown et al. 1998) of spatial modes that are optimized by other algorithms.

A key point in this approach is that the identification of communities within a functional imaging network contains information on both segregation *and* integration. A partition of the overall network into smaller subunits suggests a degree of

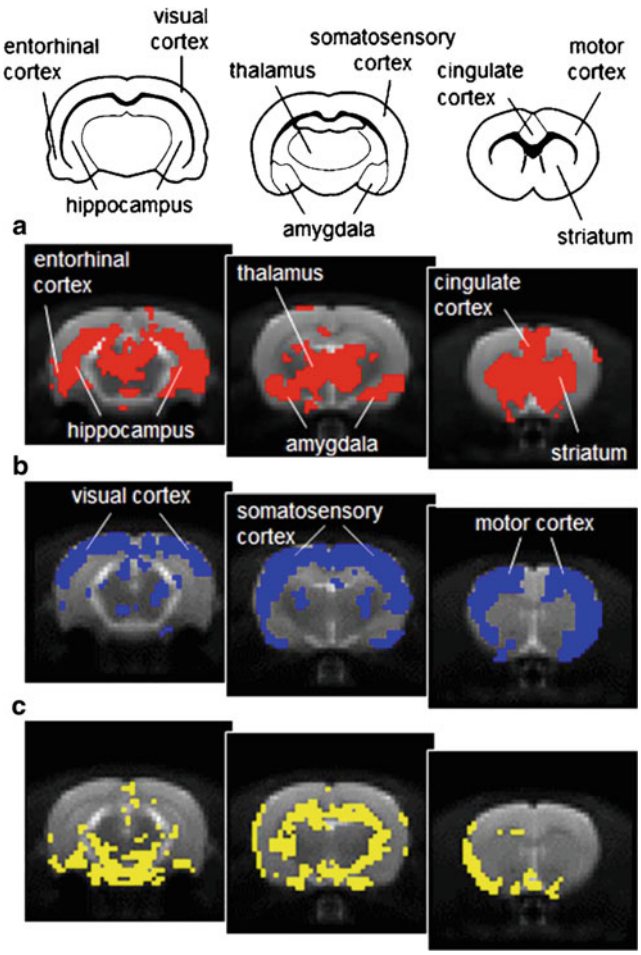

Fig. 9 Anatomical representation of the three communities identified by modularity maximization in a functional network derived from phMRI responses to fluoxetine. Adapted from Schwarz et al. (2008) with permission

functional segregation in the response, whereas the set of brain regions – not necessarily contiguous – identified within each subnetwork reflects their integrated action in response to the experimental stimulus. Importantly, the value of the modularity Q provides a measure of the degree of functional segregation in the network and may provide the basis for an operational definition of this.

This first demonstration of the potential of community structure analysis of functional connectivity in preclinical species has been rapidly translated to human functional connectivity, and several studies extending this approach to resting state fMRI have subsequently appeared in the literature (Meunier et al. 2009; Bullmore and Sporns 2009). However, a few questions remain open and are the object of active investigation. For example, binarization of the adjacency matrix was used to make

the problem computationally tractable. However, application of a threshold to retain only the strongest correlations ignores information that may be contained in the distribution of the weaker links, and the extension to fully weighted functional connectivity networks is the natural development for this approach, dependent on the availability of computationally efficient partitioning algorithms. Also, introduction of a direction to the edges linking pairs of nodes, perhaps using Granger causality or related concepts, would provide a means of creating directed graphs of connectivity that may be informative of the roles played different nodes. Finally, and most importantly, the origin of structured patterns of correlated responses is still largely unknown. Specifically, it is unclear whether the patterns of functional connectivity reflect interregional correlations induced by the drug challenge itself, or the intrinsic organization of the brain, perhaps determined by the structure of the underlying neuronal substrate.

A follow-up paper applied community structure analysis to address this question (Schwarz et al. 2009). To this end, three different pharmacological challenges (D-amphetamine, fluoxetine, and nicotine) were investigated, and the emergent communities under different pharmacological conditions were compared to discriminate between connectivity patterns that are stimulus-specific and those independent of the particular neurotransmitter system(s) engaged by the drug, which may thus correspond to general features of the rat brain functional architecture.

Interestingly, common features across all three networks revealed two groups of tightly coupled brain structures that responded as functional units independent of the drug, including a network involving the prefrontal cortex and subcortical regions extending from the striatum to the amygdala. This suggests that this network of functional connectivity may reflect a general feature of the brain organization, and is consistent with evidence showing strong intrinsic connectivity between neurons in these brain structures. Hence, structural connectivity of the neuronal substrate appears to constraint functional connectivity, and stimulus independent patterns of functional connectivity reflect the structure of large-scale neuronal wiring in the rat brain.

4 Conclusions

FMRI methods have been extensively applied over the last 20 years to study the human brain and its functional organization in healthy and disease states. A strong rationale exists for the extension of this approach to animal models as a translational tool to bridge clinical and preclinical research. In this chapter, we have reviewed methodological aspects of functional MRI in preclinical species and some of the key findings. Specifically, we have described the use of phMRI, i.e., the application of fMRI to map spatiotemporal patterns of brain activity induced by pharmacological agents. The main merits of this approach lie in its ability to elicit robust and reliable activations even under anesthesia conditions required to reduce motion artifacts and animal stress, and to enable selective stimulation of different

neurotransmitter systems, thus providing a experimental framework to study the neurochemical basis of fMRI responses. While phMRI is still a relatively young technique, a decade of methodological development and validation provides a solid basis for its application to study the complex neurochemical control of brain function at a systems level. Many of the examples reported above demonstrate drug and mechanism specific patterns of brain (de)activation, thus providing functional fingerprints of pharmacological activity. Moreover, the combination of imaging methods with neurochemical, metabolic or electrophysiological techniques in animal models provides a powerful means to relate the hemodynamic responses measured with phMRI to underlying changes in neuronal activity. Finally, we have discussed the analysis of phMRI in terms of functional connectivity, a measure of correlation in the responses in spatially remote brain areas. While conceptually different from the human resting state fMRI, which relies on spontaneous fluctuations of the fMRI signal in the brain in the absence of external stimuli, the phMRI approach has proven a powerful tool to explore functional connectivity in rodents, and to map a variety of different neurotransmitter pathways. PhMRI has also provided a useful experimental framework to explore novel statistical methods of analysis of functional connectivity represented in terms of complex networks. Particularly, network partitioning methods have made it possible for the first time to resolve biologically and anatomically meaningful patterns of correlated responses in the rat brain, thus providing a novel and powerful method to investigate the brain functional architecture in preclinical species.

While functional MRI in preclinical species has been driven by imaging applications in humans, the methodological gap between the clinical and the preclinical arenas is closing, with increasingly sophisticated approaches now available for animal studies. The few examples in which comparable studies have been performed in humans and in preclinical species, e.g., with NMDA receptor antagonists, demonstrate encouraging correspondence across species, thus supporting the use of phMRI approaches as a translational tool.

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The Pig as a Model Animal for Studying Cognition and Neurobehavioral Disorders

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Contents

Abstract In experimental animal research, a short phylogenetic distance, i.e., high resemblance between the model species and the species to be modeled is expected to increase the relevance and generalizability of results obtained in the model

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species. The (mini)pig shows multiple advantageous characteristics that have led to an increase in the use of this species in studies modeling human medical issues, including neurobehavioral (dys)functions. For example, the cerebral cortex of pigs, unlike that of mice or rats, has cerebral convolutions (gyri and sulci) similar to the human neocortex. We expect that appropriately chosen pig models will yield results of high translational value. However, this claim still needs to be substantiated by research, and the area of pig research is still in its infancy. This chapter provides an overview of the pig as a model species for studying cognitive dysfunctions and neurobehavioral disorders and their treatment, along with a discussion of the pros and cons of various tests, as an aid to researchers considering the use of pigs as model animal species in biomedical research.

Keywords Animal model · Dementia · Depression - Psychosis · Eating disorders · Magnetic resonance imaging · Neurodegenerative diseases · Pig (*Sus scrofa*) · Positron emission tomography (PET) · Translational research

Abbreviations

ADME	Absorption, distribution, metabolism, and excretion
APPsw	Amyloid precursor protein, Swedish mutation
CNS	Central nervous system
CS	Conditioned stimulus
HCA	Hypothermic circulatory arrest
HPA	Hypothalamic–pituitary–adrenal
MR	Magnetic resonance
MRI	Magnetic resonance imaging
ORT	Object recognition test
PET	Positron emission tomography
RNA	Ribonucleic acid
US	Unconditioned stimulus

1 Introduction

Biomedical research is currently predominantly performed using rodents. Mice constitute approximately 80% of all animals used in biomedical research. Interest in the pig, in particular the miniature pig (minipig) as a useful model species is growing. The scientific advantages of using pig models in biomedical research were first described in detail in the 1970s (Baldwin and Stephens 1971; Chaput et al. 1973). In various areas of pharmacological research, the pig is already an established

model species, in particular owing to the anatomical similarities to humans (body size, cardiovascular system, skin, urinary system), functional similarities (gastrointestinal system, immune system) and the availability of disease models (diabetes, atherosclerosis, gastric ulcer, wound healing) (Gad 2007; Mortensen et al. 1998; Swindle and Smith 1998). Pigs are frequently used for the characterization of cardiovascular, diabetes and dermatology drugs.

Furthermore, compared to mice, the immune system of the pig is more similar to that of humans, with 80% similarity on compared variables between pigs and humans versus 10% between mice and humans (Schook et al. 2005). The sequence and chromosome structural homology of the pig genome also shows strong similarity to that of humans (Chen et al. 2007; Lunney 2007; Petersen et al. 2009).

The biotransformation of many drugs in man and pig is similar, as shown by liver metabolism studies (Witkamp and Monshouwer 1998). Therefore, pigs are suited for pharmacokinetic and toxicological studies, mainly as a substitute for dogs and nonhuman primates (Nunoya et al. 2007). The pig is a suitable animal for most routes of administration and for the evaluation of most pharmacokinetic (absorption, distribution, metabolism and excretion; ADME) endpoints. To reduce the amount of drug needed for efficacy and safety testing, the Göttingen minipig is often selected for preclinical drug studies. Finally, the inbreeding of (mini)pig lines for xenotransplantation studies is of importance, and larger pig breeds are used for the evaluation of the efficacy and safety of developmental medical devices, such as implanted insulin pumps and cardiovascular devices and prostheses (International Organization for Standardization 2002).

As a nonendangered species, the pig may replace some areas of research, which are currently performed with other larger model animal species, such as nonhuman primates or dogs. The use of nonhuman primates, in particular, (Bailey 2005) and companion species meets strong ethical concerns and substantial opposition in a large proportion of the population (Rollin 2006). A short phylogenetic distance, i.e., high resemblance between the model species and the species to be modeled is expected to increase the relevance and generalizability of results obtained in the model species (van der Staay et al. 2009a). Moreover, the use of pigs, generally regarded as production animals, may lead to less controversy in the public view than the use of companion animals such as dogs (see Fig. 1). It is expected that the research on (mini)pigs can fill the gap between preclinical studies in rodents and clinical trials in humans (de Groot et al. 2005; Lind et al. 2007; Nunoya et al. 2007; Vodička et al. 2005).

The (mini)pig shows multiple advantageous characteristics that have led to an increase in the utilization of this species in studies modeling human medical problems, including neurobehavioral (dys)functions. The cerebral cortex of pigs, unlike that of mice or rats, has cerebral convolutions (gyri and sulci) similar to human neocortex (see Table 1). This may facilitate physiological experiments and neuropathological comparisons with the human brain (Alisky 2006). The relatively large gyrencephalic brain promotes using the (mini)pig, particularly younger animals, for (noninvasive) imaging techniques (Arnfred et al. 2004; Danielsen et al. 2001).

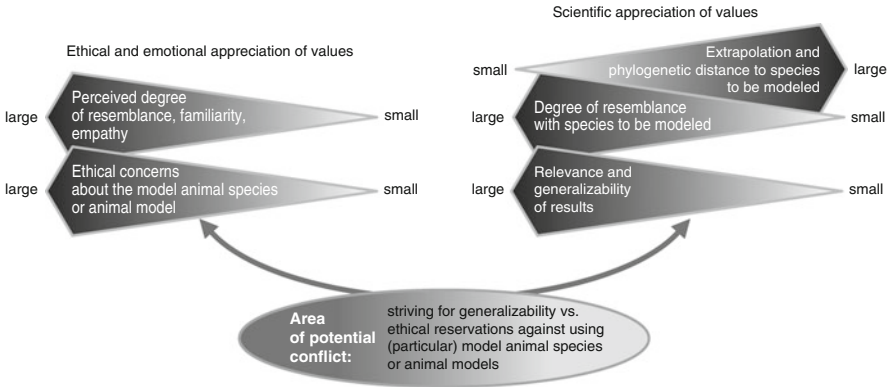

Fig. 1 Area of potential conflict between emotional and ethical values and scientific facts when selecting a model animal species for scientific research. Ethical concerns against using a particular model animal species (e.g., primates or companion animals) and the (perceived) degree of resemblance with humans or familiarity and empathy toward a species are often correlated. On the other hand, a low extrapolation distance and phylogenetic distance, and a high degree of resemblance between the model species and the species to be modeled are expected to increase the relevance and generalizability of results. Due to their high similarity with humans and the growing concerns about using nonhuman primates as model species, the pig may provide an excellent alternative. In particular, the smaller perceived similarity with humans, and the larger empathic distance to this species reduces the area of potential conflict, whereas the anatomical and physiological and genetic resemblance with humans is high

1.1 Neuroimaging in Pigs

Imaging research has increasingly been conducted using pigs in recent years, both as a technique to further investigate pigs as model animals (particularly developmental models) and as a step beyond rodents when characterizing PET ligands. A few salient studies are discussed here and a more comprehensive review has recently been published by Sauleau (2009).

Studies using functional MRI have been used to locate functional homologues of primate cortical areas, including the visual (Gizewski et al. 2007) and somatosensory (Duhaime et al. 2006) cortex. By stimulating the animals either visually, using a flashing light, or with sensory stimuli such as snout stimulation, one can determine the brain areas which are activated by these stimuli. Thus far, these studies have shown a high similarity between pigs and humans in terms of stimuli needed to produce activation and in the cortical areas activated. Interestingly, pain sensitivity was shown to become increasingly focused in piglets when followed from 2 to 6 months, with low reactivity in 2-month-old piglets, generalized cortical activity at 4 months and focused responses in the contralateral sensory cortex and brainstem at 6 months of age (Fang et al. 2005). The study reveals developmental changes in mammalian pain response pathways, which would be difficult to investigate in man due to ethical constraints, thus underlining the utility of the developmental model.

Table 1 Brain weights and sizes of adult humans, pigs, dogs, cats, rats, and mice

	Species					
	Human	Pig	Dog	Cat	Rat	Mouse
Brain size						
Approximate brain weight (grams)	1,300–1,400	80–180 (large variation between breeds)	70–130 (large variation between breeds; e.g., ≈72 in Beagles)	≈30	≈2	≈0.5
Gyri and sulci	Gyrencephalic	Gyrencephalic	Gyrencephalic	Gyrencephalic	Lissencephalic	Lissencephalic

Note the large variation between breeds of pigs and dogs as a consequence of strong selection on body size and appearance. Brains redrawn to scale from "Major National Resources for Studying Brain Anatomy," of the University of Wisconsin, Michigan State University, and the National Museum of Health and Medicine (see: http://www.brainmuseum.org/index.html). The brains of gyrencephalic animals have rounded elevations or convolutions or gyri, and grooves or sulci, on the surface of the hemispheres, whereas the brains of lissencephatlic animals such as rats and mice do not

Pigs have also been favored animal models for testing tracers being developed for human PET studies, including tracers for nicotinic receptors (Brust et al. 2008; Patt et al. 1999, 2005, 2010), histaminergic receptors (Plisson et al. 2009), serotinergic receptors (Ettrup et al. 2009; Kornum et al. 2007b), serotonin transporters (Brust et al. 2003) and type 1 glycine transporters (Passchier et al. 2010). With their relatively large, gyrencephalic brain, and PK characteristics highly similar to humans, pigs are frequently the species of choice as an intermediate between rodents and humans when testing tracers.

A number of initiatives are actively evaluating and promoting the pig as a model animal species, for example the National Swine Resource and Research Center (NSRRC; http://www.nsrrc.missouri.edu/), established in 2003; the initiative "Advantages of Domestic Species as Dual-Purpose Models that Benefit Agricultural and Biomedical Research" (http://www.adsbm.msu.edu/Home/tabid/54/Default.aspx), and the EU Sixth Framework Program project RETHINK (http://www.rethink-eu.dk) that evaluates the potential impact of toxicity testing in the minipig as an alternative approach in regulatory toxicity studies. Due to its smaller body size, the minipig is more suited to be housed and tested under laboratory conditions than the domestic pig (see Fig. 2).

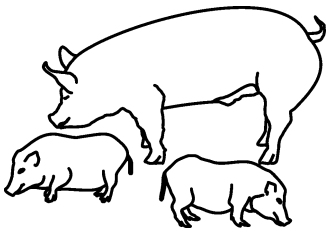

Fig. 2 Their smaller size and low weight make minipigs more suited for biomedical research than the domestic pig

However, the potential of (mini)pig-based animal models for investigating (dys) functions of the nervous system and their consequences for the regulation of normal and abnormal behavior has not yet been fully explored (Nielsen et al. 2008; Schook et al. 2005). There even appears to be a recent trend toward reducing the number of studies using nonrodent species in biomedical research (Reynolds 2009; Roberts et al. 2003). In particular, mice are the dominant animal model species in biomedical research, absorbing the bulk of all funding. However, a range of animal models, based on different model animal species is required for extrapolating and translating results from animal studies to humans (Roberts et al. 2003).

1.2 Genetically Modified Pigs

A broad range of genetically engineered rodent models has been developed to gain new insight into the brain–behavior functions and into the neuropathological processes, which underlie neurodegenerative and neuropsychiatric disorders (Branchi et al. 2003; Cryan and Holmes 2005). Many recent reviews have addressed the experimental techniques to construct transgenic (Sachs and Galli 2009; Wheeler and Walters 2001), knockout, antisense oligonucleotides, and most recently, interfering RNA animal models (Hoyer 2007; Salahpour et al. 2007). However, the translational value of rodent models in the field of neurosciences has come under criticism, mainly for the apparent lack of translational value of a large body of mouse-based research for predicting the clinical efficacy of therapeutics to treat neurobehavioral disorders in humans (Benatar 2007; Dirnagl 2006).

This critique has stimulated the development of genetically engineered pig models of neurobehavioral disorders (Kragh et al. 2009; Prather et al. 2008). Despite ongoing progress in technical developments, the production of transgenic pigs remains a demanding task (Niemann and Kues 2007). However, new approaches have proven that the development of transgenic pig models needs not be more time consuming than that of murine transgenic models (Aigner et al. 2010). Progress in swine genomics, coordinated by the Swine Genome Sequencing Consortium (Amaral et al. 2009; Ramos et al. 2009), transcriptomics (Tuggle et al. 2007) and proteomics (Miller et al. 2009) are expected to make the pig an interesting and valuable model species for biomedical research. The majority of studies

with genetically modified pigs are performed in the area of xentotransplantation (Klymiuk et al. 2010).

A research group at the University of Aarhus (Denmark) is developing a transgenic pig model of Alzheimer's disease, in which the APPsw mutation has successfully been expressed in the pig brain (Kragh et al. 2009). However, it has not yet been shown that these transgenic pigs develop Alzheimer's disease-like neuropathology and associated behavioral dysfunctions. A wide range of behavioral tests designed for pig studies is already available for this purpose and additional tests are now being developed and validated (see below). Although the genetically modified pig as a large animal model of behavioral and neuropsychiatric disorders is still in its infancy, we expect that both the recent developments in genetic methods and behavioral tests will boost its use in the near future.

However, this chapter is not a plea for deciding to work with pigs. The choice of a model animal species should be multifactorial weighing theoretical, practical, and ethical factors. Weighing the scientific evidence relating to the predictive and construct validity of the animal model (van der Staay et al. 2009a), against the adversity of housing and testing under laboratory conditions is an appropriate basis for selecting the model animal species for biomedical research (Webster et al. 2010). Nevertheless, it is anticipated that appropriately chosen pig models will yield results with high translational value, although this claim needs to be substantiated empirically, as pig research in this area still is in its infancy. An overview is provided of the pig as model species for studying cognitive dysfunctions and neurobehavioral disorders and their treatment, along with discussion of pros and cons of various tests, as an aid to researchers considering the use of pigs in biomedical research.

2 The (Mini)Pig in Cognitive Neurobehavioral Research

There is a wealth of tests for assessing learning and memory in rodents, and to be suitable for cognitive biobehavioral research, validated cognitive tests are needed which assess cognitive functions in pigs. Cognitive studies using pigs have been classified according to the underlying mode of learning, briefly outlining the most interesting and promising studies to give an overview of this field of research. Some of the studies described are designed to gain relatively fundamental knowledge but can also be used as a basis for future applied cognitive biomedical research.

2.1 Classical Conditioning Studies

The first category is a form of associative learning. Pigs can be easily conditioned (Kratzer 1971) using classical (Pavlovian) conditioning, but only a small number of studies have applied this training method. Moreover, the majority of these tasks are

aversively motivated. For example, Noble and Adams (1963) examined the effect of interval length between a conditioned stimulus (CS) and an unconditioned stimulus (US) on classical conditioning performance (bracing posture). In several experiments, domestic pigs were exposed to different interval times (1–8 s) between a conditioned (increase in illumination and/or vibratory-auditory cue) and unconditioned stimulus (electric shock) while confined in a crate or placed in a sling. The bracing posture was found to be more pronounced with increasing CS and US interval.

2.2 Operant Conditioning Studies

Operant conditioning procedures using aversive or appetitive reinforcers have been applied more frequently than classical conditioning procedures in pig cognition studies. The first experiments applying operant panel-switching (lever pressing) in pigs were performed in the 1970s (Kennedy and Baldwin 1972). At the beginning of the twenty-first century, panel-switching was applied in studies on the effects of housing and gender (Sneddon et al. 2000). Ferguson et al. (2009) applied various lever pressing tasks in mini-pigs and compared the results with those of rats and nonhuman primates. Acquisition of the task was found to be poor, though this was likely caused by the physical difficulty of operating the apparatus with the hooves and by the relatively short duration of the study.

Croney et al. (2003) showed that pigs were able to discriminate between the colors orange and green and the olfactory cues coconut and almond using an operant task (clicker training; a click sound followed by a food reward for a correct choice). Results showed that pigs are able to discriminate between both cues even when multiple choices (2–10 smells or colors) were presented simultaneously.

In another multiple choice task, Wang et al. (2007) trained pigs to discriminate between two different visual cues (one or two versus three black dots) in a (non-spatial) radial arm maze task, with reward located at the end of the correct arm. Two days after training on this task (40 trials), memory was tested by presenting the same task again in one trial. Pigs were able to acquire the task even faster (fewer trials) when supplemented with sialic acid (possibly a conditional nutrient during rapid brain growth). Sialic acid was also found to positively influence memory performance; a single memory test trial was performed 2 days after completion of a set of learning trials. This study shows the potential utility of the task for studying the effects of putative cognition enhancers on memory and/or motivation.

More difficult are the operant discrimination tasks in which Moustgaard et al. (2004, 2005) shaped pigs to place their snouts in a response hole for a reward in which an incorrect choice or decision led to a punishment (20 s of darkness). The pigs were trained to discriminate between visual (black–white) and spatial (left–right) cues in a go-no go task. After the pigs acquired the discriminations, an extra-dimensional shift was applied (visual to spatial or the other way around). Boars and sows reached the same level of performance and no important role of

attention to stimulus dimensions was found. In the go-no go task, approximately all animals reached behavioral criterion (>90% correct choices per session).

Aversive operant tasks were performed a few times during the late 1960s and 1970s. The negative stimuli applied were mainly electric shocks and, in one study, a water-maze (Barnes et al. 1969; Chaput et al. 1973; Hammell et al. 1975; Kratzer 1969). A more recent experiment making use of negative reinforcement studied time perception and anticipation of future events (Spinka et al. 1998). In this preference test, pregnant sows were trained to enter one of two rooms each containing several feeding crates. Once the sow entered a crate, food was provided and the crate closed automatically. In the left room, the crate opened automatically again after 30 min but in the right room the pig was confined for 240 min. After a training period in which sows were allowed in the left or right room variably, the sows were allowed to choose freely. In general, they chose the short confinement crates in the left room, suggesting that pigs have perception of time.

2.3 Spatial Learning and Memory Studies

Mazes have been useful in assessing the spatial abilities of pigs. Mazes can be divided into the so-called "*alley*" *mazes'* (one correct route to a fixed goal) and "*free-choice*" *mazes* (rewards can be found in different places and those places can be (re)visited in every desired order). Only a few studies using alley-mazes have been reported in pig research, but de Jong and colleagues studied the influence of housing enrichment on long-term memory in an alley maze (based on a Hebb–Williams maze) (de Jong et al. 2000). After the pigs had been trained to perform the task, their retention performance was tested 9 weeks later. Based on the increased number of line crossings and the longer latency to reach the food reward, de Jong et al. concluded that pigs raised in a barren environment showed impaired long-term memory compared to those pigs housed in an enriched environment (de Jong et al. 2000). Siegford et al. (2008) used an alley maze to cognitively enrich young piglets. Five-day-old piglets were released into the maze (increasing in complexity). Returning to the home pen with the sow was their reward. The piglets that were maze-trained, i.e., exposed to cognitive challenges, were found to show a decreased fear of unfamiliar persons compared to controls housed under the same conditions after weaning. Learning performance of the cognitively enriched animals was also observed: in a spatial water maze for pigs, maze enriched pigs escaped faster to the platform compared to piglets that had previously been subjected to a treatment of short lasting social isolation. However, the normal controls (housed with the sow without any further treatment) performed similarly to the trained animals and therefore enhancement of cognitive performance cannot unequivocally be ascribed to the early cognitive enrichment of the piglets.

Foraging arenas in which several food rewards can be found, the so-called free choice mazes, are used to study the susceptibility of pigs' spatial memory

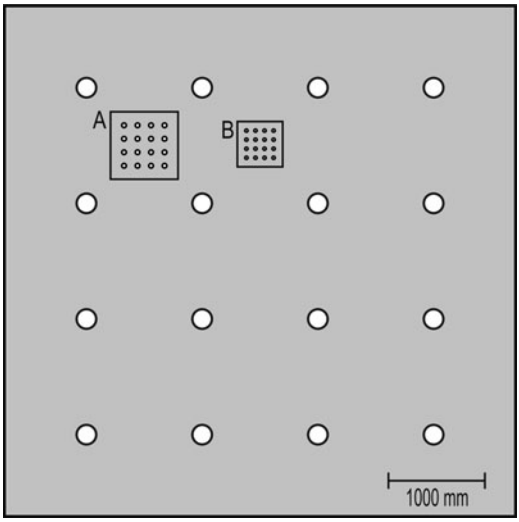

Fig. 3 Dimensions of the holeboard apparatus with a 4 × 4 matrix of holes for assessing spatial working and reference memory in pigs, rats (**A**) and mice (**B**)

performance to disruptions in the environment (Mendl et al. 1997), environmental enrichment and learning performance (Sneddon et al. 2000) and to investigate the pigs' abilities to discriminate between food of different value (Held et al. 2005). One of these mazes is the spatial holeboard, in which spatial working and reference memory performance can be assessed simultaneously.

In a study by Arts et al. (2009), the test arena consisted of 16 food buckets placed in a 4 × 4 pattern (see Fig. 3). Every animal was assigned its own configuration of rewarded buckets (4 out of 16) and was trained to collect the baits. Working memory or short-term memory errors (revisits to already visited baited buckets) and long-term or reference memory errors (visits and revisits to never baited buckets) were measured. The pigs quickly learned the task to criterion, with performance at or above the level of rodents. Mild mixing stress did not influence working and reference memory performance.

Instead of an open arena with baited places, spatial tests in pigs have also been designed using tasks with baited and nonbaited arms or rooms. In 1999, Laughlin and colleagues used a radial-arm maze (8 arms, 4 arms baited) to show that environmental stimuli could cause some disruption of memory of many food sites (Laughlin et al. 1999). They demonstrated this by applying a disturbance factor (e.g., weighing in a crate) during the 10-min retention interval between sample and choice trials. Also win-shift/win-stay strategies have been studied in a radial-arm maze (Laughlin and Mendl 2000).

A learning impairment study in pigs that suffered from induced hypothermic circulatory arrest (HCA) was performed in a multi-room maze by Hagl et al. (2005). Six out of eight rooms contained a reward. During each trial, an animal was allowed to visit only one of the rooms (all other doors were closed immediately after the first entry into one of the rooms) with time between trials being 30 s. Later task difficulty

was increased by only rewarding all the rooms situated on the left side and reversing the rewarded rooms during the subsequent trials. This experiment found impaired performance of the HCA animals but only for the second, more difficult test.

2.4 Recognition Studies

Another type of task applied in pigs assesses recognition (social or object recognition). The rodent-oriented "object recognition test" (ORT) (Ennaceur and Meliani 1992; Ennaceur and Delacour 1988; Ennaceur et al. 1989) is one of those tasks and can provide memory performance measures as well as noncognitive effects of experimental manipulations (Sik et al. 2003). Where object recognition generally deals with exposure and reexposure (successive trials), social recognition (recognition of conspecifics or humans or social learning) is often studied in a simultaneous setting.

The ORT has not yet indisputably proven its relevance in pigs (Gifford 2005; Gifford et al. 2007), although some studies point in this direction (Kornum et al. 2007a; Moustgaard et al. 2002). In theory, this test could be promising and potentially fulfill several of the criteria that will be discussed in Sect. 5 "Experimental and Practical Implications for Biomedical Research." Up till now, the main question to be answered is whether pigs show a preference for exploring an unfamiliar object when presented together with a familiar one.

Several social recognition studies have found pigs to be able to discriminate between familiar and unfamiliar congeners (de Souza et al. 2006; Kristensen et al. 2001; McLeman et al. 2005) based on tactile, visual, and/or olfactory information. In the domain of socially cued behaviors, also observational learning-like studies in pigs have been conducted. Held and colleagues studied this phenomenon (Held et al. 2000, 2001) and described it as "the exploitation of knowledge of an individual by another pig." In a spatial arena with hidden food rewards, noninformed pigs were allowed in together with an informed individual or could choose between following an informed or noninformed individual. Results suggested that the noninformed individuals did follow informed individuals, rather than engaging in an alternative task examining the food buckets at random. Results cautiously suggest knowledge exploitation in pigs and abilities to appreciate visual perspective, i.e., the ability to appreciate what others can and cannot see. Further research is needed.

An awareness study in pigs was very recently performed by Broom et al. (2009). They investigated whether piglets are able to locate a rewarded food bowl with the use of information from a mirror. One group of pigs was allowed in a pen with a mirror for 5 h and a control group was not. Later that day, the test was performed. Pigs were individually allowed into the room (controlled for smell) with the mirror. Only through the mirror could a food bowl (familiar to the animals) be seen, present on the other side of a barrier. Most animals familiar with a mirror looked at the mirror and then directly walked around the barrier and reached the food bowl.

Almost all control animals first approached the mirror and then walked behind it. When the mirror was replaced by a wire mesh and the food bowl placed behind it, mirror experienced pigs directly walked around the wire mesh. To go around the barrier and get access to the food, a map of the environment and awareness of an animal's own movement abilities are needed. So pigs' abilities indicate assessment awareness i.e., an ability to assess and deduce the significance of a situation in relation to itself over a short time span.

3 Pig Models of Psychiatric Disorders

3.1 Schizophrenia

The similarity of the pig brain to the human brain has led to the exploration of the pig as a model animal for psychiatric disorders in humans. Recently, the amphetamine-treated minipig as putative animal model of schizophrenia has been investigated. Amphetamine treatment has been used to mimic symptoms of psychosis and to provide a model system that enables assessment of the efficacy of putative antipsychotics. The majority of these studies used rodents as subjects. Lind and colleagues found that amphetamine caused hyperlocomotion in minipigs in the open field test (Lind et al. 2005a) and evoked an increased release of dopamine in the pig brain (Lind et al. 2005b). In an acoustic startle test, amphetamine disrupted prepulse inhibition in minipigs (Lind et al. 2004). These observations raise the possibility of using pigs to evaluate the antipsychotic-like actions of compounds.

We investigated the effects of antipsychotic drugs on the spontaneous behavior and on the D-amphetamine-induced increase in locomotor activity in an open field, using young adult male Göttingen minipigs as subjects (van der Staay et al. 2009b). Using both a within-subjects design and a between-subjects design, we showed that the open field behavior of minipigs is highly reproducible when measured after administration of drug vehicles. Whereas amphetamine causes huge increases in locomotor activity in rodent species (Nordquist et al. 2008), only a mild (approximately 30%) increase was found in minipigs injected s.c. with 0.4 or 0.7 mg kg^{-1}. Higher or lower doses were less effective or ineffective in the minipig. The effective D-amphetamine dose range in rats appears to be wider than that measured in minipigs, and the dose response curve has shifted to the right in rodents. The effective dose of 0.4 mg kg^{-1} in minipigs is similar to that shown to induce restlessness in nonhuman primates (Peacock and Gerlach 1999; Peacock et al. 1999). The decrease of behavioral activity of minipigs after administration of the highest dose tested (2 mg kg^{-1}) suggests that this dose causes motor side effects in these animals. These observations indicate that the moderate D-amphetamine-induced increase in locomotion better reflects the effects of psychostimulants on primate behavior than the large increase in locomotion evoked by D-amphetamine in mice or rats.

The subcutaneous administration of the antipsychotic therapeutics haloperidol and risperidone (dose 0.04 mg kg^{-1}) reduced spontaneous locomotion (distance moved, mean velocity, and total duration of moving) of minipigs in the open field. The lowest effective dose of haloperidol and risperidone to decrease spontaneous behavior in rats is higher: 0.08 mg $kg^{-1,}$ and 0.62 mg kg^{-1} respectively (Bardin et al. 2007). The effective doses in our pig study are within the range used for human dosing, i.e., 0.01–0.03 mg kg^{-1} for haloperidol and 0.03–0.05 mg kg^{-1} for risperidone (van der Staay et al. 2009b). This suggests that minipigs may be more sensitive to antipsychotics than rats, but comparative pharmacokinetic studies are lacking.

The amphetamine-induced increased activity of minipigs in the open field was dose dependently antagonized by administration of haloperidol and risperidone (0.01 and 0.04 mg kg^{-1}). The high dose (0.04 mg kg^{-1}) of both antipsychotics fully antagonized the amphetamine-induced behavior (van der Staay et al. 2009b). The minimal effective dose of the antipsychotic drugs for reversal of D-amphetamine-induced behavior in minipigs is in the range of doses used in nonhuman primates (0.015 mg kg^{-1} s.c.) (Peacock and Gerlach 1999). This dose does not suppress D-amphetamine-induced locomotion in rodents. Taken together, the data from these minipig experiments indicate that the pharmacological homology between minipigs and primates is greater than between rodents and primates. Furthermore, the data suggest that the D-amphetamine-treated minipig may be suited to characterize novel antipsychotics, a notion that needs experimental support from future studies.

3.2 Depression

It has been suggested, based on the similarity of platelet fatty acids in humans suffering from depression and in (stressed) pigs, and on a review of the relevant literature, that the pig may provide a model of human depression (Cocchi et al. 2008). Stress is believed to be a major etiological factor in depressive illness and chronic stress appears to elicit major depression (Kendler et al. 2003), although some investigators disagree (Holmes 2003). The hypothalamic–pituitary–adrenal (HPA) axis is activated by stressful stimuli and chronically enhanced activity of the HPA system is the main neuroendocrine characteristic of depression (Belmaker and Agam 2008). We have shown that pairing two unfamiliar piglets for a period of 2 h results in a rapid threefold transient increase of the saliva cortisol concentration as a consequence of the acute social stress that is elicited by rank order fights (van der Staay et al. 2007). However, in a study in which female piglets underwent repeated defeat, a form of recurrent psychosocial stress, we found only a transient, but no long term, increase in saliva cortisol levels, with no effects on organ weights, number of GR and MR in the ventral hippocampus, or on serotonin turnover in the dorsal hippocampus (van der Staay et al. 2008). Increasing the duration, frequency, and/or intensity of the stressful events (e.g., prolongation of the period and increase in the number of defeat experiences) might induce depression-like

symptoms but an alternative approach was chosen. Previous studies have shown that tethering – a common farming practice until its recent banning in Europe – is a chronic stressor to sows (van der Beek et al. 2004). We investigated whether long-lasting, recurrent tethering stress over a period of approximately 2 or 4 years in sows leads to enduring effects on measures that may be indicative of (major) depression (van der Staay et al. 2010). We found that the pituitary gland was lighter in tethered sows than in loose sows. Furthermore, plasma cortisol levels were higher in the tethered sows than in the loose sows. The older, but not the younger, tethered sows had heavier adrenal glands than their loose counterparts. Microarray analyses revealed an increased expression of β-globin mRNA in the hippocampus and to a lesser extent in the frontal cortex of the older tethered sows, compared with the older loose sows. Taken together, the findings indicate that pigs experiencing chronic stress develop some depression-like symptoms. Consequently, pigs exposed to chronic stress may be considered an animal model of some symptoms of depression. However, further studies are needed to extend and validate this putative model.

3.3 Eating Disorders

Pigs, like humans, are omnivores. Their gut transit time is similar to that of humans and enzyme activity in the gut and absorption show many similarities. Therefore pigs are extensively used for testing the safety, and to a lesser extent, the efficacy of food additives. Pigs have a high motivation for food intake and a natural propensity to develop atherosclerosis and obesity (Koopmans et al. 2009; Skold et al. 1966; van der Meulen et al. 2009). We recently investigated whether pigs are suitable for the investigation of the efficacy of CNS-active anorectic drugs that are developed for the treatment of obesity. Six pigs of a large breed (production animals), body weight approximately 60 kg, were housed individually and fed two meals a day. Two clinically effective anorectic drugs, the serotonin reuptake inhibitor Sibutramine®, and the cannabinoid receptor-1 antagonist Rimonabant®, were mixed with 25 g of mashed feed that were offered as a small "pre meal" to the pigs 1 h before the regular morning meal. Drug doses were 0.3, 1.0 and 3.0 mg kg^{-1} and were escalated during the study. The oral administration of the two test drugs alternated. All six pigs received the six drug treatments (within subject design). One hour after drug intake the standard diet was available unrestricted for 60 min. Food consumption was measured every 90 s by weighing the food trough. Morning meal size on drug test days was compared to meal size of control days (no drug in the 25 g pre meal). Morning meal size under nondrug conditions was 1.0–1.2 kg. Administration of Rimonabant® and Sibutramine® reduced morning meal size dose dependently. Maximum reduction of food intake during the morning meal was 250–300 g. We concluded that Rimonabant® and Sibutramine® have short term effects on central mechanisms of satiety. The doses in this pig study are comparable to the effective doses in humans. This again illustrates the pharmacological homology between pigs

and humans. Pigs may be a useful species in satiety research and for the preclinical evaluation of nutritional and pharmacological strategies aiming at body weight reduction.

4 Developmental Pig Models (Prenatal, Perinatal and Postnatal Development)

4.1 Hypoxia–Ischemia Models

Piglets have been extensively used to model hypoxia–ischemia in neonates. The model was established and used by several groups simultaneously starting in the late 1980s. Perfusion of neonate piglet brains was demonstrated to be adversely affected by either hypoxia (produced by inhalation of low percentages of oxygen), ischemia (by tourniquet or surgically implanted ligatures around the carotid artery) (Leffler et al. 1989), or combinations of both (Odden et al. 1989). These effects were measurable during the procedures, but long-lasting effects 24 h post insult were seen on constriction and dilation responses of blood vessels as well as prostanoid synthesis.

Much attention has been focused on examining the effects of hypoxia–ischemia on the brain acutely in the hours following hypoxia–ischemia, with follow up measures up to 7 days post hypoxia–ischemia. Improved imaging techniques have allowed for extensive mapping of damage caused by hypoxic–ischemic events, including edema visible at 24 h (Cheng et al. 2005) and up to 7 days post insult (Vial et al. 2004). In addition, near-infrared spectroscopy was introduced as a method that is portable (thus more useful in clinical settings for assessing neonate hypoxia–ischemia) and reliably able to measure responses to hypoxic–ischemic events in piglets (Kurth et al. 2002; Winter et al. 2009).

A number of parameters have been developed in the piglet model that can function as early markers for clinical outcome. These include near-infrared spectroscopy, which is predictive of edema development when measured at 30 min, long before edema appears on MRI images (Winter et al. 2009), phosphocreatine recovery in the first hours after hypoxia–ischemia, which is a good predictor of secondary energy failure at 24–48 h (Cady et al. 2008; Iwata et al. 2008), and diffusion-weighted imaging and proton MR spectroscopy, which provided earlier prognostic information than traditional MRI or diffusion-tensor imaging (Munkeby et al. 2008).

Recent studies have focused on using the piglet model of hypoxia–ischemia to develop treatments to prevent or reduce effects of hypoxia–ischemia on damage to various areas of the brain. Cooling has been studied in piglets, producing good results with both whole-body cooling (O'Brien et al. 2006) and head-only cooling (Robertson and Iwata 2007). Pharmacological interventions, including allopurinol, deferoxamine (Peeters-Scholte et al. 2002a, 2003), 2-imminobiotin (Peeters-Scholte et al. 2002b, c)

and cannabidol (Alvarez et al. 2008) have also been shown to have protective effects when administered immediately after hypoxia–ischemia in newborn piglets.

Building upon the traditional newborn piglet model, several studies have modified the protocol to test related issues. Intrauterine ischemia induced by umbilical cord clamping has been demonstrated to cause increased glial formation and white matter lesions after 48 h, when the ischemia is induced at the beginning of the third trimester (Lyng et al. 2006). Interestingly, however, umbilical cord clamping does not seem to produce strong asphyxia in term piglets (van Dijk et al. 2008). Another extension of the model has been the inclusion of birth weight as a potential index for intrauterine growth restriction. Multiple births seen in pigs allow for comparisons between low birth weight piglets and normal birth weight siblings (Burke et al. 2006; Moxon-Lester et al. 2007). The addition of intrauterine ischemia and variables such as birth weight provide an interesting avenue for expanding the utility of the model.

While these studies provide strong evidence that the piglet model can provide information for development of prognostic criteria for outcomes and development of interventions in human neonates, the follow up times are generally very short, ranging from a few hours to, at maximum, a week. More long-term follow up studies are needed to validate the model for long-term outcome parameters into childhood and adulthood. Neuroprotection has been a difficult field for animal studies, with few successful translations from animal studies to human clinical use. It remains to be seen whether the piglet model will improve the number of candidate drugs which make it to the clinic. Clinical studies in humans which are currently underway will aid in determining the predictive validity of the piglet model for neuroprotection in neonates (Kaandorp et al. 2010).

4.2 Traumatic Brain Injury Models

Piglets have also been used in several laboratories as a model species for traumatic brain injury. A device, termed the scaled cortical impact device, has been developed to standardize percussion injuries produced in piglets. This device was used successfully to demonstrate that structural abnormalities detected with MRI following the injury follow a specific time course that varies with age, with animals tested at ages ranging from 5 days to 4 months (Duhaime et al. 2000, 2003; Durham et al. 2000; Grate et al. 2003). In a fluid percussion injury model, SPECT was shown to reliably detect hypoperfusion at 2 h post injury (McGoron et al. 2008) and the hemoglobin-based oxygen carrying solution HBOC-201 reduced the number of degenerated neurons observed histologically after 6 h. As with the models for hypoxia–ischemia, traumatic brain injury models in piglets show translational value at time points shortly after the hypoxic–ischemic event, but data on long-term effects are lacking. Follow up studies would add considerable value to this model.

5 Experimental and Practical Implications for Biomedical Research

Size matters! Many nonrodent model animal species are larger than mice or rats. This has a number of implications for animal housing and managing and handling (Holtz 2010). Moreover, the breeding, rearing and housing conditions may affect the experimental unit, in particular when working with farm animals (Festing 2006) such as pigs. A systematic overview of the experimental units is provided in Fig. 4. Experiments with large animals can be logistically challenging, in particular if the experimental unit/test site is the pen, barn, or farm.

On the other hand the size of pigs may provide advantages above smaller, rodent species, if parts of the animal are designated as an experimental unit (e.g., skin patches in dermatological studies), or when tissue samples of small brain structures must be collected or experimentally manipulated. Sampling can be more precise, and the samples can be larger, i.e., pooling of samples from different individuals in order to obtain sufficient material for analysis is not necessary.

When designing a cognitive behavioral task for pigs, several criteria should be kept in mind depending upon the specific research questions. It is important to consider that the task should, ideally, be suitable for unimpaired animals of all ages, suitable for repeated testing, and allow a detailed analysis of behaviors from different domains, i.e., cognition, sensation, locomotion, motivation (Wainwright and Colombo 2006). The task should tap ethologically relevant behaviors in a stress-free environment. Standardization and automation to a level comparable to the current state of the art in rodents are also desirable. Complexity and sensitivity are needed to be able to capture subtle differences in cognitive abilities (Friess et al. 2007; Hagl et al. 2005; Laughlin et al. 1999).

We previously outlined a classification of the tests applied so far in the field of cognitive biobehavioral research in pigs (see Sect. 2). These criteria provide a framework for assessing the advantages and disadvantages of different types of tests. Tests within a specific category all show several (dys)advantages which make them more or less suitable for answering specific experimental questions. The classical conditioning tasks overall do not fulfill a number of the criteria mentioned above and are therefore not recommended unless for very specific questions. Most operant tasks (e.g., lever pressing, discrimination tasks) for pigs score high on validity and can easily be automated but might lack the opportunity to support a detailed assessment of natural, ethologically relevant behavior. However, increasing or decreasing the complexity of a task is generally feasible and therefore an advantage for this type of test. Spatial tasks such as mazes, spatial arenas and the pig holeboard, do score well against the list of criteria. Most of them not only allow for a detailed behavioral analysis, but also for the investigation of developmental effects. Adjusting the complexity of a task is, as with most operant tasks not difficult. Tests within the category of recognition tasks (e.g., object recognition,

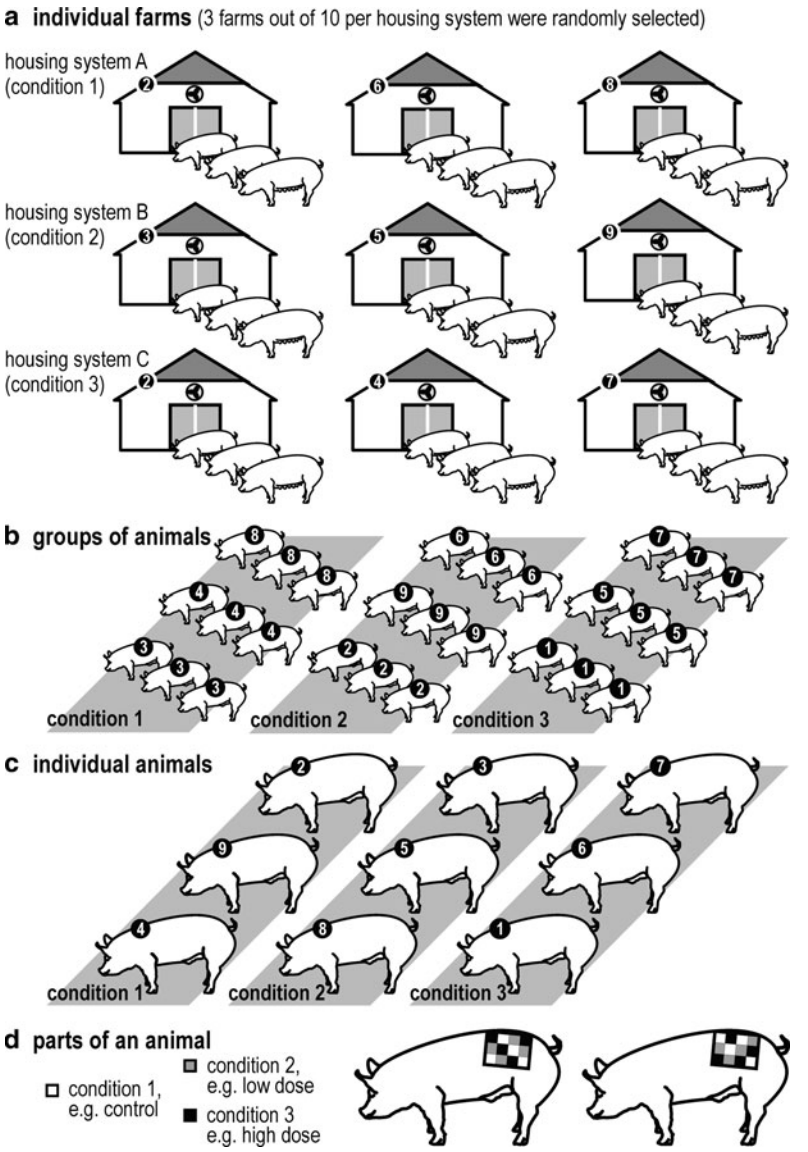

Fig. 4 Experimental units in animal research. *Panel A*: From 10 farms per housing condition, three were randomly selected to participate in the study. Note that the pigs within farm are automatically assigned to the housing condition of the particular farm. *Panel B*: In research on the effects of prenatal stress on the offspring, all piglets of a litter can only be assigned to one of the stress conditions (first group consisting of 3 animals each from litters 8, 4, and 3; second group consisting of three animals each from litters 6, 9, and 2, and third group consisting of 3 animals each from litters 7, 5, and 1). In the example, three litters (out of nine different litters) were randomly assigned to one of three experimental conditions. Within litter, three piglets were randomly selected and used in the study. *Panel C*: Individual animals are randomly assigned to one of three experimental conditions. *Panel D*: Randomly assigned skin areas are treated with different (doses of) putative therapeutics or allergens

certain Y-mazes and social tasks) do also allow for most of the criteria mentioned above, although these kinds of tasks can be useful to answer very specific questions.

One of the practical issues to consider is cost. The size of an apparatus for pigs exceeds the normal rodent-apparatus size by several times (see, for example, Fig. 3), and costs are much higher than in rodent research (although lower compared to primate research). When able to measure multiple variables and to test differing hypotheses within one apparatus the cost and space demands could be reduced.

6 Conclusions

Pigs are established model animals in biomedical research and the first choice animal species in the areas xenotransplantation, cardiovascular, and dermatological research. The use of pigs in preclinical safety research, in particular toxicity testing, is rapidly increasing. Pigs are mainly used in research that traditionally has been performed using dogs and nonhuman primates and they may provide an appropriate substitute for these species. Up to now pigs are less represented in neurobehavioral and CNS research in general.

Several types of tasks appear to be suitable for cognitive studies in the pig. However, caution should be taken when choosing or designing tasks because some gaps in our knowledge of pig behavior and pig sensorimotor abilities need still to be closed. Too little is known about sensory capacities of the pig in general and there is a lack of replicated experimental findings and a lack of studies to optimize experimental approaches using pigs as subjects. The pig is expected to be a model species with putatively higher translational ability than the commonly used rodent species. Therefore pigs should be considered as valuable species for this type of biomedical research. Of course, valid and solid test systems are a precondition for reliable research (van der Staay 2006; van der Staay et al. 2009a). Meanwhile, biomedical researchers using pigs to study behavior and brain functions should be aware of the gaps in knowledge when choosing and designing their experiments.

The size and complexity of the brain of pigs offers opportunities for neuroimaging research that exceed those with rodent species. First studies with psycho-active drugs in pigs support the notion that the pharmacological homology between pigs and (nonhuman) primates may be greater than between rodent species and humans. New techniques enable development of genetically modified pigs in a time frame that does not exceed the time required to develop transgenic or knockout rodents. Pigs may be especially suited to perform perinatal research and stroke research.

Pigs, especially minipigs have a great, and not yet fully explored, potential in CNS research. Further applications of this model species needs to be explored. In particular, tests to assess the consequences of experimental manipulations of the CNS of pigs on behavior need to be developed and validated.

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Index

J.J. Hagan (ed.), *Molecular and Functional Models in Neuropsychiatry*,
Current Topics in Behavioral Neurosciences 7, DOI 10.1007/7854_2010

E

F

O

P

Q

R

S