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The Norepinephrine Transporter in Physiology and Disease

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Abstract The norepinephrine transporter (NET) terminates noradrenergic signalling by rapid re-uptake of neuronally released norepinephrine (NE) into presynaptic terminals. NET exerts a fine regulated control over NE-mediated behavioural and physiological effects including mood, depression, feeding behaviour, cognition, regulation of blood pressure and heart rate. NET is a target of several drugs which are therapeutically used in the treatment or diagnosis of disorders among which depression, attention-deficit hyperactivity disorder and feeding disturbances are the most common. Individual genetic variations in the gene encoding the human NET (hNET), located at chromosome 16q12.2, may contribute to the pathogenesis of those diseases. An increasing number of studies concerning the

identification of single nucleotide polymorphisms in the hNET gene and their potential association with disease as well as the functional investigation of naturally occurring or induced amino acid variations in hNET have contributed to a better understanding of NET function, regulation and genetic contribution to disorders. This review will reflect the current knowledge in the field of NET from its initial discovery until now.

Keywords Norepinephrine transporter · Re-uptake · Storage · Synthesis · Disease · Genetic variation

1 Introduction

The neurotransmitter norepinephrine (NE), released from noradrenergic neurons of the peripheral (PNS) or central nervous system (CNS), is rapidly removed from the synaptic cleft by means of the NE transporter (NET) located in the plasma membrane of noradrenergic neurons. After re-uptake, NE is then either deaminated by mitochondrial monoamine oxidase (MAO) or taken up into the storage vesicles by means of the vesicular monoamine transporter 2 (VMAT2). The re-uptake of NE ensures fast termination of synaptic transmission; this fast inactivation enables NET to exert a fine control over its effector system.

The neuronal NE uptake system was first detected in the periphery. The availability of radiolabelled catecholamines enabled Axelrod and co-workers (Axelrod et al. 1959, 1961) to examine the fate of these amines after intravenous injection in laboratory animals. They observed a selective accumulation of radiolabelled epinephrine (EPI) and NE in sympathetically innervated organs (e.g. spleen and heart) which was dependent on intact sympathetic nerve terminals and which was inhibited by cocaine.

In 1963 Iversen described in more detail some pharmacological properties of the neuronal uptake of tritiated NE by the isolated perfused rat heart (Iversen 1963). After the discovery of a further and completely different extraneuronal NE uptake process, the neuronal NE uptake system was designated as "uptake1" and the extraneuronal transport system as "uptake2" (Iversen et al. 1965). The latter has come to be known as EMT (extraneuronal monoamine transporter) or OCT3, a member of the organic cation transporter (OCT) family. Since catecholamines are largely protonated at physiological pH (Mack and Bönisch 1979), they do not readily cross the blood–brain barrier; thus, it was not surprising that only small amounts of radiolabelled catecholamines were detected in the brain in the early experiments by Axelrod and co-workers.

In 1969 uptake of EPI and NE was described for the first time in brain tissues, namely in homogenates (Coyle and Snyder 1969) and in synaptosomes (Snyder and Coyle 1969), i.e. in brain nerve terminals isolated with a newly available method. By means of (1) radiolabelled NE, (2) the usage of synaptosomes, (3) slices of sympathetically innervated tissues, and (4) isolated perfused organs

(e.g. hearts), a great deal of our present knowledge about the physiological and pharmacological properties of the neuronal uptake of NE—such as the dependence on sodium and chloride ions, reversal of transport by indirectly acting sympathomimetic amines or its inhibition by a variety of drugs—was established (for reviews see e.g. Iversen et al. 1967; Paton 1979; Bönisch and Trendelenburg 1988; Trendelenburg 1991).

With the availability of tritiated desipramine (DMI), a tricyclic antidepressant which specifically inhibits the NET, and subsequent binding studies using this radioligand (Langer et al. 1981; Raisman et al. 1982) or the selective NET blocker nisoxetine (Tejani-Butt 1992)—as well as the identification of clonal cell lines natively expressing the NET, such as PC12 and SKN-SH cells—it was possible to examine the NE transport system at a more molecular level (for review, see Bönisch and Brüss 1994).

A milestone in the field of NET research was the expression cloning of the human NET (hNET) from complementary DNA (cDNA) originating from the human neuroblastoma line SKN-SH by Amara and co-workers (Pacholczyk et al. 1991). This was possible due to the availability of new techniques in molecular biology. The knowledge of the cDNA sequence of the hNET and that of the related transporter for γ-aminobutyric acid (GABA) (Guastella et al. 1990) enabled cDNA cloning of the related transporters for dopamine (DAT) and serotonin (SERT) and of species homologues of the NET (Lingen et al. 1994; Brüss et al. 1997).

A further step forward was the construction of NET/DAT transporter chimeras (Buck and Amara 1994; Giros et al. 1994), chromosomal mapping of the hNET gene (Brüss et al. 1993) and the characterization of the genomic structure (Pörzgen et al. 1995) and promoter of the hNET gene (Meyer et al. 1998) and its naturally occurring hNET variants (Runkel et al. 2000), as well as the establishment of NET knockout (NET-KO) mice (Xu et al. 2000). These and further recent findings—such as the elucidation of rapid changes in transporter surface expression and new techniques to measure transport (Blakely et al. 2005) or the recent description of the crystal structure of a bacterial homologue of Na^+/Cl^--dependent neurotransmitter transporters (Yamashita et al. 2005)—resulted in new insights in the structure and function of the NET and in its role in physiology and disease summarized in this review.

2
Properties, Physiology and Pharmacology of the NET

2.1
Basic Properties and Mechanisms of Transport

Transport of the naturally occurring substrate NE by the NET is saturable and characterized by a half-saturation constant (K_m) of about 0.5 μM (Table 1).

Table 1 Substrates and inhibitors of the NET[a]

Substrates			
Catecholamines	(K_m, μM)	Other substances	(K_m, μM)
Dopamine (DA)	(∼0.1)	Tranylcypromine	(∼2.0)
Norepinephrine (NE)	(∼0.8)	Selegiline	(∼4.0)
Epinephrine (EPI)	(∼3.0)	Amezinium	(∼0.5)
Other amines		Bretylium	(∼10)
Metaraminol	(∼0.3)	Guanethidine	(∼4.0)
Tyramine	(∼0.5)	MIBG	(∼3.0)
Phenylethylamine	(∼0.5)	DSP-4	(∼3.0)
d-Amphetamine	(∼0.5)	Xylamine	(∼3.0)
Ephedrine	(∼3.0)	MPP^+	(∼2.0)
Serotonin (5-HT)	(∼20)	ASP^+	(∼2.0)
Inhibitors			
Antidepressants	(K_i, nM)	Other substances	(K_i, nM)
Desipramine	(∼4)	Nisoxetine	(∼4)
Nortriptyline	(∼8)	Sibutramine	(∼180)
Reboxetine	(∼8)	Atomoxetine	(∼1)
Maprotiline	(∼10)	RTI-55 (β-CIT)	(∼1)
Nomifensine	(∼10)	Cocaine	(∼900)

ASP^+, 4-(4-(dimethylamino)styryl)-*N*-methyl-pyridinium; MIBG, *m*-Iodobenzyl-guanidine; MPP^+, *N*-methyl-4-phenylpyridinium [a]K_m and K_i values are means of published values taken from: Iversen 1965; Paton 1976; Bönisch and Harder 1986; Schömig et al. 1988; Graefe and Bönisch 1988; Pacholczyk et al. 1991; Cheetham et al. 1996; Apparsundaram et al. 1997; Eshleman et al. 1999; Rothman and Baumann 2003; Schwartz et al. 2003; Mason et al. 2005; Owens et al. 1997; Tatsumi et al. 1997; Olivier et al. 2000

In hNET cDNA-transfected cells, Apparsundaram et al. (1997) demonstrated that dopamine (DA) was transported by the NET with an eightfold lower K_m and an about two-fold lower V_{max}, resulting in a fivefold higher V_{max}/K_m value for DA than for NE. The V_{max}/K_m value is a measure of effectiveness of transport (Graefe and Bönisch 1988) or an indicator of the catalytic efficiency of translocation. That DA is a better substrate of the NET than NE was also seen by Giros et al. (1994) and Buck and Amara (1994); however, no difference in the kinetic constants of uptake of NE and DA by the NET was observed by (Burnette et al. 1996). EPI, the third endogenous catecholamine, is transported by the NET with an about fourfold higher K_m (Table 1), an about twofold lower V_{max} and an about ninefold lower effectiveness than NE (Apparsundaram et al. 1997). Thus, the presence of a β-hydroxyl group is not essential for transport by the NET, whereas alkylation of the primary amino group reduces transport effectiveness, and isopropyl-NE (isoprenaline) seems not to be transported

by the NET but is an excellent substrate of the extraneuronal monoamine transporter (OCT3).

Introduction of a methyl group at the α-carbon of the side chain of catecholamines or phenylethylamines does not affect effectiveness of transport (Graefe and Bönisch 1988), since α-methyl-NE (the metabolite of the antihypertensive drug α-methyl-dopa) and metaraminol are well-transported NET substrates. Introduction of a methyl group at one of the phenolic hydroxy groups of catecholamines, as it occurs by *O*-methylation through catechol-*O*-methyl transferase (COMT) results in amines such as normetanephrine which are not transported by the NET. As shown in Table 1, the NET also transports many other amines such as serotonin (5-hydroxytryptamine, 5-HT) and the indirectly acting sympathomimetic amines tyramine, ephedrine and *d*-amphetamine. It has been questioned whether the lipophilic *d*-amphetamine is transported or whether it only competitively inhibits the NET; however, using tritiated *d*-amphetamine, specific uptake by the NET (in PC12 cells) has been clearly demonstrated (Bönisch 1984).

The list of "other transported compounds" includes drugs such as MAO inhibitors (tranylcypromine, selegiline, amezinium), adrenergic neuron-blocking agents (guanethidine, bretylium), meta-iodobenzylguanidine (MIBG), the covalently binding NET suicide substrates and noradrenergic neurotoxins DSP-4 and xylamine, the dopaminergic neurotoxin MPP^+ (which is also a substrate for the DAT and SERT), and the fluorescent model substrate ASP^+ (Table 1).

2.1.1 Co-substrates and Direction of Transport

Substrate transport by the NET is dependent on Na^+ and Cl^- and the Na^+-gradient (Na^+ outside high) across the plasma membrane is the main driving force, dictating the transport direction (normally from outside to inside; for review, see e.g. Masson et al. 1999; Rudnick 1997; Sonders et al. 2005). The inside negative membrane potential created by the K^+ gradient also contributes a driving force. Both ion gradients are maintained by the Na^+/K^+-ATPase, and inhibition of this enzyme (by e.g. ouabain) or a lack of ATP causes a suppression of NE uptake and a reversal of the transport direction (see below), a phenomenon observed e.g. in ischemia (Schömig et al. 1991). Outward transport can experimentally also be induced by an increase of intracellular Na^+ evoked by e.g. the sodium channel opener veratridine (Graefe and Bönisch 1988, Chen et al. 1998) or by a reduction of extracellular Na^+ (Graefe and Bönisch 1988; Pifl et al. 1997). In the latter situation, monovalent cations other than Na^+ are not able to take over the unique role of Na^+ (Graefe and Bönisch 1988); this seems to hold true also for the DAT and SERT (Shank et al. 1987; Bryan-Lluka and Bönisch 1997).

Since both Na^+ and Cl^- change K_m and V_{max} of NE transport, and since both ions are needed for binding of competitive NET inhibitors such as de-

sipramine, we had proposed a transport model based on a single centre-gated pore mechanism with alternating access of the solute to the binding site. In this model, the empty and mobile carrier loses mobility by first binding (from the extracellular site with high Na^+) the co-substrate Na^+. In the next step, the substrate NE and the co-substrate Cl^- are bound, resulting in a regain of mobility. After translocation of substrate and co-substrates and dissociation at the inner face of substrate and co-substrates, the carrier returns in its unloaded state (to become "fixed" again by binding of extracellular Na^+) and thus catalyses net transport, or it returns in a substrate-loaded state catalysing exchange, i.e. carrier-mediated outward transport (Harder and Bönisch 1985; Bönisch and Trendelenburg 1988; Graefe and Bönisch 1988; Bönisch 1998). The model considers that NE is translocated as positively charged NE^+ and that coupling of NE^+ transport to co-transported ions occurs at a stoichiometry of 1 NE^+:1 Na^+:1 Cl^-. The turnover rate has been estimated to be between 1 and 2.5 transport cycles per second (Bönisch and Harder 1986; Gu et al. 1996). In our model, which is an extension of the "facilitated exchange diffusion model" proposed by Fischer and Cho (1979), the co-transported Na^+ facilitates substrate binding from the internal site, and thereby also facilitates carrier-mediated NE efflux by inward transport of substrates (together with Na^+) such as indirectly acting sympathomimetic amines (Bönisch 1986; Langeloh et al. 1987). In fact, substances which are NET (or DAT or SERT) substrates can be identified (and distinguished from transporter inhibitors) by their ability to induce outward transport (Bönisch and Trendelenburg 1988; Wölfel and Graefe 1992; Burnette et al. 1996; Chen et al. 1998).

The extended model of facilitated exchange diffusion is also supported by the fact that (1) a preload of noradrenergic neurons (Graefe and Bönisch 1988) or of NET-expressing cells (Chen et al. 1997) with a NET substrate causes saturation of carrier-mediated efflux and (2) IC_{50} or K_i values for competitive inhibition of NE uptake by NET substrates are identical (or very similar) to EC_{50} values for induction of carrier-mediated efflux (Bönisch and Trendelenburg 1988; Chen et al. 1997).

Although our model is compatible with the crystal structure and features of the recently described bacterial Na^+/Cl^--dependent leucine transporter related to Na^+/Cl^--dependent neurotransmitter transporters (Yamashita et al. 2005), questions still remain concerning e.g. the role of Na^+ for substrate binding and for its coupling to substrate transport as well as reversal of transport induced by amphetamine. Using real-time, spatially resolved analysis and the fluorescent substrate ASP^+, Schwartz et al. (2003, 2005) showed that substrate binding is in a narrow pore deep within the transporter and is independent of Na^+, and that binding rates by far exceed transport rates. The NET has also a channel mode (Galli et al. 1998), suggesting the appearance of an aqueous pore through the transporter protein during the transport cycle.

Amphetamine-induced reverse transport of DA by the DAT has recently been shown to occur by a slow carrier-like and a rapid channel-like mode involving protein kinase C (PKC)-mediated phosphorylation of the DAT (Kahlig et al. 2005). Furthermore, Seidel et al. (2005) provided evidence at a concatamer of the GABA transporter and SERT that substrate-induced efflux relies on sequential rather than concomitant counter-transport, and that the switch from concomitant to sequential mode is evoked by amphetamine-induced activation of PKC. It remains to be shown whether this holds true also for NET oligomers.

2.1.2 NET Inhibitors

As shown in Table 1, the NET is inhibited with potencies in the nanomolar range by various antidepressants (ADs) such as the tricyclic ADs desipramine and nortriptyline, the tetracyclic AD maprotiline, and the ADs nomifensine and reboxetine. Reboxetine is the most selective NET inhibitor among all of these drugs, while desipramine is the most selective among the tricyclics. Other high-affinity NET inhibitors listed in Table 1 are nisoxetine, atomoxetine [the (−)isomer of tomoxetine], sibutramine, cocaine and the cocaine analogue RTI-55 (also known as β-CIT). Among these compounds, nisoxetine is a very selective NET inhibitor (Tejani-Butt 1992); this holds true also for atomoxetine (Tatsumi et al. 1997; Bymaster et al. 2002), whereas sibutramine also inhibits the SERT (Luque and Rey 1999), while cocaine and RTI-55 additionally block the DAT and the SERT (Eshleman et al. 1999).

The high-affinity NET inhibitors desipramine and nisoxetine are experimentally used as radioligands to label the NET (e.g. in autoradiography studies) and to examine the density of transporter sites in tissues and cells as well as the binding properties of the NET, and inhibition of radioligand binding can be utilized to examine affinities of unlabelled compounds to the NET (Bönisch and Harder 1986; Michael-Hepp et al. 1992; Tejani-Butt 1992; Graham and Langer 1992, and see references cited in the legend to Table 1). These studies showed that binding to the NET of desipramine and nisoxetine is dependent on Na^+ and Cl^- and is displaced by NET substrates; this was interpreted to indicate a common binding site for substrates and inhibitors at the NET. However, differences in sodium dependence, in K_i values for inhibition of NE uptake and for inhibition of radioligand binding by NET substrates, as well as differences in sensitivity to exchanges of certain amino acids of the NET, indicate that these NET inhibitors bind to a site at the NET which is not identical with the substrate recognition site but may overlap with that site. It should be noted that, in the micromolar range, NET inhibitors also interact with ligand-gated ion channels such as the nicotinic acetylcholine (nACh) receptor (Hennings et al. 1999) or the 5-HT_3 receptor (Eisensamer et al. 2003).

2.2 Physiological Importance and Knockout of the NET

NE is an important neurotransmitter in the PNS and CNS. The major noradrenergic nucleus in the brain is the locus coeruleus (LC) in the brain stem. Projections from the LC innervate virtually all areas of the brain including the spinal cord. Projections to the prefrontal cortex (an area involved in drive and motion) and the hippocampus (involved in learning and memory) may play an important role in depression. LC activity is sensitive to environmental stimuli and changes in internal homeostasis. LC activity is involved in flight-or-fight responses, arousal, sleep–wake cycle and modulation of the sympathetic nervous system, including pulse rate and blood pressure. LC firing with NE release potentiates the firing of dopaminergic cells in the ventral tegmental area that project to the limbic forebrain, and projections to raphe nuclei influence the firing rate of 5-HT neurons. Thus, because of these interactions between the brain stem modulatory systems, any (pharmacological) intervention directed toward one system will most likely lead to changes within the others (for review, see Ressler and Nemeroff 1999; Anand and Charney 2000).

The physiological importance of the NET located on noradrenergic nerve terminals is the re-uptake of released NE; thus, the NET is important for the fine-tuning of the noradrenergic neurotransmission. The NET, which also transports DA (as mentioned already in Sect. 2.1), has been shown to be also implicated in the clearance of DA in brain regions with low levels of the DAT (Burnette et al. 1996; Carboni and Silvagni 2004; Moron et al. 2002).

The targeted disruption of the NET gene in NET-KO mice (Xu et al. 2000) has provided an opportunity to examine a NET defect in vivo. NET-KO mice show profound alterations in NE homeostasis. In prefrontal cortex, hippocampus and cerebellum the NE level was up to 70% lower than in wild-type mice, and the clearance rate of released NE was at least sixfold slower, indicating that diffusion becomes the main mechanism for NE clearance in NET-KO mice (Xu et al. 2000). The depletion of intraneuronal stores, in spite of increased activity of tyrosine hydroxylase (Xu et al. 2000) underlines the importance of the NET in the maintenance of a physiologically high intraneuronal NE content and in the prevention of volume transmission by NE escaping (by diffusion) from the synaptic cleft to reach more distant brain areas.

In NET-KO mice, α_1-adrenoceptor expression was decreased in the hippocampus (Xu et al. 2000) as well as in other brain regions (Dziedzicka-Wasylewska et al. 2006). On the other hand, in the spinal cord, expression of α_2-adrenoceptor tended to increase and morphine-induced analgesia was increased (Bohn et al. 2000), whereas in several other brain regions α_2-adrenoceptor expression was clearly elevated in NET-KO mice (Gilsbach et al. 2005). This is in accordance with an increased modulation of NE release by presynaptic α_2-adrenoceptor observed by Vizi et al. (2004) in NET-KO mice. In antidepressant tests, NET-KO mice behaved like antidepressant-treated wild-

type mice. Furthermore, in NET-KO mice no additional effects of paroxetine and bupropion, antidepressants interacting primarily with the SERT and DAT, respectively, were observed (Xu et al. 2000). NET-KO mice showed enhanced responses to the psychostimulant cocaine, which correlated with suppression of presynaptic DA function and supersensitivity of postsynaptic D2 and D3 receptors (Xu et al. 2000). A reduced effectiveness of cocaine for inhibition of DA uptake in the nucleus accumbens of NET-KO mice has been interpreted as an indication that DA uptake in this brain region might primarily depend on the NET (Gainetdinov et al. 2002). On the other hand, after selective inhibition of the NET by reboxetine, results of microdialysis experiments indicate that NE is taken up and stored in striatal dopaminergic neurons (Gobert et al. 2004). That the DAT accepts NE as substrate had already been shown previously (Buck and Amara 1994; Giros et al. 1994). In NET-KO mice it was furthermore shown that NE is also taken up and stored in serotonergic neurons (Vizi et al. 2004). Uptake and storage of monoamines by heterologous monoamine transporters may contribute to effects at other monoaminergic targets of antidepressants which selectively inhibit a defined monoamine transporter.

NET-KO mice show reduced locomotor responses to novelty; they also show reduced temperature and reduced body weight (Xu et al. 2000), indicating that NE and the NET may be involved in their regulation. In addition, NET-deficient mice exhibit decreased vulnerability to seizure (Kaminski et al. 2005), and they show excessive tachycardia and elevated blood pressure with wakefulness and activity (Keller et al 2004).

In the periphery, the NET is not only expressed at noradrenergic nerve endings but also in the placenta. In the human placenta, the NET, together with further monoamine transporters (SERT, VMAT2 and OCTs) keeps low the concentration of circulating vasoactive monoamines as a protective mechanism preventing vasoconstriction in the placental vascular bed and thereby securing stable blood flow to the fetus (Bottalico et al. 2004).

3 Tissue Expression

The expression of the mRNA of the NET is, like the mRNAs for other monoamine transporters such as the DAT or the SERT, localized to monoaminergic cell bodies rather than to nerve terminals (Povlok and Amara 1997; Backs et al. 2001). Furthermore, mRNA expression is generally restricted to cells that synthesize the corresponding monoamine. Thus, in the brain, NET mRNA expression is an indicator for noradrenergic pathways with cell bodies primarily located in the brain stem, and there in the locus coeruleus complex in the dorsal pons, especially in the nucleus locus coeruleus proper (Lorang et al. 1994; Eymin et al. 1995). NET mRNA is additionally expressed (together with dopamine-β-hydroxylase mRNA) in the lateral tegmentum of the medulla and

pons. All these regions encompass most of the noradrenergic cell bodies in the CNS.

Since noradrenergic neurons originating from the locus coeruleus project to many different brain regions, the NET can influence many neural pathways involved in e.g. autonomic and neuroendocrine regulation, arousal, attention, and complex behaviours that are associated with affect, emotion and depression. In the periphery, NET mRNA is expressed in sympathetic ganglia, in the adrenal medulla and the placenta. No mRNA could be detected in peripheral noradrenergic nerve endings (Ungerer et al. 1996; Backs et al. 2001; Li et al. 2001), indicating that NET mRNA is expressed only in noradrenergic cell bodies in which the synthesis of the transporter protein also takes place. The NET protein is expressed at noradrenergic cell bodies, dendrites, axons and nerve endings, and obviously not directly in the active zones of synapses but more laterally; this has at least been demonstrated for the closely related DAT (Nirenberg et al. 1996; Schroeter et al. 2000).

NET protein can be detected either functionally by its transport activity or by specific labelling of the NET by means of specific anti-NET antibodies (Brüss et al. 1995; Savchenko et al. 2003), or by radioligand binding using specific and high-affinity NET inhibitors such as desipramine or nisoxetine (Raisman et al. 1982; Michael-Hepp et al. 1992; Tejani-Butt 1992; Sucic et al. 2002; Distelmaier et al. 2004). NET protein expression was demonstrated in the brain not only in noradrenergic somata but also at dendrites and axons of noradrenergic fibres, e.g. within the cortex and hippocampus (Tejani-Butt 1992; Hoffman et al. 1998; Schroeter et al. 2000), and in the periphery in the plasma membrane of sympathetic ganglia and noradrenergic nerve terminals within sympathetically innervated tissues such as heart and blood vessels (Eisenhofer 2001).

Examination of ontogeny of NET expression showed that the NET appears early in the young embryo (Sieber-Blum and Ren 2000; Ren et al. 2003), and its postnatal expression in the brain stem changes during maturation (Sanders et al. 2005). The NET protein is also expressed in neuroendocrine tumour cell lines such as rat PC12 pheochromocytoma cells or the human neuroblastoma cell line SKN-SH and in some non-neuronal cells, e.g. in endothelial cells of small vessels of the lung, fibroblast-like cells of the dental pulp, myometrial cells, syncytiotrophoblasts and in cultured glial cells (Bönisch and Brüss 1994). However, NET does not seem to be expressed in situ in glia (Hoffman et al. 1998).

4 Regulation of NET Function and Expression

The NET (or its function) can be regulated acutely or chronically, and regulation may be due to changes in gene transcription, mRNA translation or stability,

posttranslational modifications such as phosphorylation, protein trafficking, cytoskeleton interaction and oligomerization (Zahniser and Doolen 2001).

The membrane turnover rate of the NET is not known, but it may be relatively long if one assumes that it is similar to that of the closely related DAT whose turnover rate, determined by intraventricular administration of an irreversible DAT inhibitor, was characterized by a half-life of about 2 days (Kimmel et al. 2000). Long-term NET regulation by de novo synthesis may be relevant during long-term blockade of the NET by e.g. NET-inhibiting antidepressants. Thus, repeated administration of desipramine was shown to produce up-regulation of NET mRNA in the rat locus coeruleus (Szot et al. 1993), and up-regulation of the NET protein in placenta was observed during treatment of rats with cocaine (Shearman and Meyer 1999). However, in an autoradiographic study using tritiated nisoxetine, repeated administration of desipramine was shown to cause a reduction in the number of nisoxetine binding sites in rat hippocampus, amygdala and thalamus but not in other regions such as frontal cortex, hypothalamus or locus coeruleus (Bauer and Tejani-Butt 1992). In addition, in a recent and similarly designed study, no regulation of brain nisoxetine binding sites was observed (Hebert et al. 2001).

In in vitro studies on PC12 cells constitutively expressing the rat NET (rNET), prolonged exposure to desipramine or nisoxetine reduced the number of nisoxetine binding sites and produced a parallel reduction in NE uptake (Zhu and Ordway 1997). A similar antidepressant-induced down-regulation of the NET was also observed in HEK293 cells stably expressing the hNET (Zhu et al. 1998); this down-regulation was not accompanied with changes in NET mRNA levels. Similar results were also obtained in several other studies (for review, see Zahniser and Doolen 2001). The mechanism underlying this antidepressant-induced NET regulation remains unclear. However, Zhu and co-workers recently showed that it could also represent an experimental artefact, at least when expression is measured by radioligand binding, since antidepressants such as desipramine cause long-term NET occupancy by persistent membrane retention (Zhu et al. 2004; Ordway et al. 2005). Long-term regulation of the NET in cultured cells induced by extremely high concentration of NET substrates reported in the literature may also largely be due other effects such as toxic side effects (for details see: Zahniser and Doolen 2001). Administration to rats of reserpine, an inhibitor of VMAT2, was shown not only to increase tyrosine hydroxylase expression but also to decrease NET mRNA expression in locus coeruleus and adrenal medulla (Cubells et al. 1995), indicating a certain role of NET substrates in the regulation of the NET.

In cultured neurons from superior cervical ganglia of newborn rats, NET expression is reduced (concomitantly with tyrosine hydroxylase expression) by long-term cell treatment with the cholinergic differentiation factor/leukaemia inhibitory factor and ciliary neurotrophic factor, neurokines known to induce a switch from adrenergic to cholinergic phenotype, whereas retinoic acid increased NET expression (Matsuoka et al. 1997). In cultured neurons of the rat

adrenal medulla, NET expression was decreased by long-term treatment with the glucocorticoid dexamethasone but increased by nerve growth factor (NGF) (Wakade et al. 1996). However, in PC12 cells, long-term exposure to NGF reduced NE uptake and mouse NET expression (Ikeda et al. 2001). Long-term exposure to insulin, another growth factor, has been reported to cause a reduction in NET mRNA expression in the locus coeruleus (Figlewicz et al. 1993). In cultured quail neural crest cells, fibroblast growth factor, neurotrophin-3 and transforming growth factor-β1 caused an increase in NET mRNA expression and function (Ren et al. 2001). In cultured sympathetic neurons, the inflammatory cytokine cardiotrophin-1 was shown to decrease NET mRNA expression and NET function (Li et al. 2003).

Acute and rapid changes in NET function were observed by B- and C-type natriuretic peptides which both caused enhanced NE uptake in rat hypothalamus and adrenal medulla (Vatta et al. 1996, 1997). These peptides activate specific membrane-bound guanylyl cyclase receptors, thus modulating cellular functions via the intracellular second messenger cyclic guanosine monophosphate (cGMP) and activation of protein kinase G. However, neither short-term nor long-term cGMP elevation had an effect on NE transport in rat PC12 and human SKN-SH cells constitutively expressing the NET or in cells transfected with human or rat NET cDNA (Bryan-Lluka et al. 2001). In the same study, elevation of cyclic AMP (cAMP), which activates protein kinase A, also had no effect in these cell systems, except at PC12 cells, where short-term cAMP elevation caused a decrease in NE uptake and long-term elevation additionally evoked a decrease in mRNA expression, indicating a cell type-specific regulation (Bryan-Lluka et al. 2001).

We were the first who showed that phorbol ester-induced activation of PKC causes a relatively fast reduction in NE uptake and membrane expression (measured by nisoxetine binding) in cells constitutively expressing the NET (SKN-SH) and in hNET cDNA-transfected cells. We also showed that this rapid down-regulation was observed in transfected cells expressing an hNET Ser^{259}Ala mutant in which the canonical PKC phosphorylation site had been destroyed (Bönisch 1998). PKC activation-induced down-regulation of the NET (in SKN-SH cells) by activation of muscarinic receptors was shown by Blakely and co-workers, who also demonstrated that this effect was due to NET internalization, a new type of trafficking-dependent regulation of monoamine transporters (Apparsundaram et al. 1998a; 1998b; Blakely et al. 2005).

PKC-triggered reduction in NET surface expression was recently shown not to involve dynamin- or clathrin-mediated internalization but to occur via lipid rafts, and thus to be similar to the regulation of the glucose transporter GLUT4 (Jayanthi et al. 2004). Interestingly, insulin had been shown to cause in SKN-SH cells a rapid stimulation of NE uptake with an increase in V_{max} but without an alteration of K_m or NET surface expression (Apparsundaram et al. 2001). The insulin-mediated stimulation of intrinsic NET activity was dependent on extracellular calcium and on p38 mitogen-activated protein kinase (p38

MAPK) and phosphatidylinositol 3-OH kinase (PI3K); this study also provided evidence that NET surface expression under basal conditions is dependent on a PI3K-linked pathway (Apparsundaram et al. 2001). In hypothalamus-brainstem neuronal cultures from rat brains, angiotensin II (Ang-II) causes both acute and chronic stimulation of NE uptake.

Long-term Ang-II-mediated activation of AT1 receptors induces an up-regulation of NET mRNA and enhanced NE uptake through activation of the Ras-Raf-MAP kinase, including nuclear signalling via SRE (serum-response enhancer element) and AP-1 (activator protein-1), whereas in neurons from SHR (spontaneous hypertensive rats), signalling through PI3K (including protein kinase B and AP-1) contributes to the stimulation of NET gene transcription (Yang and Raizada 1999). Acute Ang-II-mediated increase in NE uptake is a posttranscriptional event (Lu et al. 1996) leading to a rapid increase in NET surface expression, an effect also observed after brief potassium-induced depolarization (Savchenko et al. 2003; Blakely et al. 2005). Thus, the movement of the NET [and of the other monoamine transporters (Torres et al. 2003)] to and from the plasma membrane provides a new and interesting regulation of the transport capacity. This process seems to be supported by a close localization of surface NETs to synaptic vesicle pools (Kippenberger et al. 1999; Blakely et al. 2005). In this rapid regulation of NET surface expression, several proteins associated with plasma membranes and storage vesicles have been shown to be involved, including: syntaxin 1A, a presynaptic soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein, which colocalizes with the NET (Sung et al. 2003) and protein phosphatase 2A anchoring subunit, as well as 14-3-3 proteins (Sung et al. 2005).

Finally, acute regulation of the NET (as for all other Na^+/Cl^--dependent transporters) occurs also through the alteration of the electrical and chemical gradients that drive substrate transport.

5
Structure–Function Relationship

The NET, DAT and SERT belong to the family of Na^+/Cl^--dependent monoamine transporters. Hydropathy analysis of the deduced amino acid sequences suggests the presence of 12 putative transmembrane domains (TMs), intracellular localization of the amino and carboxyl termini and a large extracellular loop positioned between TM3 and TM4 with several potential *N*-glycosylation sites (Fig. 1). A comparison of the primary sequences of the human (h) monoamine transporters demonstrates 80% and 69% homology between hNET and hDAT and hDAT and hSERT, respectively. Figure 1 indicates amino acids conserved between hNET and hDAT as well as those conserved between all three transporters. This topological model has been confirmed for the NET (Brüss et al. 1995) and also for the DAT and SERT (see Torres

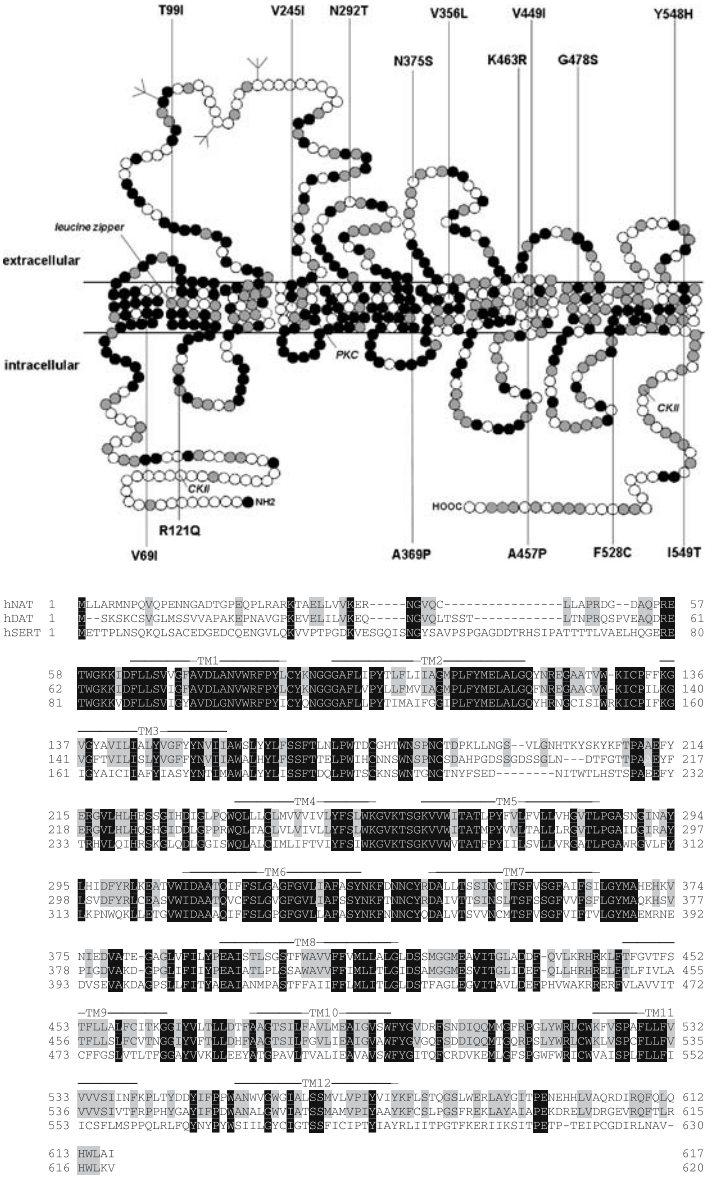

Fig. 1 Proposed topology of the human NET depicting 12 transmembrane domains (TMs) (*upper part*), and alignment of the human NET with the human DAT and human SERT (*lower part*). The *upper part* also indicates naturally occurring hNET variants, a leucine zipper in TM2, *N*-glycosylation sites within the second extracellular loop and intracellular residues which are potential sites for phosphorylation by protein kinase C (*PKC*) or casein kinase II (*CKII*). Amino acids conserved between hNET and hDAT are indicated in both parts in *grey* and those conserved between all three transporters in *black*

et al. 2003). Two cysteine residues located in the large extracellular loop are conserved among all members of the family; they may form a disulfide bond thus maintaining a functional transporter conformation (Povlok and Amara 1997). TM2 contains several conserved leucine residues resembling a leucine zipper, which may be implicated in mediating protein–protein interactions.

Residues and domains important for substrate binding and translocation have been identified by means of transporter chimeras, accessibility of substituted cysteines, and site-directed mutagenesis. Using chimeric constructs between NET and DAT, Giros et al. (1994) reported that regions from the amino-terminal through the first five TMs are likely to be involved in the uptake mechanism and ionic dependence. They postulated that regions within TMs 6–8 determine tricyclic antidepressant binding and cocaine interactions, and they said that the carboxyl-terminal region encompassing TM9 to the C-terminal tail appears to be responsible for the stereoselectivity and high affinity for substrates. However, Buck and Amara (1994), using similar chimeras to examine domains influencing substrate selectivity and translocation, proposed that the region from TM4 to TM8 is involved in substrate translocation.

The region between TM5 and TM8 of the NET has been examined in more detail for residues important for desipramine binding, using hNET mutants in which amino acids not conserved in the desipramine-insensitive hDAT were exchanged against those of the hDAT. Replacement in TM8 of serine (in position 399) and glycine (in position 400) against proline and leucine, respectively, resulted in an about 3,000-fold reduction in desipramine affinity without a change in the affinities for NE, DA and cocaine (Roubert et al. 2001). In a search for residues involved in binding and translocation of substrates and co-substrates, we exchanged residues in TM1 (Trp80 and Arg81) and TM2 (Glu113) of the hNET, which are absolutely conserved in all Na^+/Cl^--dependent neurotransmitter transporters including the glycine transporter (GLYT) and GABA transporter (GAT). In the rat GAT, replacement of these residues had been shown to cause an almost complete loss of transport without a change in membrane expression (Pantanowitz et al. 1993; Kleinberger-Doron and Kanner 1994; Keshet et al. 1995). None of the three hNET mutants (Trp80Ser, Arg81His and Glu113Asp) exhibited NE transport (Bönisch et al. 1999), indicating that the positive charged arginine may be involved in Cl^- binding, while tryptophan and the negatively charged glutamate are involved in binding of Na^+. In addition, several other glutamate residues conserved in monoamine transporters (beside Glu113) have been identified as being of functional importance in the hNET (Sucic et al. 2002). Mutation of an aspartate residue in TM1 (Asp79 in hNET), which is highly conserved in all monoamine transporters, abolished transport activity of hNET and hSERT (Bönisch et al. 1999; Barker et al. 1999), indicating that its carboxyl group may interact with the positive charge of the amine group of monoamines. For the DAT, further aspartate residues (Asp13, Asp435 and Asp476) have been shown to play a potential role in substrate recognition (Chen et al. 2001).

N-Glycosylation of asparagine residues in the large extracellular loop of the NET has been shown to be important for hNET protein stability, surface trafficking, and transport activity but not for ligand recognition (Melikian et al. 1996). There are still further conserved amino acids which may play a potential role in substrate and inhibitor binding (see Torres et al. 2003b).

Monoamine transporters might exist as oligomers (Sitte and Freissmuth 2003). In the DAT, mutation of the leucine zipper in TM2 was shown to abolish transporter delivery to the plasma membrane and interaction with wild-type

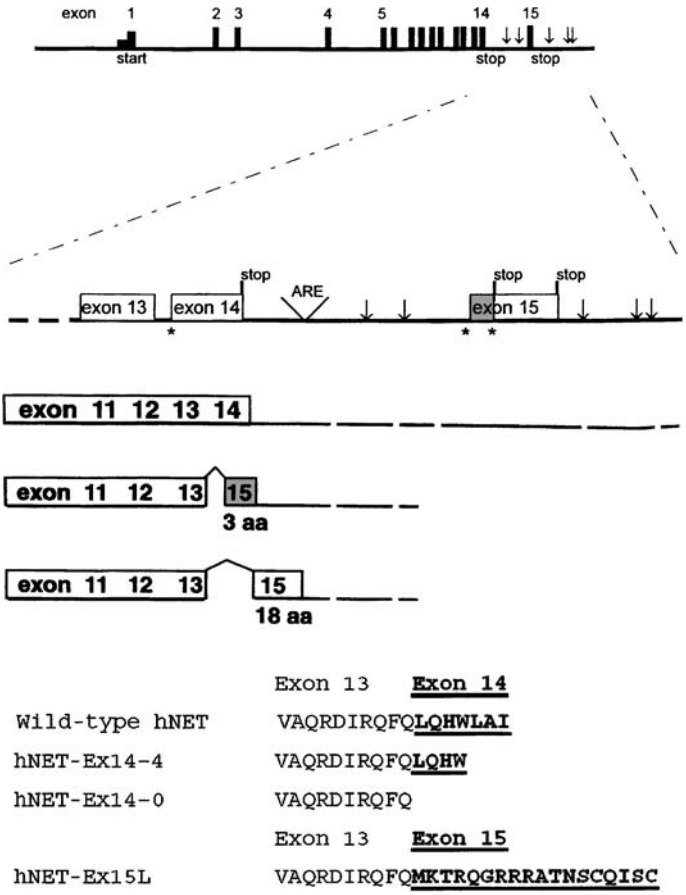

Fig. 2 Schematic representation of the hNET gene organization and of alternative splicing in the 3′ region of hNET transcripts. *Upper part* of Fig. 2, hNET gene structure and alternative splicing; *black vertical bars*, exons 1–15; *start*, translational start codon; *stop*, translational stop codon; *ARE*, adenylate-rich region; *arrows*, polyadenylation signals; *aa*, amino acid. *Lower part* of Fig. 2, deduced C-terminal amino acid sequences of wild-type hNET and two artificial (*Ex14-4* and *Ex14-0*) C-terminal hNET variants as well as the long (*Ex15L*) hNET splice variant (see Distelmaier et al. 2004 for further information)

DAT; therefore, it has been proposed that TM2 (of the DAT) is important for transporter assembly and oligomerization is essential for cell surface trafficking (Torres et al. 2003a).

An interaction between the PDZ domain-containing protein PICK1 and the amino acid motif Leu-Ala-Ile in the carboxyl termini of DAT (Torres et al. 2001) and NET (Distelmaier et al. 2004) has recently been shown to play an important role in transporter trafficking. A lack of this motif (in an hNET splice variant, see Fig. 2) or a mutation of these amino acids caused a strong reduction in hNET surface expression. In addition, the expression of the mutant hNET exerted a dominant-negative effect on plasma membrane expression of the wild-type hNET, indicating a physiological role of PICK1 interaction in the regulation of hNET surface expression. Since PICK1 has been shown to interact with PKC, it may also be involved in PKC-mediated trafficking of monoamine transporters.

6
Gene Structure, Promoter and Alternative Splicing

The human NET is encoded by a single gene which has been localized to chromosome 16q12.2 by somatic panel hybridization of fluorescence in situ hybridization (Brüss et al. 1993; Gelernter et al. 1993). The hNET gene consists of 14 coding exons and spans about 45 kb (Pörzgen et al. 1995). Downstream of exon 14, five consensus polyadenylation sites and a new coding exon (exon 15) which can be alternatively (by skipping exon 14) spliced to exon 13 have been identified. Due to alternative usage of two splice acceptor sites in exon 15, either 3 or 18 amino acids will be expressed at the C-terminus of hNAT following exon 13 (Pörzgen et al. 1998) (Fig. 2).

Functional expression of the long exon 15 splice variant (hNET-Ex15L) and two artificial hNET variants in which either three or all seven amino acids of exon 14 were deleted showed that all variants exhibited no difference in comparison to the wild-type hNET concerning the K_m of NE uptake and K_d of nisoxetine binding, but all variants were affected by reduced V_{max} of NE transport and a diminished B_{max} of nisoxetine binding (Distelmaier et al. 2004). The short hNET variant containing only three amino acids encoded by exon 15, and thus lacking the PICK1 recognition-motif, has been shown to be functionally inactive (Kitayama et al. 2001).

In addition to the coding exons, a noncoding exon 0 has been identified in the 5′-region of the hNET gene which is preceded by the promoter region (Meyer et al. 1998; Wiedemann et al. 1998; Kim et al. 1999). By primer extension and 5′ rapid amplification of cDNA ends (RACE) experiments, it was found that exon 0 is located from −343 to −765 upstream from the translation start codon. Through alternative splicing in exon 0, two transcripts which differ by 183 base pairs may be generated. The promoter of the hNET gene is located upstream of the transcription start site at −765 with respect to the translation

start site. Functional analysis by means of luciferase assays of a 4-kb promoter fragment revealed a robust promoter-driven expression of the reporter gene only in hNET-expressing cells (SKN-SH) but not in hNET-negative JAR or COS-7 cells. The basal promoter is located in a short stretch (123 bp) upstream of the transcription start site. A cAMP response element (CRE) located upstream of the core promoter confers elevated hNET expression in a cAMP-dependent manner (Meyer et al. 1998). Kim et al. (1999) reported that when intron 1 (located between exon 0 and exon 1) is included in reporter gene constructs of hNET gene 5′ constructs, this intron confers elevated expression of reporter constructs. The highest promoter activity was found when a 9-kb upstream fragment including intron 1 was used to transfect SK-N-BE(2)C cells. Later it was shown that an E-box motif within intron 1 is a least partly responsible for the promoter-enhancing activity of hNET intron 1 in hNET-positive and hNET-negative cell lines (Kim et al. 2001).

7
hNET: Significance in Disease, Therapy and Diagnosis

7.1
Genetic Variations

In the CNS, NE is involved in the regulation of mood, sleep, behaviour and the central control over the endocrine and sympathetic system (Young and Landsberg 1998).

Since the hNET is responsible for NE clearance in the periphery and in CNS, variations in the coding sequence affecting functional properties of the transporter or noncoding variations leading to elevated or decreased expression levels of hNET may be associated with a variety of physiological/pathophysiological consequences. The CNS hNET is a target of tricyclic antidepressants, and selective NE re-uptake inhibitors (SNRIs) are being used as antidepressants and in the therapy of attention deficit hyperactivity disorder (ADHD)—as well as in eating disorders. It is also notable that central hNETs are affected by addicting drugs such as cocaine and amphetamines (see Sect. 1). It is therefore not surprising that more studies than ever are concerned with the identification of single nucleotide polymorphisms (SNPs) or other genetic variations in the hNET gene of varying psychiatric-patient collectives and with corresponding association studies (see Table 2 and also Fig. 1).

The same is true for the periphery with respect to several autonomic dysfunctions such as orthostatic intolerance syndromes, essential hypertension and congestive heart failure, which are related to disturbances of the homeostasis of the sympathetic nervous system. Sympathetic homeostasis mainly depends on a fine-regulated concerted action of NE synthesis, release and re-uptake into sympathetic nerve endings or other tissues endowed with hNET as

Table 2 Naturally occurring amino acid variations of hNET as deduced from hNET gene SNPs

Variant	Position (aa)	Functional effect	Known association to disease	rs-number	Reference(s)
Asn → Lys	7	nd	None	rs11568323	1
Ala → Pro	31	nd	None	rs13306039	2
Val → Ile	69	None	None	rs1805064	3, 4
Thr → Ile	99	None	None	rs1805065	3, 4
Arg → Gln	121	TR ↓ ; SF ↓	None	rs13306041	5, 6
Asn → Lys	146	nd	None	na	1
Val → Ile	160	nd	None	na	1
Thr → Arg	193	nd	None	na	1
Val → Ile	244	None	None	na	6
Val → Ile	245	None	None	rs1805066	3, 4
Val → Ile	247	nd	None	rs11568341	7
Thr → Arg	283	nd	None	rs11568325	7
Asn → Thr	292	TR ↓; SF ↓	None	rs5563	6, 8
Val → Leu	356	None	None	rs5565	6, 8
Ala → Pro	369	TR ↓↓; SF nd	None	rs5566	6, 8
Asn → Ser	375	None	None	rs5567	6, 8
Val → Ile	449	None	None	rs2234910	3, 4
Ala → Pro	457	TR ↓↓; SF ↓↓	OI	Swiss:Var 010022	9
Lys → Arg	463	None	None	rs5570	6, 8
Gly → Ser	478	NE-K_m ↑	None	rs1805067	3, 4
Phe → Cys	528	TR ↑; SF ↑	None	rs5558	6, 8
Tyr → His	548	TR ↓; SF ↓	None	rs5559	6, 8
Ile → Thr	549	SF ↓	None	rs3743788	6, 10

Abbreviations: aa, amino acid position in hNET protein; na, not available; nd, not determined; NE-K_m, Michaelis–Menten constant for NE-uptake; OI, orthostatic intolerance; SF, surface expression; TR, NE transport rate; References: 1, http://www.pharmgkb.org/; 2, the Japan metabolic disease database (JMDBase); 3, Stöber et al. 1996; 4, Runkel et al. 2000; 5, Iwasa et al. 2001; 6, Hahn et al. 2005; 7, http://www.mutdb.org/; 8, Halushka et al. 9; Shannon et al. 2000; 10, NCBI-SNP-database (dbSNP)

the adrenal medullary (for review, see Blakely 2001). Of course, not only hNET variations but also other variations in the genes encoding adrenergic receptors or NE synthesizing as well as metabolizing enzymes and related genes encoding proteins interfering with the signalling pathways of adrenergic receptors may play a role in the often complex and multigenic genesis of such diseases.

Table 2 gives an overview of the currently identified hNET-gene nonsynonymous coding variations and their proven or putative functional consequences and possible associations to diseases, of which some are discussed in more detail elsewhere in this volume.

7.2 NET and Dysautonomia

The autonomic nervous system, which is divided into a sympathetic and a parasympathetic part, is responsible for the maintenance of body homeostasis. While the main transmitter of the parasympathetic system is ACh, postganglionic sympathetic neurons use NE as their main neurotransmitter, along with noradrenergic neurons in the CNS brainstem which are involved in the regulation of sleep–wake rhythm, food ingestion, blood pressure, learning and mood (Foote et al. 1983). Dysautonomia is a pathophysiological condition caused by abnormal function of the autonomic nervous system which may be transiently induced by drugs or may be permanent due to genetical disturbances of the autonomic system. The concentration of catecholamines in the sympathoadrenal system normally is relatively constant as a result of a balanced concerted action of catecholamine biosynthesis, storage, release, re-uptake and degradation. NE released at central and peripheral synapses is effectively inactivated by re-uptake via hNET, which recaptures 70%–90% of released NE, leaving the rest to spill over to into the circulation or uptake in other tissues expressing uptake2 (Iversen 1961; Esler et al. 1990). Re-uptake of NE by hNET in the heart is most noticeable, since in the heart noradrenergic synaptic clefts are narrower compared to other tissues (Novi 1968) and uptake2 is mostly absent (Eisenhofer et al. 1996).

Impaired function of NET through NET blockade by drugs (e.g. antidepressants) or through genetic alterations in the NET gene should affect the sympathoadrenal balance. Indeed, a missense mutation in hNET has been identified recently in a patient suffering from orthostatic intolerance (Shannon et al. 2000). This is the only currently identified variation in hNET which is definitely disease-related (Table 2). Orthostatic intolerance is an autonomous dysfunction characterized by elevated heart rate, light-headedness, weakness, sweating and other symptoms which generally occur after trying to get a standing position (for review, see Goldstein et al. 2002; Lu et al. 2004). The heterozygous missense variation found in hNET of this patient was exchange of alanine 457 to proline in TM9 of hNET protein. Functional analysis of the mutant hNET showed that this variant exhibited a 98% loss of function (Shannon et al. 2000). The functional impairment of this variant hNET has been shown to be the result of disturbed trafficking of hNET A457P to the plasma membrane and a pronounced increase in the K_m of NE (Paczkowski et al. 2002). Combined expression of this variant with wild-type hNET resulted in a dominant-negative

effect on wild-type hNET expression, an explanation for the dramatic in vivo effect of this heterozygous mutation (Hahn et al. 2003).

Despite this genetic evidence of hNET involvement in dysautonomia, several other reports show more or less controversial effects of NET blockade on cardiovascular regulation. When NET was blocked by desipramine, female rats demonstrated elevated supine heart rate, reduced tyramine response and a diminished plasma ratio of the NE-metabolite dihydroxyphenylglycol relative to NE. DMI-treated rats exhibited an attenuated tachycardia after stimulation of the baroreflex by nitroprusside, indicating that a reduction of baroreflex function and sympathetic outflow are the main effects of NET inactivation in orthostatic intolerance (Carson et al. 2002).

7.3 NET and Hypertension

Several reports indicate that hNET may be involved in essential hypertension. A substantial fraction of patients suffering from essential hypertension show a neurogenic origin of disease as indicated by high NE spillover from heart and kidney (Esler et al. 1988) or from increased muscle sympathetic nerve activity measured at the tibial nerve (Yamada et al. 1989). These findings may be attributable to an elevated neuronal firing rate or increased density of sympathetic innervation but may also partly be related to impaired re-uptake of NE by the NET. This view is supported by the finding that selective blockade of hNET by reboxetine induces a slight but significant rise in blood pressure (Schroeder et al. 2002). In a study with overweight normotensive, obesity hypertensive and lean patients with essential hypertension, only the group of lean hypertensive patients displayed an elevated NE spillover from the heart. In addition, only in this group a reduced cardiac release of the NE metabolite dihydroxyphenylglycol was found, indicating impaired NET function (Rumantir et al. 2000). In a large study in which the nature and frequency of SNPs in 75 candidate genes for blood-pressure homeostasis and hypertension had been investigated by chip hybridization, seven nonsynonymous coding SNPs were detected in the hNET gene (Halushka et al. 1999). Functional investigation of these variants revealed varying effects on transporter function, expression and regulation (Hahn et al. 2005; see Table 2). Furthermore, a genetic association study including 1,950 Japanese subjects indicated an association between a variation in the hNET promoter and essential hypertension (Ono et al. 2003).

7.4 NET and Myocardial Ischemia

In protracted myocardial ischemia, the induced hypoxia, ATP depletion and pH decrease affect NE storage and elevate the activity of the Na^+/H^+-exchanger (NHE), which leads to increased intracellular NE and Na^+ concentrations. This condition leads to the reversal of NET, resulting in a massive carrier-mediated

NE release (Schömig et al. 1991; Kübler and Strasser 1994). In guinea-pig heart, it has been demonstrated that drugs which inhibit or stimulate NE overflow lead to reduced or prolonged time of ventricular fibrillation (Imamura et al. 1996; Hatta et al. 1999).

In a deduced cell-culture model stably expressing hNET, it was shown that e.g. desipramine inhibited NET-mediated NE efflux and that inhibition of NHE abolished this efflux, whereas inhibition of Na^+/K^+ ATPase by ouabain potentiated NE efflux (Smith and Levi 1999). In another cell culture model (SKN-SH cells stably transfected with angiotensin II AT1 receptor), it has been shown that Ang-II, which is known to be elevated in myocardial ischemia, activates NHE through AT1 receptors and thus should stimulate NET-mediated NE release during myocardial ischemia (Reid et al. 2004). It may be concluded from these findings that polymorphisms in the hNET gene may influence the risk and outcome of myocardial ischemia.

7.5 NET and Obesity

Several lines of evidence suggest that NE and thus NET may be implicated in feeding behaviour and eating disorders (see also the following section). Hypothalamically administered NE reduces food intake in animals (Grossman 1960). LY368975 [(*R*)-thionisoxetine], a selective inhibitor of NET, has been shown to decrease food intake of food-deprived rats by up to 44% (Gehlert et al. 1998). While selective 5-HT re-uptake inhibitors are able to reduce the frequency of binge eating, they were unable to reduce long-term body weight. Sibutramine, an inhibitor of 5-HT and NE re-uptake, is efficient in the promotion and maintenance of weight reduction and is well tolerated (Milano et al. 2005). In a recent study, 15 healthy probands were treated with 8 mg reboxetine or placebo. In the reboxetine group, carbohydrate oxidation and adipose tissue blood flow was higher compared to placebo. The isoproterenol-induced increase in interstitial NE concentration was higher in the placebo group compared to reboxetine-treated probands. It was concluded that NET blockade by reboxetine may sensitize adipose tissue to β-adrenergic stimulation (Boschmann et al. 2002). Thus, one may be tempted to speculate that genetic disturbances leading to elevated hNET expression or function also may be associated with an obese phenotype. This assumption would be consistent with the finding that only in lean hypertensive patients an elevated NE spillover from the heart was observed (see Sect. 7.3).

7.6 NET and Anorexia Nervosa

The fact that long-term weight-restored patients suffering from anorexia nervosa (AN) have reduced NE plasma levels compared to controls (Kaye et al.

1985; Pirke et al. 1992) and the finding that NET-KO mice exhibit a reduced body weight (Xu et al. 2000; see also Sect. 2.2) indicate that NET may be involved in the pathogenesis of AN. Recently a novel 343-bp GA-rich repeat containing six islands of consensus AAGG tetranucleotide repeats has been detected in the upstream region of hNET promoter (Urwin et al. 2002).This region was shown to be highly polymorphic in that a long and short variant of repeat 4 (L4 and S4) were found with frequencies of 0.74 and 0.26, respectively, in 50 DNA samples. Another deletion of an AAGG (S1) was found in repeat island 1 of few AN patients. Both S1 and S4 lead to the abolishment of a putative Elk-1 transcription factor binding site. Investigation of 87 trios (patients and parents) with DSM-IV AN restrictive type exhibited a significant association of the L4 genotype, or another variation in linkage disequilibrium with L4, with AN restrictive type (Urwin et al. 2002). Later the same group reported that the risk for the development of AN restrictive type more than doubles when individuals are homozygous for the L4 allele and additionally carry a functional polymorphism in the promoter of the MAO-A gene, which is X-chromosomally located (Urwin et al. 2003). We have analysed the polymorphic hNET region (about 500 bp containing the repeat islands) with reporter gene constructs and dual-luciferase assays in hNET-negative HEK293 cells and in hNET-expressing SKN-SH cells. None of the constructs containing the different repeat alleles exhibited a significant promoter activity in HEK293 cells and in SKN-SH cells, whereas a silencing activity was found when the constructs were tested in 5′ to the SV40 promoter (M. Brüss, A.K. Wübken, H. Bönisch unpublished). Since there was no striking difference between the particular alleles, it seems more likely that the polymorphism is in linkage disequilibrium to another variation in this chromosomal region.

7.7 NET and ADHD

ADHD is one of the most frequent (8%–12% worldwide) psychiatric disturbance in childhood. By means of family, twin and adoption studies, the predisposition to ADHD seems to be genetically determined. By genome-wide genetic scanning and association studies of candidate genes, several groups have tried to find significant association of genetic variation with ADHD (for review, see Faraone et al. 2005). Among the candidate genes—which include nearly all catecholaminergic receptors and transporters—hNET was included in some studies, not least because NET inhibitors are effective in the treatment of ADHD. In most studies, no association between hNET polymorphisms and ADHD was found (De Luca et al. 2004; Xu et al. 2005) but a significant association of two NET SNPs was reported recently (Bobb et al. 2005). Furthermore, an association between a NET polymorphism (G1287A) and responsiveness to methylphenidate therapy has been reported (Yang et al. 2004). Methylphenidate (Ritalin) and other psychostimulant drugs such as amphetamine are among

the most commonly used drugs in the treatment of ADHD. This drug is thought to achieve its therapeutic benefit mainly by inhibition of the dopamine transporter and facilitation of dopamine release (Spencer et al. 2000). Nevertheless, there are strong indications that the noradrenergic and serotonergic systems are involved in ADHD. Neonatal rats in which dopaminergic neurons were destroyed by 6-hydroxydopamine show increased motor activity and learning deficits. Enhanced motor activity and learning deficiency respond to treatment with *d*-amphetamine, as well as to methylphenidate, in these dopamine-depleted rats (Shaywitz et al. 1978). In this animal model of ADHD, it has been shown that selective inhibitors of NET (desipramine and nisoxetine)—as well as of SERT (citalopram and fluvoxamine) but not DAT inhibitors—were able to reduce motor activity (Davids et al. 2002). The results of these animal studies are supported by the effective therapy of ADHD with SERT and/or NET inhibitors. Recently a large multicentre study has been conducted in which a combination of fluoxetine (a selective SERT inhibitor) and atomoxetine (a selective NET inhibitor) was compared with atomoxetine monotherapy in the effectiveness of therapy of ADHD with concurrent symptoms of depression and anxiety. The results of the study indicate that atomoxetine monotherapy was almost as effective as a combination therapy of both drugs (Kratochvil et al. 2005). These and other data indicate that disturbances of the noradrenergic system are involved in the aetiology of ADHD (for review, see Biederman and Spencer 1999). Regarding the fact that NET-inhibitors are effective in ADHD therapy, it seems likely that genetic variations which would either lead to enhanced NET function or expression (such as in the promoter region) or to diminished NE efficacy at target receptors may be involved in the pathogenesis of this disorder.

7.8 NET and Depression

The human NET is one of the main targets of commonly used antidepressants including non-selective and selective NET inhibitors. Elevation of central extracellular NE and/or 5-HT is thought to be the primary antidepressive action of such compounds. So it is self-evident that variations in the hNET gene may contribute to the pathogenesis of depression. A number of studies have reported changes in the level of NE and its metabolites in cerebrospinal fluid, plasma and urine, indicating the involvement of the NE system in depression (Lake et al. 1982; Yehuda et al. 1998). With respect to this view, a number of studies have been performed in which SNPs in the hNET gene were identified and the possible association to depression and related psychiatric disorders have been examined. In patients affected by bipolar disorder or schizophrenia, 13 NET SNPs have been identified but no associations to the disorders were found (Stöber et al. 1996). Investigation of a silent G1287A polymorphism in exon 9 of hNET in a collective suffering from major depression did not reveal an association to this disease (Owen et al. 1999). No genetic linkage of hNET

SNPs was found in patients suffering from Tourette's syndrome (Stöber et al. 1999) and manic disorder (Hadley et al. 1995). However, a recent report indicated a positive association of the T182C polymorphism in the hNET promoter to major depression in a Japanese and Korean population (Inoue et al. 2004; Ryu et al. 2004), a finding that previously could not be established in a German collective with major depression (Zill et al. 2002). Taken together, these data indicate that psychiatric disorders are complex and that population-specific multigenic contributions in addition to environmental factors determine the pathogenesis of these malignancies. Whole genome scanning of large patient collectives and controls of defined populations could provide further insight into the genetic factors which elevate the risk for the development of psychiatric disorders such as major depression.

7.9 NET and Addiction

Cocaine is a potent blocker of DAT, SERT and NET, and elevation of dopamine is thought to be the main mechanism of cocaine's psychotropic effects (Kuhar et al. 1991). Other drugs of abuse such as nicotine, opioids, amphetamine and ethanol may possess particular effects at various receptor systems, but the dopaminergic system seems to be involved in any rewarding effect and thus in the addictive state of drug abuse. Nevertheless, there is growing evidence that SERT and NET may play a complex role in the altered brain physiology in chronic drug consumption.

It is known that depressed patients are more often drug abusers than healthy controls and that antidepressants including NET inhibitors may reduce this addictive state. Rats treated for 1 month with cocaine exhibited elevated SERT density in the infralimbic cortex, nucleus accumbens and some other brain regions, and these changes were abolished after 4 days of cocaine withdrawal. In contrast, NET density was reduced in cocaine-treated rats in the bed nucleus of stria terminalis, lateral parabrachial area and inferior olive, but upon drug cessation a strong upregulation of NET has been observed in the paraventricular nucleus of the hypothalamus. In these experiments, no change in dopamine transporter level was seen (Belej et al. 1996).

In contrast to these data—which were determined by radioligand binding—another study investigated mRNA levels in chronically cocaine-treated rats by in situ hybridization. Interestingly, in this study NET and SERT mRNA levels were found to be unchanged during cocaine administration and after withdrawal, whereas DAT mRNA was upregulated in the ventral tegmental area upon cocaine cessation (Arroyo et al. 2000). In rhesus monkeys, chronic cocaine treatment led to strong upregulation of NET protein in the bed nucleus of the stria terminalis (Macey et al. 2003); and in postmortem human brains of cocaine abusers, upregulated NET protein was found in the insular cortex (Mash et al. 2005). In the conditioned place-preference test, DAT-KO mice and

SERT-KO mice still exhibited cocaine-conditioned place preference (Sora et al. 1998); but in double-knockout mice without DAT and SERT, this conditioned place preference was abolished (Sora et al. 2001). This finding indicates the complex nature of addiction and reward and implicates a special role of 5-HT in cocaine's reward action.

A known chronic cocaine effect is the down-regulation of prodynorphin (PDYN) expression in hypothalamus and an increase in caudate putamen. In rats, the selective NET inhibitor nisoxetine induced an increase in PDYN expression in hypothalamus and other brain regions but a decrease of PDYN in caudate putamen (Di Benedetto et al. 2004); thus, it may counteract some cocaine effects. Reboxetine, another selective NET inhibitor, has been shown to reduce nicotine self-administration in rats; but this effect may partly be a consequence of reboxetine's non-competitive inhibition of nACh receptors (Rauhut et al. 2002).

7.10 NET and Pain

It is generally accepted that, in addition to standard analgesic therapy with e.g. opioids in the management of chronic pain, the addition of antidepressants such as tricyclic compounds in antinociceptive therapy may improve the benefit for patients with chronic or neuropathic pain (Sindrup and Jensen 1999). 5-HT and NE are known to be mediators of endogenous analgesic mechanisms in the descending pain pathways which project to the spinal dorsal horn (Jones 1991; Willis and Westlund 1997). NET-KO mice have been shown to have increased extracellular NE levels (Xu et al. 2000), and these mice exhibit a potentiated analgesic effect of opioids compared to wild-type mice (Bohn et al. 2000). Recently it has been shown that intra-peritoneally administered duloxetine, a dual 5-HT and NE re-uptake inhibitor, is a potent analgesic to reduce late phase paw-licking behaviour in a rat formalin model of persistent pain (Iyengar et al. 2004). In addition, it was shown that orally applied duloxetine was an efficacious analgesic in the L5/L6 spinal nerve ligation model in contrast to a very low efficacy in the tail-flick model of acute nociceptive pain (Iyengar et al. 2004). In contrast, intrathecal NE has been shown to have antinociceptive effects in acute pain in tail-flick and hot-plate tests, and these effects are thought to be mediated through activation of α_2-adrenoceptors in spinal cord neurons (Reddy et al. 1980; Howe et al. 1983). In rats, the selective competitive NET inhibitor reboxetine and Xen2174—a structural analogue of the cone snail-derived peptide Mr1A (Sharpe et al. 2001), a non-competitive NET inhibitor—reduced tactile hypersensitivity after surgery when they were intrathecally administered. This effect could completely be blocked by the α_2-adrenoceptor antagonist idazoxan but only party by atropine (Obata et al. 2005). In contrast to this, an analgesic clonidine effect could completely be blocked by atropine in a rat model for neuropathic pain (Pan et al. 1999),

indicating that NET-inhibition suppresses post-surgery hypersensitivity by a mechanism different from that in neuropathic pain. Taken together, these data demonstrate that variations in the hNET gene as well as in the pain-modulating and transmitting receptor systems may influence the interindividual pain sensitivity and the efficacy of applied analgesics.

7.11 NET Ligands in Diagnosis and Therapy

Substrates and inhibitors of the NET are used as drugs with various indications (Iversen 2000). Indirectly acting sympathomimetic amines which are not able to penetrate the blood–brain barrier (e.g. tyramine and related amines) are used as vasoconstrictors, while lipophilic amines (e.g. amphetamine and derivatives) possess not only misused psychostimulatory and anorectic properties, they are also utilized to treat ADHD. With the exception of cocaine—which possesses local anaesthetic and pronounced psychostimulatory properties—and RTI-55, all NET inhibitors are used, or have at least been developed, as antidepressants. However, NET inhibitors have also been shown to be useful drugs in the treatment of other disorders. Thus, atomoxetine is used to treat ADHD (Simpson and Plosker 2004), and sibutramine as anti-obesity drug (Luque and Rey 1999). Furthermore, high-affinity transporter ligands may also be utilized as diagnostic tools for single photon emission computed tomography (SPECT) or positron emission tomography (PET) (for review, see Laakso and Hietala 2000; McConathy et al. 2004; de Win et al. 2005).

An increasing number of studies underline the usefulness of hNET substrates for diagnosis and therapy of several tumour types. Cancer tissues overexpressing hNET such as pheochromocytoma and several neuroendocrine gastrointestinal tumours are sensitive to therapy with radiolabelled [^{131}I]-meta-iodobenzylguanidine (^{131}I-MIBG) (Hoefnagel et al. 1987; Hadrich et al. 1999; Zuetenhorst et al. 1999; Höpfner et al. 2002, 2004). In a bladder cancer cell line (EJ138) transfected with NET-cDNA under control of a tumour-specific telomerase promoter, ^{131}I-MIBG dose-dependently led to cell death (Fullerton et al. 2005). Thus, NET expression under tissue-specific promoters is a new tool for suicide gene therapy. Recently it has been shown that the combined application of interferon-ɣ and unlabelled MIBG was more effective than interferon-ɣ alone in its antiproliferative and apoptotic effects on neuroendocrine gastrointestinal and pancreatic carcinoid tumour cell lines. MIBG which is taken up by NET led to additive cytotoxic effects and increased S-phase arrest in these cells, indicating that a combined application of interferon-ɣ and cold MIBG may be a suitable treatment option in neuroendocrine gastrointestinal cancer therapy (Höpfner et al. 2004). In addition to these therapeutic implications of hNET, radiolabelled hNET substrates are also useful in diagnosis, e.g. in the detection of metastases of the above-mentioned tumour types. Myocardial uptake of ^{123}I-labelled MIBG reflects the relative distribution of sympathetic

neurodensity and function in the myocardium (Bourachot et al. 1993; Hattori and Schwaiger 2000). This approach has also been shown to be useful in staging of diabetic peripheral nerve degeneration (Kiyono et al. 2005).

PET is a valuable tool for in vivo imaging of a growing number of malignancies. Since NET is involved in a variety of diseases including cardiovascular and CNS-related mood disorders, the usage of PET probes for in vivo monitoring of hNET density would be a valuable method for diagnosis and staging of several disorders, as well as for the understanding of disease pathology. So far, the measurement of NET levels by PET has been hampered by the lack of suitable and specific PET radioprobes for NET, due to the short half-life or defluorination of the ligand (Wilson et al. 2003; Schou et al. 2004). A recent study in monkeys showing successful PET imaging of NET by the usage of (*S*,*S*)-[18F]FMeNER-D2, a fluorinated reboxetine derivative, has suggested the usefulness of this compound in humans which, however, may be restricted due to radiation dose limitations by law (Seneca et al. 2005).

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