CHAPTER 9

Clonal Unit Architecture of the Adult Fly Brain

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Abstract

During larval neurogenesis, neuroblasts repeat asymmetric cell divisions to generate clonally related progeny. When the progeny of a single neuroblast is visualized in the larval brain, their cell bodies form a cluster and their neurites form a tight bundle. This structure persists in the adult brain. Neurites deriving from the cells in this cluster form bundles to innervate distinct areas of the brain. Such clonal unit structure was first identified in the mushroom body, which is formed by four nearly identical clonal units each of which consists of diverse types of neurons. Organised structures in other areas of the brain, such as the central complex and the antennal lobe projection neurons, also consist of distinct clonal units. Many clonally related neural circuits are observed also in the rest of the brain, which is often called diffused neuropiles because of the apparent lack of clearly demarcated structures. Thus, it is likely that the clonal units are the building blocks of a significant portion of the adult brain circuits. Arborisations of the clonal units are not mutually exclusive, however. Rather, several clonal units contribute together to form distinct neural circuit units, to which other clones contribute relatively marginally. Construction of the brain by combining such groups of clonally related units would have been a simple and efficient strategy for building the complicated neural circuits during development as well as during evolution.

Introduction

The fly brain consists of a complicated meshwork of neural circuits.[1,2] Each neuron projects to and arborises in its distinct subareas. Visualisation of specific subtypes of neurons, either by antibody staining or by expression of reporter genes, suggests that, although certain variability is observed in the number of the labelled cells, the projection patterns of the labelled neurons are rather stereotyped in the adult brain.[3-5] Molecular mechanisms underlying the formation of such complicated but stereotyped neural architecture have been studied extensively during the past few decades. Neurons are generated by asymmetric division of the stem cells called neuroblasts.[6,7] Each neuroblast gives birth to a series of clonal progeny during neurogenesis. The brain is therefore composed of "families" of clonally related cells. In this chapter, we examine how such lineage-dependent groups of neurons contribute to the formation of the elaborated neural circuits of the adult fly brain.

Structure of the Adult Brain

Before discussing the relationship between clones and neural network, we will briefly overview the general structure of the adult fly brain (for structure and development of the larval brain, see chapter by V Hartenstein et al). The adult brain is a mass of neurons that is about 500 μm wide, 200 μm thick and 250 μm tall. It consists of three parts, the central brain and an optic lobe on either side. The latter is the lower-order sensory centre specialised for visual information processing,[8,9] whereas the former contains lower-order centres of other sensory modalities (olfactory,

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etc.) as well as integrative and associative centres and higher-order motor control centres. Figures 1A,B show sections of a silver-stained adult fly brain. The area near the brain surface is occupied by the rind, or cortex, where cell bodies of all the neurons are confined (yellow areas). Unlike vertebrates, insect neurons have no synapses around their cell bodies. Thus, there are no synapses in the rind. All the brain neurons are monopolar, sending single neurites (cell body fibres) deeper into the brain and form synaptic connections[2] (Fig. 1C). The area occupied by these fibres and synapses is called the neuropile.

The thickness of the rind is different depending on the area of the brain. It is thickest in the area called the lateral cell body region (LCBR), which is between the central brain and the optic lobe (Fig. 1A,B). The rind is thin in the areas where the underlying neuropiles are protruded. Especially, there are essentially no cell bodies in the anteriormost surface area of the suboesophageal ganglion (SOG), antennal lobe (AL), ventrolateral protocerebrum (vlpr) and the anterior inferiorlateral protocerebrum (aimpr) (Fig. 1D). The ventral area of the posterior brain has no cell bodies, either, because this area is occupied by the cervical connective that houses the descending and ascending neural fibres to and from the thoracic ganglion (Fig. 1E). The diameter of the neural cell bodies tend to be smaller in the optic lobe and in the area above the calyx (ca) of the mushroom body (MB) than in other areas of the central brain (Fig. 1E).

Neurites generally form arborisations in several areas along their trajectories (Fig. 1C). The arborisations that are closest from the cell bodies are called the primary arborisations and those that are farthest are the terminal arborisations. In a simplistic view, the primary arborisation is often regarded as "postsynaptic dendrites" or "input areas," whereas the terminal arborisation is often called "presynaptic axon terminals" or "output areas." Though this is true in some cases, the situation is often more complicated. For example, many projection neurons that convey olfactory information from the AL to the second-order olfactory centres (the MB and the lateral horn, LH) have presynaptic sites not only in their terminals in the MB and LH but also in their dendrites in the AL (R Okada and KI, unpublished observation). Kenyon cells of the MB have postsynaptic sites not only in the calyx, which is supposed to be the input area of the MB, but also in the lobes, which is regarded as its output area.[10] Thus, pre and postsynaptic sites may in various cases co-exist in the same branches of neurites. Presynaptic sites in the primary arborisations may function for emitting local feedback signals and postsynaptic sites in the terminal arborisations might receive local modification signals for their output. On the other hand, there are indeed some neurons in which pre and postsynaptic sites are preferentially distributed in the proximal and distal areas of the neurites, respectively.[8] The direction of information therefore is not self evident from the projection pattern alone. Because the term "dendrite" often infers its role as input sites, care should be taken when using this word for referring to certain primary arborisations.

The brain consists of neurons and glial cells. Figure 1F,G show cross sections of the brain labelled for synaptic areas (with monoclonal antibody nc82[11]) and glial processes (with GFP driven by the glial specific repo-GAL4 driver.) The rind is contributed extensively by the processes of cell body glia (or cortex glia),[12] which ensheath each neural cell body. As explained before, synapses exist only in the neuropile. By comparing Figure 1A and 1G, which show the sections of the same level of the brain, it is clear that the neuropile areas that are occupied by large tracts of neural fibres (bundles of thick lines in Fig. 1A) are devoid of synapses (black areas in Fig. 1G). These tracts are covered by the processes of the neuropile glial cells.

The neuropile glia also separate the borders between major brain areas. For example, the borders around the AL, MB and the central complex, as well as the border between the suboesophageal ganglion (SOG) and the supraoesophageal ganglion, are covered by the glial sheath. Glial processes, however, do not always demarcate borders between functional areas of the neuropile. For example, although the MB is covered extensively by glial processes, there is no glial sheath structure between the LH—the other second-order olfactory centre—and the surrounding neuropiles. Similarly, although the anterior half of the ventrolateral protocerebrum (vlpr) is clearly demarcated by glial processes, the border between its posterior half and neighbouring neuropiles is more ambiguous.

Whereas three particular regions of the central brain, the AL, MB and the central complex, have clear glial sheaths that demarcate their borders and simple and organised circuit structures within them, neural fibres in the rest of the central brain do not form clearly distinguishable unit structures. These areas are often collectively called "diffused neuropiles." Short of a comprehensive knowledge about the circuit structures in the diffused neuropiles, it is not possible to determine the functional areas unambiguously in these brain areas. Therefore we here rely on a simple block-based terminology system to describe the subregions in these neuropiles (Fig. 1H-N).[2,5]

The central brain is divided into two parts: the supraoesophageal and suboesophageal ganglia. They are separated clearly in insect species that appeared earlier during evolution, but in flies they are fused with no clear external border (Fig. 1A,D). The supraoesophageal ganglion is divided into three neuromeres, the proto-, deuto- and tritocerebrum. The protocerebrum occupies most area of the supraoesophageal ganglion. The deutocerebrum is a small, flat area that lies beneath the protocerebrum and spans on both sides of the SOG. The neuropiles that receive sensory projections from the antennae, i.e., the AL and the antennal mechanosensory and motor centre (AMMC), are parts of the deutocerebrum (Fig. 1M,N).[13-15] Evolutionary studies and analyses of early embryogenesis suggest that the animal body anterior to the oesophagus is likely to consist of three segments (Chapter 2). Thus, the third supraoesophageal neuromere, the tritocerebrum, should exist somewhere between the deutocerebrum and the SOG. Such neuromere is not clearly discernible in the adult fly brain, however (Fig. 1A,G).

The SOG can also be divided into three neuromeres: the mandibular, maxillary and labial neuromeres. They derive from the three head segments posterior to the oesophagus and each neuromere receives peripheral nerves from the corresponding head segment. The internal borders between these neuromeres within the brain, however, are difficult to identify. The SOG consists mainly of the terminals of sensory neurons from the mouth and the surface of the head capsule and dendrites of the motor neurons for the head muscles. Judging from its primary role that is closely associated with the peripheral nervous systems, the SOG is functionally more similar to the thoracic ganglion than to the supraoesophageal ganglion. For this reason, the term "brain" sometimes refers specifically to the supraoesophageal ganglion.

As this example shows, the definition of the word "brain" is somewhat ambiguous in the insect nervous system (Table 1). Depending on the context, it refers to either all the central nervous system that resides in the head capsule, the supraoesophageal ganglion including the optic lobes, the combination of the SOG and the central part of the supraoesophageal ganglion, or only the central part of the supraoesophageal ganglion. To avoid confusion, in this chapter we use the word "brain" to refer to all the central nervous system in the head and use the words shown in parentheses of Table 1 to refer to each specific part of it.

Techniques for Visualising Clonally Related Progeny

Neuroblasts divide asymmetrically to generate their progeny (Fig. 2A). The proliferation pattern is rather different between the optic lobe and the central brain (inset photograph in Fig. 2A). In the optic lobe, precursor cells arranged in the two optic anlagen first divide symmetrically to increase their number and than asymmetrically, to produce large numbers of progeny[16] (see Chapter by KF Fischbach and PR Hiesinger). In the central brain, the proliferation pattern is essentially the same as in the thoracic ganglion (the ventral nerve cord), where a limited number of neuroblasts

Table 1. Classification of the brain areas

Suboesophageal ganglion	Supraoesophageal ganglion without optic lobe	Optic lobe
Brain		
SOG	**Brain** (→ *supraoesophageal ganglion*)	
Brain (→ *central brain*)		Optic lobe
SOG	**Brain** (→ *cerebrum*)	Optic lobe

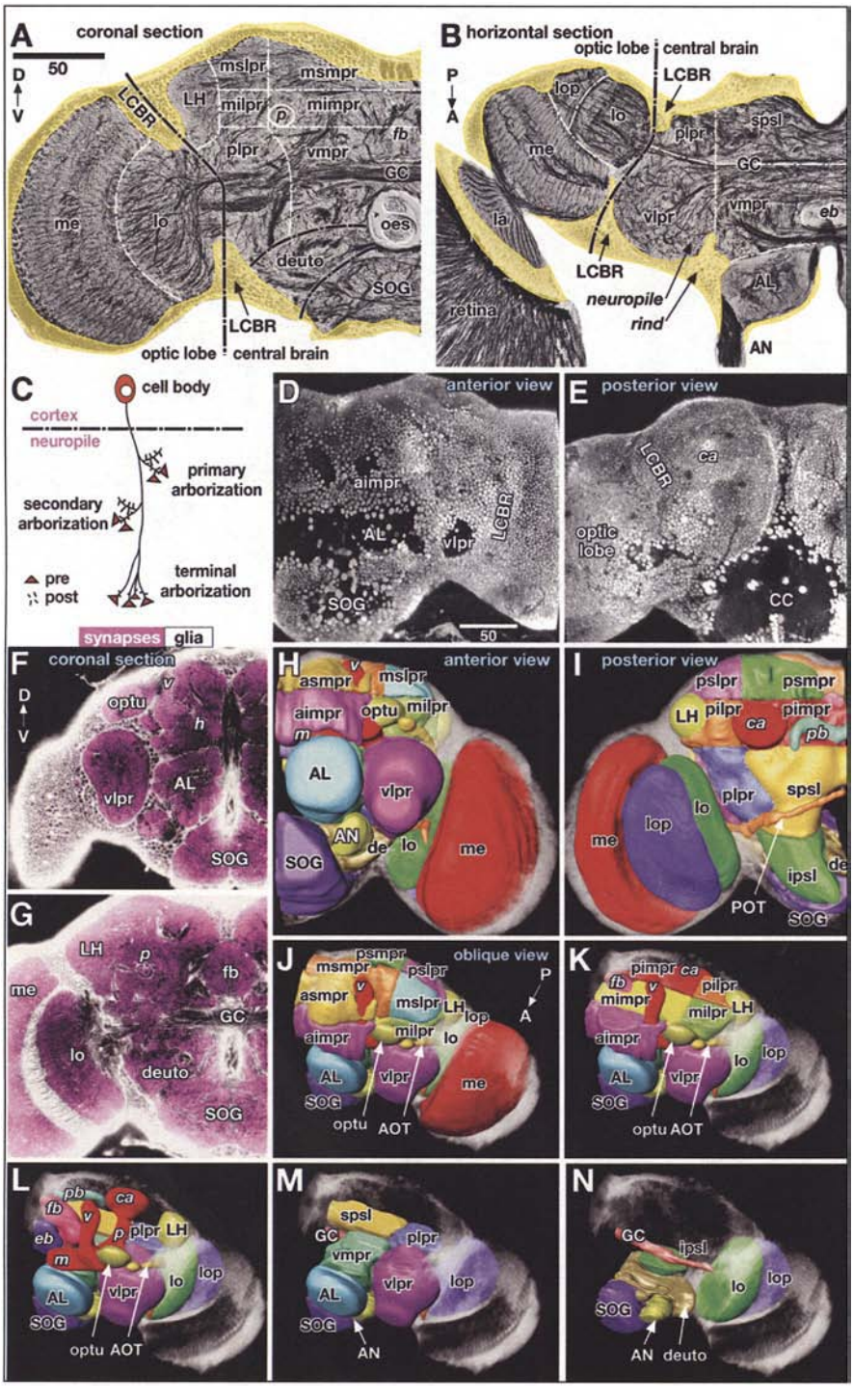

Figure 1, legend viewed on following pages.

Figure 1, viewed on prvious page. Overall structure of the adult fly brain. A,B) Coronal (frontal, A) and horizontal (B) sections of silver-stained brains (A, B, D and N modified from ref. 5 with permission from John Wiley and Sons, Inc. ©2006). Areas with yellow overlay represent the rind, or cortex. Black dashed lines show the border between the central brain and the optic lobe and between neuromeres (A). White lines show the arbitrary border of the neuropile regions. C) Scheme of a neuron in the brain. D,E) Distribution of the neural cell bodies, showing the anterior (D) and posterior (E) views of the brain. Three-dimensional (3D) reconstruction of the confocal optical sections of the brain expressing nuclear-specific reporter UAS-*NLS-lacZ* driven by elav-GAL4 enhancer trap strain c155 (modified from ref. 10). F, G) Confocal optical sections, showing the anterior (F) and middle (G) areas (Data by H. Otsuna). Magenta represents the synaptic areas visualised by the monoclonal antibody nc82, which recognises the active zone protein, Bruchpilot.[11] White represents the glial processes visualised with UAS-*GFP* driven with glia-specific repo-GAL4 driver. H-N) Neuropile regions defined for indicating the positions in the brain (modified from ref. 5). 3D reconstruction of the anterior (H), posterior (I) and anterior-dorsal oblique (J-N) views of the brain showing neuropile regions at different dorsoventral levels. Because analysis of the function and neural architecture of the diffused neuropiles remains scarce and spotty, our current knowledge is not enough for making conclusive regional map that reflects the functional organisation of this area. To provide a way to describe neuropile regions unambiguously under this situation, borders of the neuropile regions are here defined arbitrarily with simple planes that are defined in association with easily recognisable landmarks such as the MB and the great commissure (GC). This nomenclature system is introduced by Strausfeld[2] and expanded by Otsuna and Ito.[5]

List of the neuropile regions: The dorsal area of the protocerebrum is divided into two areas: the superiormedial protocerebrum (smpr) and the superiorlateral protocerebrum (slpr). The sagittal border between smpr and slpr is defined by the lateral surface of the MB pedunculus (p). The horizontal border between the superior protocerebrum and the inferior protocerebrum is defined with the 50% height between the ventral surface of the pedunculus and the tip of the MB vertical lobe. *asmpr* (anterior superiormedial protocerebrum): the asmpr is the anteriormost area of the smpr, between the two vertical lobes (v) of the MB. The area slightly lateral to the MB vertical lobe but dorsomedial to the lateral pedunculus surface is included in the aimpr, because many neurons around the vertical lobe arborise also in its lateral side. The posterior border of the asmpr is defined with the posterior surface of the MB vertical lobe. *msmpr* (middle superiormedial protocerebrum): the middle area of the smpr, directly posterior to the asmpr. Its posterior border is defined with the plane above the GC. The pars intercerebralis—the area near the midline with many large cell bodies of neurosecretory cells—lies in the medilalmost region of the msmpr. *psmpr* (posterior superiormedial protocerebrum): the posteriormost area of the smpr, spanning above and anterodorsal to the MB calyx (ca). *mslpr* (middle superiorlateral protocerebrum): the area lateral to the msmpr. Note that there is no area called the aslpr, because there is no neuropile anterolateral to the MB vertical lobe (see Fig. 1J). *pslpr* (posterior superiorlateral protocerebrum): the area lateral to the psmpr, dorsolateral to the MB calyx. The area below the superior protocerebrum and above the ventral surface of the pedunculus is the inferiormedial protocerebrum (impr) and inferiorlateral protocerebrum (ilpr). *aimpr* (anterior inferiormedial protocerebrum): The anteriormost area of the impr, above the antennal lobe and in front of the posterior surface of the MB vertical lobe. The medial lobe of the MB is embedded in this area. *mimpr* (middle inferiormedial protocerebrum): The area of the impr behind the MB lobes, anterior to the plane above the GC and medial to the lateral surface of the pedunculus. The dorsal half of the ellipsoid body (eb) and the fan-shaped body (fb) of the central complex is contained in this area. *pimpr* (posterior inferiormedial protocerebrum): The area between and anteromedial to the calyx. The protocerebral bridge (pb) of the central complex lies in this area. *optu* (optic tubercle): The anteriormost area of the ilpr, lateral to the aimpr. Though this area could be called as ailpr, it is occupied by the structure that is traditionally called as the optic tubercle, which is contributed by the terminals of the visual projection neurons from the optic lobe via the anterior optic tract (AOT). *milpr* (middle inferiorlateral protocerebrum): The area lateral to the mimpr. *pilpr* (posterior inferiorlateral protocerebrum): The area lateral to the pimpr, between the calyx and the lateral horn. *LH* (lateral horn): The area protruded in the lateral area of the central brain, between the milpr and pilpr. This area contains the terminals of the olfactory projection neurons from the AL. *AL* (antennal lobe). Legend continued on following page.

Figure 1, viewed on page 138. The anterior protrusion of the medial cerebrum, receiving projections of the sensory neurons of the antennae via the antennal nerve (AN). It is a part of the deutocerebrum. *vmpr* (ventromedial protocerebrum): The area just posterior to the AL, in front of the GC and ventromedial to the MB pedunculus. Unlike the AL, it is a part of the protocerebrum. It houses the ventral half of the ellipsoid body and the fan-shaped body as well as the lateral accessory lobe (also called the ventral body), an annex of the central complex that is important for motor control. *spsl* (superior posterior slope): Dorsal part of the area in the posterior brain surrounding the oesophagus foramen. It receives projections from the ocellar nerve and is also contributed by the dendrites of descending neurons. *ipsl* (inferior posterior slope): The area of the posterior slope ventral to the oesophagus foramen, which also houses dendrites of descending neurons. *vlpr* (ventrolateral protocerebrum): A large area in the lateral cerebrum in front of the GC. It is also called the anterior optic foci, because it receives many visual projections from the optic lobe. Their terminals in this area form several glomerular structures called the optic glomeruli. *plpr* (posteriorlateral protocerebrum): The area behind the vlpr, which is also called as the posterior optic foci. Like vlpr, many visual projection neurons terminate in the plpr. *de* (deutocerebrum): The area posterior ventral to the AL. It houses the antennal mechanosensory and motor centre (AMMC), which receives projections of auditory and mechanosensory neurons from the antennae. The AL is actually also a part of the de. *SOG* (suboesophageal ganglion): The neuromere ventral to the oesophagus. Other labelled structures: *la*: lamina, *me*: medulla, *lo*: lobula, *lop*: lobula plate, *AOT*: anterior optic tract, *POT*: posterior optic tract, *LCBR*: lateral cell body region.

distributed around the surface of the nervous system each generates a large number of neurons.[6,7] Each cell division yields a neuroblast and a ganglion mother cell (GMC). It is generally believed that a GMC divides once more to generate two neural progeny. Most neuroblasts proliferate at two separate periods during neurogenesis.[7] The first proliferation occurs during mid to late embryonic stage, whereas the second proliferation starts from between the late first and late second larval instar and ends during the first day of the pupal stage. Thus, the clonal progeny of most neuroblasts consists of embryonic and postembryonic neurons (Fig. 2A).[17]

In the larval brain, there are about 100 neuroblasts per hemisphere in the cerebrum[7,18,19] and about 80 per hemisphere in the SOG (R Urbach and GM Technau, personal communication). There are therefore in total about 180 neuroblasts in a central brain hemisphere. Counting of cell bodies in the nuclear-labelled brain samples suggests that there are about 18,000 cells per hemisphere in the adult central brain including the SOG (T Shimada and KI, unpublished observation). Considering that some neuroblasts, such as those that generate the MB Kenyon cells, give birth to several hundred progeny,[7] the number of progeny of most other neuroblasts should be less than a hundred.

How, then, does each family of clonally related neurons contribute to the formation of the adult neural circuits? One possibility is that each neuron differentiates and sends its neurites independently from cell lineage (left panel of Fig. 2B). The other possibility is that neurons of a particular clone form distinct subcomponents of the neural circuits (right panel of Fig. 2B).

To determine which is more likely, a technique is required to visualise the projection pattern of all the progeny of one neuroblast in the adult nervous system. This has not been an easy task. Cell lineage can in principle be traced by injecting dyes to a cell early during development.[20-24] Though this worked well for analysing cell lineage in embryos, postembryonic progeny could not be labelled with this technique, because injected dye is diluted below detection level as neuroblasts repeat cell division. To circumvent this problem, transplantation of genetically labelled neuroblasts was developed.[17,25] In this technique, a neuroblast is picked out from an embryo expressing a reporter gene (e.g., *lacZ*) under control of a ubiquitous promoter. The neuroblast is then transplanted to a host embryo that does not carry the reporter gene. Though this system is versatile,[26-28] technical expertise is required for cell transplantation and differences in the cell positions and developmental stages between donor and host embryos might affect subsequent development of the transplanted neuroblast. Thanks to the powerful *Drosophila* genetics, however, several techniques that are easier to label clonally related cells were developed during the last decade. They use genetic mosaic

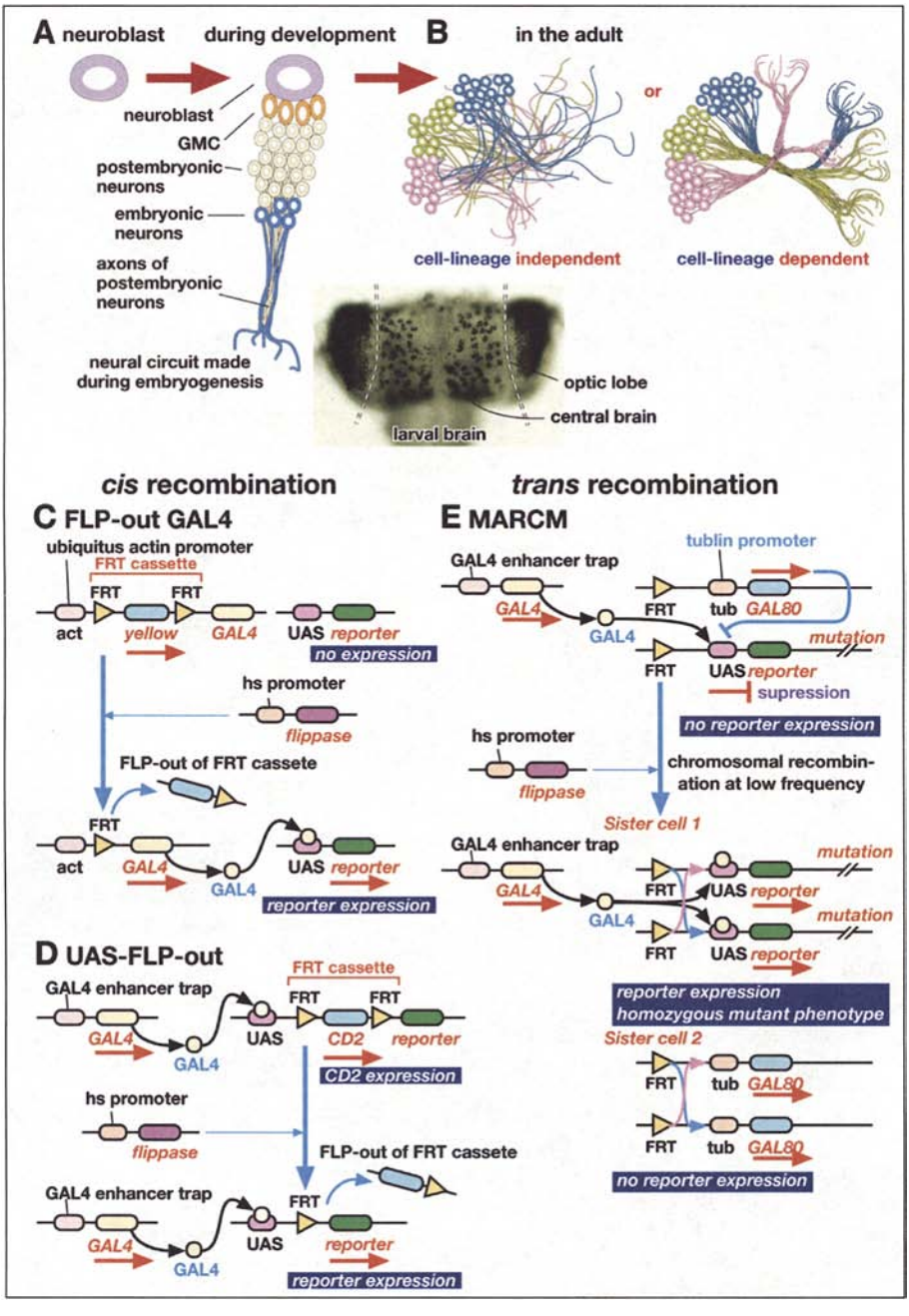

Figure 2. Neuroblast proliferation and techniques for labelling clonally related cells. A) Scheme of the neuroblast and its progeny in the larval brain. *GMC*: ganglion mother cell. Photographic inset: Larval brain shortly before puparium formation. Proliferating cells are labelled with bromodeoxyuridine (BrdU) incorporation by larval feeding and visualised with anti-BrdU antibody. Figure legend continued on next page.

Figure 2, viewed on previous page. B) Two possible strategies for constructing the neural cicuits of the adult brain. C) FLP-out-GAL4 (FRT-GAL4) system. In the first component, a marker gene *yellow* flanked by a pair of FRTs (FRT cassette) is inserted between the ubiquitous actin promoter and the transcription activator *GAL4*. Because there is no promoter directly in front of *GAL4*, only *yellow* is expressed. By applying mild heat shock to the animal during development, the heat-shock (hs) promoter activates the expression of the second component, hs-*flippase*, in some cells. Flippase protein induces recombination between the two FRTs, excising the *yellow* gene between them (FLP-out). In the cells in which this recombination occurred and also in its progeny, the actin promoter starts driving the expression of *GAL4*. The expression of the third component, a reporter gene such as *GFP* under control of the GAL4-target sequence UAS, is activated only in these cells. D) UAS-FLP-out system: The *GAL4* gene is expressed in a cell-type specific manner using certain promoter or GAL4 enhancer-trap strains. The second component features UAS and a reporter gene separated by an FRT cassette containing the *CD2* gene. GAL4 activates the expression of only *CD2*. A mild heat shock activates *flippase*, which excises the FRT cassette. This enables the expression of the reporter gene in the cell and its progeny. E) MARCM system: A ubiquitous tublin promoter drives constitutive expression of yeast-derived *GAL80*, which suppresses expression of the UAS-linked reporter gene even in the presence of *GAL4*. The tublin-*GAL80* and the reporter gene are put in the homologous chromosome in trans and the FRT sequence is put in the locus close to the centromere of each chromosome. Upon mild heat shock, *trans* recombination between two chromosomes occurs in some of the cells during mitosis. One of the daughter cell becomes homozygous for the UAS-reporter. Because GAL80 no longer exists in the genome of this cell and its progeny, the cells are visualised by the reporter.

analysis combined with yeast-derived GAL4-UAS[29-31] and flippase-FRT systems[32-34] and can be categorised into two groups.

cis-*Recombination Systems*

Flippase is the enzyme that induces recombination between two sequences called the flippase recognition targets (FRTs). The first group of techniques label cells by inducing *cis*-recombination between two FRT sequences on the same chromosome. First, a gene or a stop-codon sequence is placed between the two FRTs. This "FRT cassette" is then put between a reporter gene and a promoter of a ubiquitous house-keeping gene, e.g., actin or tublin. Because of the inserted FRT cassette, the ubiquitous promoter cannot drive the expression of the reporter gene. By inducing the expression of *flippase* transiently during development, e.g., by putting the *flippase* gene under the heat-shock promoter and giving temporal heat shock to the transgenic animals, recombination between the two FRTs would occur in some cells. This removes the FRT cassette (flip-out or FLP-out) and connect the ubiquitous promoter and the reporter gene directly. The reporter gene would be expressed specifically in these cells as well as in their progeny. If the recombination occurs in the GMC or in the postmitotic cells, single or a few scattered cells would be labelled. If the recombination occurs in the neuroblast, on the other hand, a group of clonally related cells can be visualised.

Such system was first developed by putting the *lacZ* gene after the FRT cassette.[35] An improved version featured *GAL4* instead of *lacZ*, which can activate the expression of diverse types of reporter genes to visualise different aspects of the labelled cells (FRT-GAL4, or FLP-out GAL4 system, Fig. 2C).[36] Because GAL4 can activate multiple UAS targets, genes that affect the function or development of the cells—so called effector genes—can be expressed simultaneously with the reporter genes, enabling the functional analyses of the expressed genes using this system.

Another approach is to put the FRT cassette between the UAS and the reporter gene (the UAS-FLP-out system, Fig. 2D).[37] This system can be combined with a wide variety of promoter-GAL4 lines and GAL4 enhancer-trap strains currently available, in which GAL4 is expressed specifically in particular cells. Depending on whether the recombination occurred in the neuroblast or in the postmitotic cells, the UAS-FLP-out system visualizes a clonally related subset or the morphology of the single cells out of the *GAL4*-expressing cell population.[15,38]

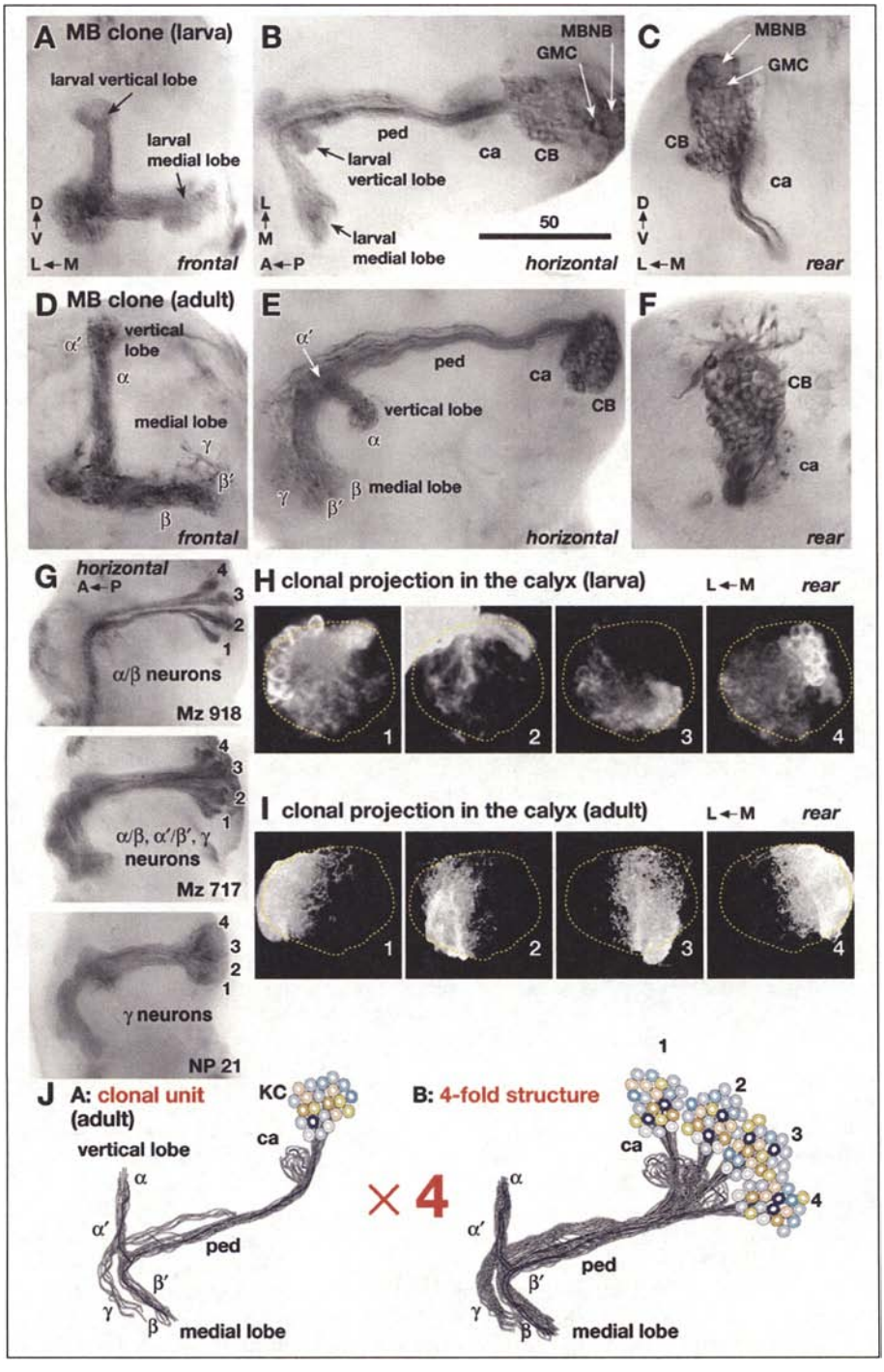

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Figure 3, viewed on previous page. Clonal units in the mushroom body. A-F) A clone labelled in larvae just after hatching and visualised at the end of the larval stage (A-C) and another clone visualised in the adult (D-F), respectively (modified from ref. 36). (FLP-out-GAL4 clones visualised with UAS-*tau* reporter. UAS-*tau* and UAS-*GFP* reporters label essentially similar structures, except that Tau labels dendritic arborisations more weakly and occasionally causes mild disturbance of the neural function.) Optical sections at different levels were taken with Nomarski optics and montaged. Frontal, horizontal and rear views of the same clone was visualized by rotating the specimen. *MBNB*: mushroom body neuroblasts, *CB*: cell bodies, *ca*: calyx, *ped*: pedunculus. G) Four-fold labelling pattern of enhancer-trap strains labelling subsets of the MB Kenyon cells. (Modified from ref. 36, UAS-*tau* reporter). H) Cross section of the calyx in the larval brain, showing areas of arborisations of each of the four clonal units. (Modified from ref. 42, ©2005 National Academy of Sciences, U.S.A., MARCM clone with UAS-*GFP* reporter.) I) Cross section of the calyx in the adult brain, showing areas of arborisations of each of the four clonal units. (Data by Nobuaki Karl Tanaka. MARCM clone with UAS-*GFP* reporter.) J: Scheme of the four-fold clonal units in the MB.

Note on the name of the lobes: The names of the lobes have been changed drastically during the last few years of the last century. The terms of the α and β lobes originated from the study of the bee brain[60] to refer to the vertical and medial lobes. The γ lobe derived from the study of sphinx moth.[61] These terms are adopted to describe the fly MB.[2,62] Because of the apparent structural similarity, vertical and medial lobes in the larval MB had also been called as larval α and β lobes.[36]

Analysis of clones and GAL4 enhancer-trap strains revealed a characteristic subdivision of the α lobe and defined it as the α′ lobe, but failed to recognise the corresponding subdivision in the β lobe.[36] The latter subdivision was identified by the comparison of labelling pattern of various antibodies and named as the β′ lobe.[3] Until this period, it was not known that the neurons innervating the γ lobe have no vertical branches. Though such unbranched neurons had been observed in Golgi impregnated samples, the non-existence of the branch could not be determined conclusively because Golgi labelling may not always label all the branches of a neuron.

Finally, systematic flippase-mediated single-cell analyses revealed that the neurons contributing to the γ lobe, α′/β′ lobes and α/β lobes are generated in this order and that the vertical and medial lobes of the larval MB is contributed exclusively by the neurons that compose the adult γ lobe as a result of reorganization.[41] The larval vertical and medial lobes, therefore, have nothing to do with the neurons of the adult α/β lobes.

To avoid confusion, it is better not to use the term α/β lobes for the larval MB but to use the generic term vertical/medial lobes instead. Also, the adult vertical lobe should not generally be called the α lobe, as it actually consists of α and α′ lobes each of which is likely to have rather different functions.

The vertical and medial lobes are sometimes called dorsal and horizontal lobes, respectively. In various insects, however, the vertical lobe does not project dorsally but anteriorly or anterodorsally. The medial lobe projects medially (towards the midline) in all insect species, but the inclination of the lobe may not always be horizontal. Thus, the combination of "vertical" and "medial" seems more appropriate when considering cross-species compatibility. A-G, J reproduced with permission of the Company of Biologists.

trans-*Recombination Systems*

One of the classic methods for analysing lineage-associated cells is to induce somatic recombination by irradiating the animals with X ray or γ ray. Recombined cells can be identified by putting a marker gene in one of the chromosomes. As a more controllable and easy-to-use approach, FRT was put into the chromosome to induce flippase-dependent *trans*-recombination.[34] The lack of convenient reporter systems for detecting the neurons that experienced recombination has made it difficult to apply this technique for brain research. The mosaic analysis with a repressible cell marker (MARCM) system solved this problem.[39] The MARCM system features GAL80, which works antagonistically to GAL4 (Fig. 2E). GAL80 suppresses expression of the UAS-linked reporter gene even in the presence of GAL4. Flippase-induced somatic recombination between the FRT sequences removes *GAL80* gene in one of the daughter cells. UAS-linked reporter/effecter genes will be expressed specifically in this cell and its progeny.

An advantage of the MARCM system is that it can be combined with the somatic recombination analysis of recessive mutations, so that only the cells that are homozygous for the mutation can be visualised. This has been proven as highly effective tool for studying cell-autonomous roles of various genes during development.

Clonal Unit Architecture in the Adult Brain

Clonal Units in the Mushroom Body

The correlation between cell lineage and adult neural circuits was first identified in the MB. Although most neuroblasts proliferate at two separated periods in *Drosophila*, there are five neuroblasts that proliferate continuously throughout neurogenesis.[7] By administrating bromodeoxyuridine (BrdU) to larvae just after hatching, it is possible to label the nuclei of these proliferating neuroblasts and their progeny. One neuroblast lies in the anteriorlateral area of the larval brain and its progeny is distributed in the lateral side of the AL in the adult. The other four neuroblasts lie in the posterior dorsal area of the larval brain and their progeny are found lying above the MB calyx. Though BrdU can visualise only the nuclei of the labelled cells, their positions on the calyx strongly suggested that they are the MB Kenyon cells.

A more direct evidence came later with the advanced genetic analysis using flippase-mediated *cis-* or *trans-*recombination analyses, which enabled visualisation of neurites of the clonally related cells.[36,39] When clones are labelled early during development and visualised in a late larval stage, a single neuroblast, a few large GMCs and many small neurons are labelled (Fig. 3A-C). They innervate only within the MB neuropile. The cell bodies of the clonally related progeny remain in a tightly bound cluster in the adult brain, indicating that the cells do not migrate long distances from their place of origin. All the fibres deriving from this cluster innervate the MB, with no projection to other brain areas (Fig. 3D-F). Thus, these clones are indeed dedicated to the neural circuit of the MB.

There should be four different clonally-related populations each deriving from one of the four neuroblasts. Are they different from each other? The clusters of cell bodies are observed in four areas of the rind above the calyx and neurites from these clusters form four large bundles that run around the lower part of the calyx. The fibres from each cluster contribute to all the known components of the MB: the calyx, pedunculus and the α'/β', α/β and γ lobes. Thus, concerning the area of projection, the neurons of four clones are essentially identical.

The four-fold structure of the MB is further confirmed by the observation of GAL4 enhancer-trap strains. There are many GAL4 strains that label various subsets of the Kenyon cells, suggesting that the MB should consist of a heterogeneous population of neurons concerning their gene expression patterns.[36,40] These strains all label neurons in each of the four clusters, indicating that each clone essentially contains an identical repertoire of Kenyon cells. The four-fold pattern is most evident in the strains that label Kenyon cells innervating the α/β lobes, which are generated latest during development[41] (top panel of Fig. 3G). The four bundles of clonally related neurons are clearly labelled at the level of the calyx. The bundles deriving from the two medial clusters and two lateral clusters (1, 2 and 3, 4 in Fig. 3G, respectively) are fused in the middle level of the calyx. The two merged bundles further merge at the anterior end of the pedunculus. The neurites from each clonal cluster are intermingled completely in the lobe area. The four bundles are discernible but are less clear in the strains that label a variety of Kenyon cells (middle panel of Fig. 3G). The discrete pattern is more ambiguous in the strains that label neurons projecting only to the γ lobe, because their neurites run near the surface of the pedunculus (bottom panel of Fig. 3G).

There are, however, certain differences between the four clones concerning the types of information they receive. The MB receives olfactory signals from the antennal lobe, which is conveyed by the antennal lobe projection neurons (AL PNs). Many of them are uniglomerular, sending signals from one particular glomerulus of the AL to the MB (see Chapter by V Rodrigues and T Hummel). In larvae, terminals of these AL PNs form small glomerular structures in the calyx called microglomeruli[42,43] (see also Chapter by R Stocker). Their positions are reproducible among

individuals, showing that olfactory information from particular glomeruli in the larval AL is transmitted to distinct subregions of the calyx. The arborisations of the Kenyon cells of each clone occupy different, but partially overlapping, areas of the calyx (Fig. 3H).[42] Thus, each clone should receive a different repertoire of olfactory information.

Because of the much larger number of AL PNs and the number of glomeruli in the adult AL, there are numerous very small microglomeruli in the calyx of the adult MB, making their mapping more complex (see Chapter by P Laissue and L Vosshall). Nevertheless, AL PNs from particular AL glomerulus terminate in specific concentric zones in the calyx.[4] The Kenyon cells of each clone again arborise in distinct areas of the calyx (Fig. 3I),[4,44] suggesting that there may also be differences in the repertoire of olfactory information each clone would receive. For example, the two "outer" clones (1 and 4 in Fig. 3I) may have fewer interaction with the projection neurons that terminate in the central area of the calyx than do the two "inner" clones (2 and 3 of Fig. 3I).

Observations in the MB suggest that there are clonally-related unit structures in the adult brain. Progeny of a single neuroblast may contain a functionally heterogeneous population of neurons. Yet, they all innervate only a limited area of the brain and form a distinct neural circuit structure. There are four such clonal units in the *Drosophila* MB, which are essentially identical regarding their morphology and biochemical diversity but slightly different in the projection pattern in their input areas (Fig. 3J).

Clonal Unit Architecture in the Central Complex

Clonal unit is not a unique feature of the MB. They are also observed in the central complex, the neuropile that lies at the centre of the cerebrum[2,4,5] and is supposed to play important roles in motor coordination control, visual memory, etc.[46-48] The structure of the central complex is much more complex than the MB (Fig. 4A). It consists of four major components, the ellipsoid body (eb), fan-shaped body (fb), protocerebral bridge (pb) and noduli (no).[45] Whereas the cell bodies of the MB Kenyon cells are all confined in a small area just around the MB calyx, those that contribute to the central complex are distributed in various parts of the brain. Nevertheless, lineage-dependent cell labelling experiments revealed that several clones contribute specifically to the central complex, each forming distinct building units of its neural circuits.

The ellipsoid body is a round structure that forms the anteriormost part of the central complex. There is a pair of clonal units with their cell bodies in the anterior brain above the aimpr area of the cerebrum, dorsolateral to the AL (EB-A1, Fig. 4A,B). A bundle of neurites projects beneath the medial lobe of the MB and forms the primary arborisation in the vmpr part of the cerebrum, forming the structure called the lateral triangle (ltr). From the ltr, some fibres project dorsally to reach the asmpr and aimpr and others project to the ellipsoid body from its central hole to form the ring neurons of this neuropile.

The fan-shaped body consists of an array of radial projections and tangential neurons that arborise at its various dorsoventral levels. One of the clonal units that form these tangential components have the cell body cluster in the dorsolateral area of the cerebrum, posterior to the LH (FB-DL1, Fig. 4A,C). The neurons form primary arborisations near the dorsal surface of the cerebrum above the LH and secondary arborisations in the msmpr and mslpr. The fibre bundle bifurcates, enters the fan-shaped body from its anterior side at two levels (Fig. 4C) and forms extensive branches that span tangentially. There are also other clonal units that form tangential arborisations in different levels of the fan-shaped body (not shown here).

The radial component of the fan-shaped body is formed by four clonal units per hemisphere (FB-P1-4, Fig. 4A,D). A row of eight cell body clusters lies in the posterior brain right behind the fan-shaped body, flanked by the calyces of the MB. The neurites form primary arborisation in the protocerebral bridge and enter the inferior part of the fan-shaped body from its posterior side. They form two bundles that run radially in the fan-shaped body and terminate in the nodulus of the contralateral hemisphere.

The protocerebral bridge is divided into eight sections per hemisphere. Similarly, the radial component of the fan-shaped body is organized in eight radial structures called the staves.[2,45]

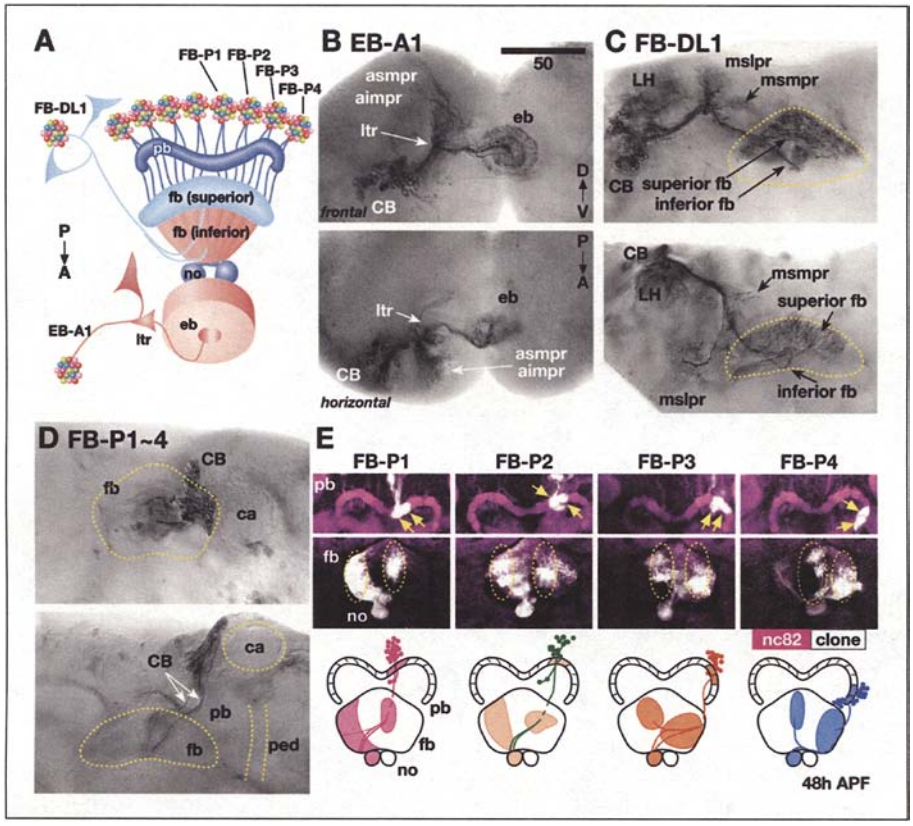

Figure 4. Clonal units in the central complex. A) Scheme of the central complex and three major types of clonally related components. There are also several other clonal units that contribute to the central complex. *pb*: protocerebral bridge, *fb*: fan-shaped body, *no*: nodulus, *eb*: ellipsoid body, *ltr*: lateral triangle. B-D) Examples of clonal units contributing to the central complex. See legend to Figure 1 for neuropile regions. FLP-out-GAL4 clones visualised with UAS-*tau* reporter in the adult brain. Top and bottom photographs of each figure show the montage of optical sections of the same clone in frontal and horizontal view, respectively. Clonal units: EB-A1 (ellipsoid body-anterior 1, B), FB-DL1 (fan-shaped body dorsolateral 1, C) and FB-P1-4 (fan-shaped body posterior 1-4, D). E) Arborisation areas of the four FB-P clonal units (Data by Mariko Kamiya). Confocal sections at the level of the protocerebral bridge (top panel), fan-shaped body and nodulus (middle panel) and the schema of the projection pattern (bottom panel). (MARCM clone with UAS-*GFP* reporter in the mid pupal brain 48 h after puparium formation, when the neuropile structure is already essentially the same as in the adult.)

Neurites of each FB-P clonal unit arborise in two sections of the protocerebral bridge (Fig. 4E, top panel) and contribute to two staves of the fan-shaped body (Fig. 4D, bottom panel). Collateral fibres deriving from these staves arborise in two areas of the fan-shaped body, one in the ipsilateral and the other in the contralateral side (Fig. 4E, middle panel). Whereas the arborisation of each clonal unit is segregated in the protocerebral bridge, there is a significantly overlap between their arborisations in the fan-shaped body. In the nodulus, fibres of all the four clonal units converge and arborise in the entire area of its neuropile (Fig. 4E, bottom panel).

Clonal Unit Architecture in Other Brain Areas

Compared to the MB and the central complex, borders between neural circuits in the rest of the central brain are much more obscure. Nevertheless, clonally related neurons innervate only limited areas of these neuropiles and form distinct unit structures.

Projection neurons from the antennal lobe innervate the MB calyx, the LH and several other areas of the brain.[13,14] GAL4 enhancer-trap strains such as GH146, NP225 and NP5288 label many of these neurons.[4,49,50] The cell bodies of these neurons form at least four clusters around the AL. The anterior dorsal cluster (AL-DA1, Fig. 5A) and a lateral cluster (AL-L1, not shown here) consists of the neurons that innervate via the inner antennocerebral tract (iACT). The cell cluster that lies ventral to the AL (AL-V1, not shown here) consists of the neurons of the middle ACT (mACT) pathway. There is yet another clone in the lateral area of the AL, which consists of the neurons that do not seem to be labelled in these GAL4 strains (AL-L2, Fig. 5B). Neurons of this clonal unit project not only to the MB and calyx but also to the SOG and the plpr.

In the MB, neurons other than the Kenyon cells also innervate its neuropile. An example of such clonal unit, MB-A1 (Fig. 5C), has the cell bodies in the anterior brain just in front of the MB vertical lobe.[10] Neurons of this clone mainly innervate the distal area of the medial lobe and project also to the neuropiles other than the MB in the aimpr and vmpr areas.

Neurons in the LH, which receives olfactory information from the AL like the MB Kenyon cells, are also organized in a clonally related manner. Several clonal units contribute to the neuropile of the LH. Their cell bodies form clusters in the LCBR. Some clones (e.g., LH-1, Fig. 5D) consist of local neurons that arborise only in the LH. The neurites of other clones (e.g., LH-2 and 3, Fig. 5E,F) arborise in the LH and project further to other areas of the protocerebral neuropiles. Depending on the clonal units, the neurites project to the LH either from inside (LH-2,3) or from outside (LH-1).

The superior lateral and superior medial protocerebrum occupies the dorsalmost area of the cerebrum. Because neural connections between these neuropiles and the neuropiles of the sensory and motor pathways are still essentially unknown, the function of the neural circuits in these areas are yet to be determined. These neuropiles are also contributed by many clonal units. Short of the knowledge of determining neural structure in these areas, these clonal units are tentatively named according to the neuropile region (Fig. 1H-N) in which they arborise most extensively. Some clonal units, e.g., PSLPR-1 and MSLPR-1 (Fig. 5G,H), arborise only in a small region of the neuropile. They tend to have simple structures, with a single bundle of neurites and arborisation in one or only a few areas. Other clones, like MSLPR-2 and MSMPR-1 (Fig. 5I,J), arborise in multiple areas. The structure of these clonal units are more complex, with bifurcation or trifurcation of neurite bundles and extensive projections that span a long distance in the brain.

The ventrolateral part of the cerebrum (vlpr and plpr) is occupied by the neuropiles that extensively receive axons of the visual projection neurons, which connect the optic lobe and the central brain.[2,5] These areas are also formed by various clonal units, whose cell bodies lie in the LCBR or in the anterior lateral area of the cerebrum. Some clonal units form circuits that connect the corresponding neuropiles of both hemispheres (e.g., VLPR-1, Fig. 5K), whereas others connect a variety of neuropile areas of the cerebrum (e.g., VLPR-2, Fig. 5L).

Formation of the Clonal Units During Development

The observations presented above suggest that a significant portion of the adult brain is composed in a cell lineage-dependent manner (Fig. 2B). Though the progeny of a single neuroblast are not as tightly packed as in the larval brain, they still form a cluster. Neurites deriving from this cluster form tight bundles and innervate distinct areas of the brain.

How, then, is such clonal unit architecture in the adult brain composed during neurogenesis? When the clones are visualised in late larval or early pupal brains, the progeny of a neuroblast form a tightly packed cluster, which sends a bundle of neurites towards the neuropile (Fig. 6A,B). The bundle either projects to a single target or bifurcates when it enters the neuropile to innervate different areas of the brain.[51] The formation of the adult clonal units should depend on this

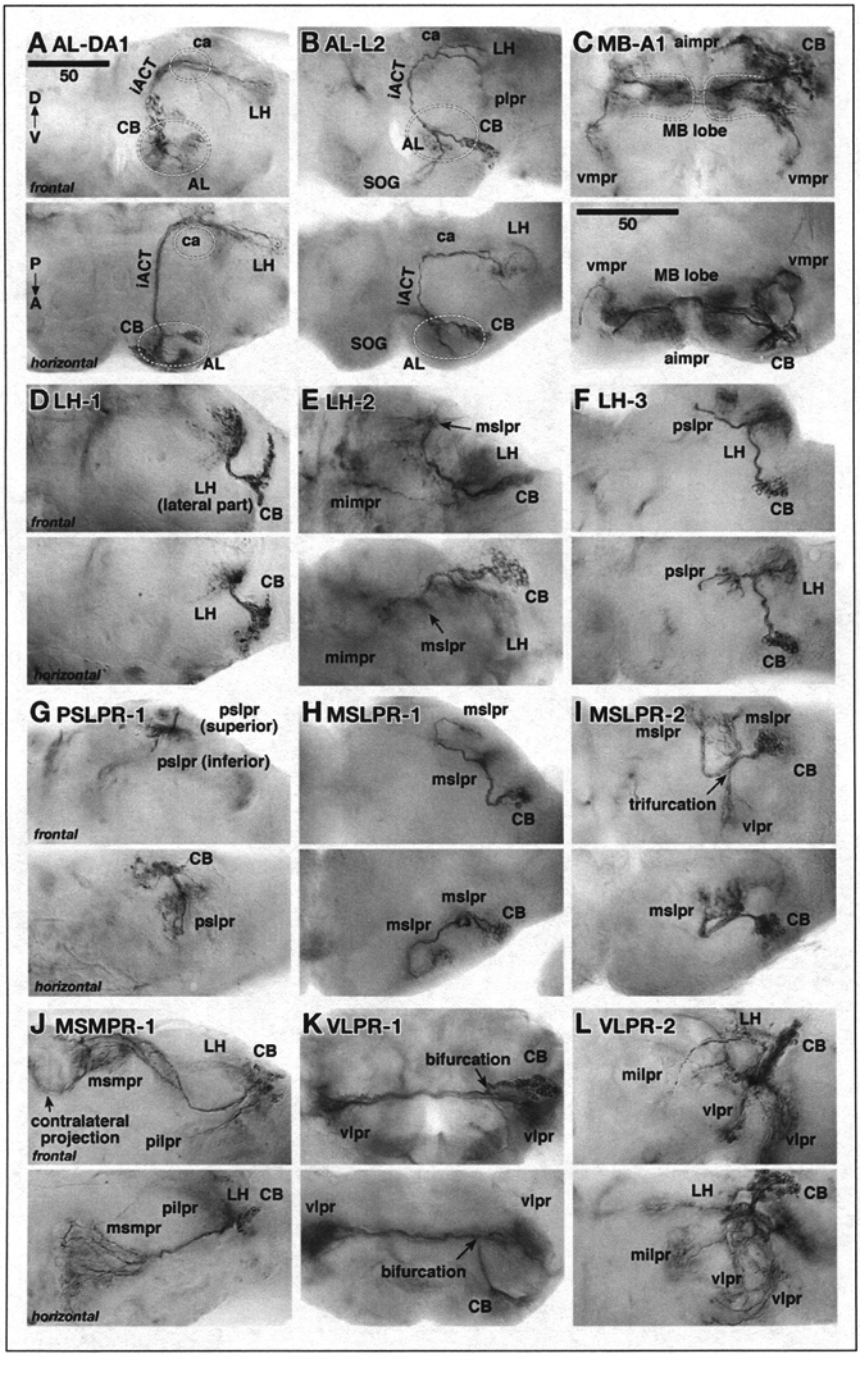

Figure 5. Clonal units in other brain areas. FLP-out-GAL4 clones visualised with UAS-*tau* reporter. Top and bottom photographs of each figure (A-L) show the montage of frontal and horizontal optical sections of the same sample, respectively. See legend to Figure 1 for neuropile regions.

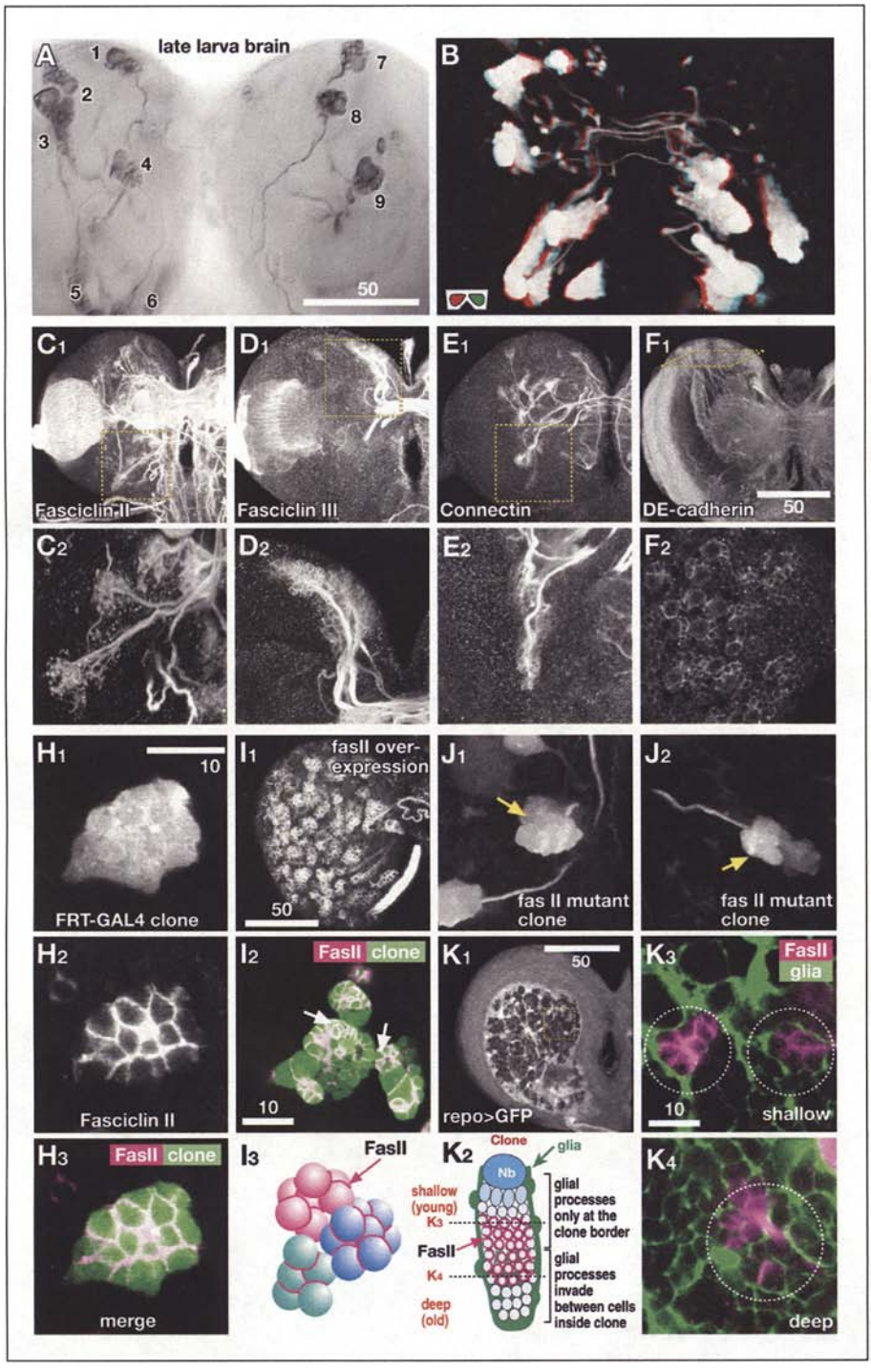

Figure 6, legend viewed on following page.

Figure 6, viewed on previous page. Formation of clonal units in the larval brain. A, B) Clonal units in late larvae. FLP-out-GAL4 clones visualised with UAS-*tau* (A, montage of optical sections) and UAS-*GFP* (B, 3D-stereograph of confocal sections). C-F) Distribution of cell-adhesion molecules in the larval brain visualised with antibodies. Overall brain (top panel) and blow-up view (bottom panel) showing the area indicated with dashed squares in the top panel. Clonal-unit dependent distribution of Fasciclin II (FasII, C), Fasciclin III (D) and Connectin (E) and pan-clonal distribution of DE-cadherin (F). H) Distribution of FasII visualised with anti-FasII antibody in the cluster of clonal cell bodies. I) Over-expression of FasII in all the neurons (using elav-*GAL4C155* driver, I1) and in the MARCM clones (I2). J) Effect of the homozygous mutation of FasII in the MARCM clones. Two examples are shown. K) Distribution of FasII (visualised with anti-FasII antibody) and glial processes (visualised with UAS-*GFP* driven with glia-specific repo-GAL4 driver).

clonal cluster formation in larvae. Because formation of the lineage-dependent structure in the larval brain is comprehensively described in the Chapter by V Hartenstein et al, here we discuss this issue only briefly.

One of the candidate mechanisms that promote binding of the clonally related cell bodies and neurites depends on homophilic cell adhesion molecules (CAMs). If such CAMs are expressed in the clonally related neurons, they would facilitate adhesion of the cells and fibre bundles.[51-53]

According to their expression patterns, the homophilic CAMs can be classified into two types. The first type is expressed only in a small subset of the clones. This includes Fasciclin II (Fas II), Fasciclin III (Fas III) and Connectin (Fig. 6C-E). Interestingly, whereas the expression patterns of these CAMs are associated with the clonal units in the developing brain, they are not related with the clonal units in the adult. This suggests that intra-clonal cell-cell adhesion would be mediated by these CAMs during the formation of certain clones.

The other group is expressed in most of the developing clonal units: this category includes CAMs like DE-cadherin (DE-cad) and Neurotactin (Fig. 6F). The role of such pan-clonal CAMs during development has been studied using the ectopic expression of the dominant negative form of DE-cad, which affected the organisation of the developing clonal clusters.[54] Although the observed abnormality was not severe, the function of DE-cad at least seems to be involved in the correct formation of the clonal architecture.

The role of the clone-specific CAMs, on the other hand, is not yet clear. When the distribution of one such CAM, Fas II, is visualised together with the clonal cluster, the protein is observed only on the cell surface that is flanked by other siblings in the same clone but not on the outer surface of the cell body cluster (Fig. 6H). To determine whether Fas II is concentrated because of the homophilic interaction with the same molecule of the neighbouring cells, we over-expressed FasII so that cells in the neighbouring clones express the same protein. Even in this case, FasII is concentrated only along the cell border within each clone but not along the cell border between clones (Fig. 6I2, I3). Ectopic expression of FasII in all the neurons, which should negate the clone-specific role of this molecule, affect neither the organised distribution of the clonal cell clusters nor the projection patterns of neurites (Fig. 6I1). Moreover, the formation of the clonal cell cluster and neurite bundles is not disturbed even when the function of FasII is removed by inducing *fasII* mutant clones using the MARCM system (Fig. 6J). Thus, removal of just one clone-specific CAM does not affect the formation and maintenance of the clonal architecture in the larval brain. It is possible that pan-clonal and clone-specific CAMs might function cooperatively to facilitate the clone-specific cell-cell adhesion.

Another factor that would be important for the organisation of the clonal unit is the cell body glial cells, which send processes between neural cell bodies.[12] The region of the rind near the surface of the larval brain is characterised by the glial processes that form large nest-like holes (Fig. 6K1).[55] Because each glial nest houses a neuroblast and its progeny, the surface of the clonal cluster is flanked by the glial sheath. This organisation explains why FasII is accumulated only in the intraclonal border of the cell bodies (Fig. 6K3). Because glial cells do not express FasII, the glial sheath physically separates the cells of the FasII-expressing clones even when they are flanked with each other.

In the deeper level of the rind, glial processes invade borders between neural cell bodies of the clones. Though FasII is still distributed along the intraclonal cell border, neighbouring cell bodies are separated by the invaded glial processes (Fig. 6K4). In the adult, all the neural cell body in the rind are each surrounded by extensive glial processes (Fig. 1F, G). Thus, the glial nest seems to be a transiently structure formed due to the time required for the extension of glial processes during larval neurogenesis. Although glial cells continue invading all the space between neural cell bodies, a temporal delay is inevitable between the period when neurons are newly formed by the GMC and the time when glial cells outside of the clonal cluster send processes between them (Fig. 6K2). This delay results in the glial nest architecture in the larval brain. Clone-specific CAMs may stabilise the clustering of sibling neurons during this time lag. Since the cell clusters are buttressed by the sheath of the glial nest and because pan-clonal CAMs may function redundantly, over-expression or lack of a particular clone-specific CAM would not lead to significantly abnormal phenotypes.

Functional Importance of the Clonal Units

Because many areas of the brain neuropile are formed by the combination of clonal units, they seem to be the fundamental building blocks of the adult fly neural circuits. There would be several advantages by organising the brain in such a clone dependent manner. Unlike in the simple nervous system of early embryos, neural fibres in the postembryonic brain must find their paths through the three-dimensional space filled with tangled fibres of other neurons. If each neuron differentiates and sends its neurite independently, a large variety of attracting and repulsive signals would be required for providing positional cues for these neurons (Fig. 7A1).[56] Because neural fibres innervating different targets would criss-cross with each other, systems for avoiding unnecessary cross-talk between these signals would be inevitable. If neurons of the same cluster, on the other hand, form fascicles to project to only distinct areas of the brain, the guidance system for the follower neurons should be much simpler (Fig. 7A2). Path finding of individual neurons will be required only in the area near the target. Projection towards an additional target is a matter of locating the branching point in the one-dimensional space along the neurite bundle. Even in such clones, the first neuron (the so called pioneer neuron) has to extend its fibre without the help of a pre-exiting fascicle. As this occurs in relatively early embryos, when the brain neuropile is still simpler and the distance between the cell body and the target is much shorter than in the adult, path finding would be relatively easy.

Although flippase-mediated labelling visualises clonal units so clearly, few molecular markers such as antibodies and enhancer-trap strains label neurons of a single clonal unit. Rather, they tend to label small subsets of neurons scattered in many clonal units. This suggests that, although neurons of each clonal unit are relatively homogeneous regarding their overall projection patterns, they are rather heterogeneous concerning properties like gene expression patterns. They are also heterogeneous in the precise arborisations within the target areas. These suggest that a single clonal unit would be a versatile functional unit in which a variety of complicated computation is possible. Organising the brain by the composition of such units might have been an economical way for developing complicated neural circuits during evolution. Just like duplication and subsequent modification of genes added new functions to the genome, addition of new clonal units by the formation of additional neuroblasts might be a convenient way of incremental evolution of the brain (Fig. 7B). The loss of certain clonal units might also have occurred during evolution. Considering that there are several clonal units contributing overlappingly to the same circuit module of the brain (discussed later), such loss of clonal units may not have jeopardised the architecture and function of the brain.

Whereas some clonal units consist of several hundreds of neurons, some have less than 50 neurons. Such significant differences in cell number may affect the computational capacity of the circuits formed by that clonal unit. Because different insect species rely on very different sensory signals depending on their habitats and life styles, computational requirements for the evolutionary comparable clonal units might vary. Not only duplication or removal of clonal units but also the change in the cell numbers of clones might have been important during evolution. Though

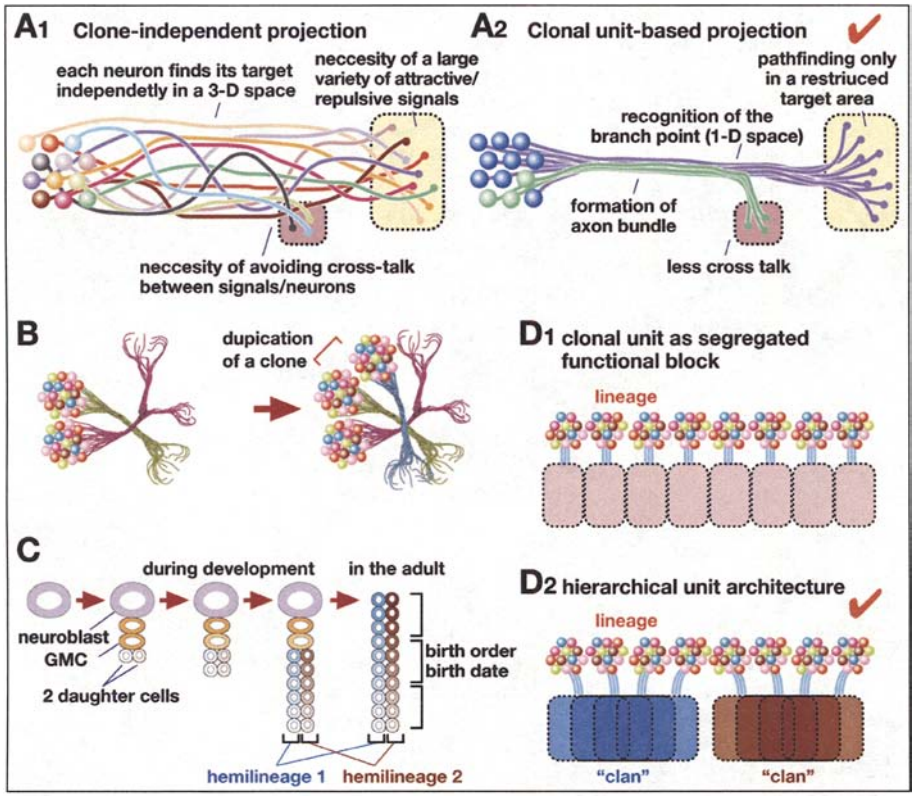

Figure 7. Clonal unit architecture of the brain. A) Comparison of possible path-finding mechanisms between clone-independent (left) and clone-dependent (right) organisation of the brain. B) Hypothetical scheme of incremental complication of neural circuits. C) Possible factors that affect the diversity of neurons within each clone. D) Scheme of the arborisation area of each clonal unit. Each clone innervates segregated areas of the neuropile (D1), or, Several clones innervate highly overlapping areas to form functional modules of the brain (D2).

visualisation of the clonal units in the adult brain is currently possible only in *Drosophila*, comparative study of clonal units across insect taxa in the future would provide important insights on the functional composition of the brain.

As for the heterogeneity within each clonal unit, there would be two candidate control factors (Fig. 7C). The first factor is the order and timing of cell generation. During embryonic development, neuroblasts change their gene expression pattern drastically and neurons that are made at each time point are characteristically affected by this.[19,57] In the postembryonic stages, expression patterns of the neuroblasts do not seem to change so quickly. Nevertheless, specific projection patterns of the adult neurons in the target area, such as the arborisation of AL-PNs in the AL and the LH and that of the MB Kenyon cells in the lobes, are dependent on the birth date of each neuron during larval stage.[41,49] A BTB zinc-finger protein gene has been identified that governs neuronal temporal identity during postembryonic fly brain development.[58] Expression levels of this molecule in the clonal neurons are reduced gradually depending on their birth timing. Temporal gradient in the activity of such genes may specify cell fate in an extended neuronal lineage.

Other factors would control the differences between the two sibling neurons made by each of the GMCs. Proteins such as Numb are distributed unevenly between the two daughter cells, activating the Notch signalling pathway in only one of them. This difference between sibling cells

of the olfactory sensory neurons made by the same precursor causes clustering of projection targets in the AL.[59] Similar differences between sister cells, each of which comprises a "hemilineage", may occur within the clonal units of the brain (Fig. 7C).

The concept of the adult brain made by the building blocks of clonal units may give the impression that each clone occupies specific and discrete areas of the brain neuropile (Fig. 7D1). Indeed, 3D reconstruction of clonal units yields images of clonally related neuronal fibres that appear to fill particular areas of the brain. This, however, might be a too simplistic view. Because the diameter of neural fibres is much smaller than the resolution of the optical microscopes, dense arborisation is visualized as a solid structure even when only a fraction of the volume is occupied by the visualised fibres. Volume- and surface-rendering algorithms of the 3D reconstruction software further remove fine detail of the visualised fibres, oversimplifying the projection pattern in the area. For the neurons of each clonal unit to communicate with neurons of other units, their arborisations have to be spatially colocalised and therefore intermingled. Thus, clonal units should in principle contribute to significantly overlapping areas of the brain (Fig. 7D2). Interestingly, the degree of overlap appears to be larger in the arborisation areas that are distal from the cell body clusters. Both in the MB and FB-P clones, arborisations of each clone occupy distinct areas in the calyx and protocerebral bridge but overlap completely in the lobes and nodulli (Figs. 3J,4E).

The degree of overlap between specific sets of clonal units is much larger than the overlap with the rest of the clones. In another word, several clonal units contribute together to form distinct neural circuit units, to which other clones contribute only marginally. In these cases, the neural circuit formed by each clonal unit may be too small and simple to represent an independent functional unit. The neural circuits in the brain are therefore organised in a hierarchical manner. Neurons deriving from several cell lineages form a "clan", which together contribute to the formation of a functional module of the brain circuit. The four clonal units of the MB, several clonal units around the AL that all arborise in the AL and form the complete set of ACT pathways, clones in the anterior and posterior brain that together compose the central complex neuropile, are examples of such clans. The clan might therefore be as important as lineage for understanding the functional dynamics of the brain, just like a clan of people, who belong to a number of tightly-associated lineages, behaved as a functional group in the dynamics of the ancient human society (Fig. 7D2).

Conclusion

Complicated neural circuits in the brain are composed by the combination of relatively simple clonal units. A group of clonal units together form a functional module of the brain. Developmental mechanisms that form such lineage- and clan-dependent structures are not yet fully understood. Guidance molecules and interactions between neurites of the same clone and between those of the neighbouring clones would play important roles in this process. More detailed analysis of the arborisation patterns and gene expression patterns of the neurons of each clonal unit would be required. Analysis of temporal aspects, not only about the order of neuron formation within each clone but also about the timing of proliferation and neurite extension among clones of the same clan, would also further our understanding about the process of the neural circuit formation.

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