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University of Turku

Faculty of Medicine
Institute of Biomedicine
Department of Pharmacology, Drug Development and Therapeutics
Drug Research Doctoral Program

Supervised by

Professor Mika Scheinin, MD, PhD
Department of Pharmacology, Drug Development and Therapeutics
University of Turku
Turku, Finland

Professor Ulrik Gether, MD
Department of Neuroscience and Pharmacology
University of Copenhagen
Copenhagen, Denmark

Reviewed by

Docent Ulla Petäjä-Repo, PhD
Research Unit of Biomedicine
University of Oulu
Oulu, Finland

Docent Petteri Piepponen, PhD
Faculty of Pharmacy, Division of Pharmacology and Pharmacotherapy
University of Helsinki
Helsinki, Finland

Opponent

Dr. Volker Eulenburg, PhD
Institute of Biochemistry, Department of Biochemistry and Molecular Medicine
Friedrich-Alexander University Erlangen-Nürnberg
Erlangen, Germany

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To Mikkel and Siiri

ABSTRACT

Anne Vuorenpää

Cellular regulation of the norepinephrine transporter

Institute of Biomedicine, Department of Pharmacology, Drug Development and Therapeutics, Drug Research Doctoral Program, University of Turku, Turku, Finland

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The norepinephrine (NE) transporter (NET) mediates high-affinity uptake of the neurotransmitter NE in central and peripheral noradrenergic neurons. NET has a critical function in controlling the synaptic levels of NE and modulating several fundamental brain and body functions, including arousal, vigilance, attention, learning and autonomic functions. In this study, the cellular regulation of NET distribution, trafficking and function were investigated in sympathetic neurons, and compared to the related transporter of dopamine (DA), *i.e.* DAT, in neuronal cell models. In addition, a kinome-wide siRNA screen was conducted in heterologous cell models to investigate the possible roles of the regulatory kinases in the functions of NET, DAT and the serotonin transporter SERT.

NET was found to undergo marked constitutive internalization and sorting mainly to Rab 11 -positive recycling endosomes in sympathetic neurons. Direct comparison in neuronal cell models demonstrated that NET was sequestered from the plasma membrane to a significantly greater extent than DAT, and was subsequently largely recycled back to the plasma membrane, whereas internalized DAT was mainly targeted to degradation. The different trafficking itineraries of NET and DAT were found to be determined by non-conserved structural elements in the intracellular N-termini of the transporter proteins. The PDZ domain protein PICK1 was shown to associate with NET and to be important for stabilizing NET in the plasma membrane of noradrenergic neurons. RNA interference -mediated depletion of PICK1 reduced both total and surface-expressed NET and decreased NET uptake capacity, but had no effect on DAT that also binds PICK1. Furthermore, the results of the kinome-wide siRNA screen indicated that the functions of monoamine transporters are likely to be regulated by several kinases, such as the cyclic adenosine monophosphate (cAMP)- dependent protein kinases, the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) and the mitogen-activated protein kinase (MAPK). In addition, protein kinase, X-Linked (PrKX) was identified as a novel kinase possible involved in the regulation of monoamine transporter function.

Keywords: transporter, norepinephrine, dopamine, serotonin, internalization, postendocytic sorting, trafficking, PICK1, PDZ domain, kinase, siRNA screen

TIIVISTELMÄ

Anne Vuorenpää

Noradrenaliinin kuljetusproteiinin säätely soluissa

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Noradrenaliinin kuljetusproteiini (NET) vastaa hermovälittäjä-aineena toimivan noradrenaliinin takaisinotosta hermopäätteeseen keskushermostossa ja sympaattisissa hermosoluissa. NET:lla on täten tärkeänä tehtävänä säädellä noradrenaliinin pitoisuutta hermovälitilassa ja osallistua useiden keskeisten aivo- ja elintoimintojen, kuten tarkkaavaisuuden, vireystason, oppimisen ja autonomisen hermoston toimintojen säätelyyn. Tämän väitöskirjan tavoitteena oli selvittää NET:n säätelyä solu- ja molekyylitasolla noradrenergisissa hermosoluissa, ja verrata NET:n säätelymekanismeja vastaavaan dopamiinin kuljetunproteiiniin, DAT:in, hermosolumalleissa. Lisäksi ihmisen kinomin kattavalla geneettisellä seulonnalla pyrittiin tunnistamaan proteiinikinaasien vaikutuksia NET:n, DAT:n ja serotoniinin kuljetusproteiinin, SERT:in, toimintaan.

Sympaattisten hermosolujen solukalvolta NET:n osoitettiin internalisoituvan jatkuvasti solun sisäisiin kuljetusrakkuloihin, minkä jälkeen NET:n osoitettiin ohjautuvan lähinnä Rab11-positiivisiin kierrätysrakkuloihin. Hermosolumalleissa osoitettiin että NET internalisoituu solukalvolta huomattavasti suuremmassa määrin kuin DAT, ja että NET lajitellaan kierrätysrakkuloihin toisin kun DAT, joka ohjataan internalisoitumisen jälkeen lähinnä lysosomeihin hajotettavaksi. NET:n ja DAT:n erot soluliikennöinnissä osoitettiin riippuvan kuljetusproteiinien aminoterminaalisien osien eroista. Sympaattisissa hermosoluissa PDZ-rakenneyksikön sisältävä ankkurointiproteiini PICK1 paikantui yhdessä NET:n kanssa hermosolupäätteisiin, ja PICK1:n osoitettiin vakauttavan NET:n ilmentymiseen solukalvolla. PICK1:n ilmentymisen heikentäminen vähensi NET:n ilmentymistä ja toimintaa, mutta ei vaikuttanut DAT:iin, jonka on myös osoitettu sitoutuvan PICK1:en, ilmentymiseen. Lisäksi koko ihmisen kinomin kattava siRNA-seulonta osoitti, että monoamiinien kuljetusproteiinien ilmentymistä ja toimintaa solukalvolla säätelevät useat proteiinikinaasit, kuten syklisestä adenosiinimonofosfaatista (cAMP) riippuvaiset proteiinikinaasit, fosfoinositidi-3-kinaasi (PI3K)/proteiini kinaasi B (Akt) ja mitogeeniaktivoitu proteiinikinaasi (MAPK). X-kromosomiin liitoksissa oleva proteiinikinaasi (PrKx) tunnistettiin uutena kinaasina, joka oletettavasti osallistuu monoaminiinien kuljetusproteiinien toiminnan säätelyyn.

Avainsanat: kuljetusproteiini, noradrenaliini, dopamiini, serotoniini, soluliikennöinti, internalisaatio, PICK1, kinaasi, siRNA seulonta

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ABBREVIATIONS

ADHD	attention deficit hyperactivity disorder
Akt	serine-threonine protein kinase encoded by the Akt proto-oncogene
cAMP	cyclic adenosine monophosphate
CAD	CATH.a. differentiated cell line
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CRE	cAMP response element
CNS	central nervous system
C-terminus	carboxyl-terminus
DA	dopamine
DβH	dopamine-beta-hydroxylase
DMEM	Dulbecco´s modified eagle medium
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
EPI	epinephrine
ER	endoplasmic reticulum
ERK	extracellular stimulus-regulated kinase
FBS	fetal bovine serum
FP	fluorescence polarization
GPCR	G protein-coupled receptor
G protein	guanine nucleotide binding protein
HA	hemagglutinin
HEK 293	human embryonal kidney cell line
Hic5	hydrogen peroxide-inducible clone-5
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
hNET-Ex15L	long form of the C terminal hNET splice variants
hNET-Ex15S	short form of the C terminal hNET splice variants
5-HT	serotonin
HS	horse serum
KD	knock-down
KO	knock-out
LC	locus coeruleus
LDCV	large dense core vesicle
LeuT	leucine transporter
LSM	laser-scanning microscope

L-dopa	L-dihydroxyphenylalanine
MAPK	mitogen activated protein kinase
MEK	MAP/ERK kinase
NE	norepinephrine
NRI	norepinephrine reuptake inhibitor
NSS	neurotransmitter:sodium symporter
N-terminus	amino-terminus
PBS	phosphate buffered saline
PC12	rat pheochromocytoma cell line
PDZ	postsynaptic density protein 95 (PSD-95)/ Discs large homolog 1 (Discs large)/ Zonula occludens-1 (ZO-1)
PICK1	protein interacting with C kinase 1
PI3K	phosphoinositide 3-kinase
PKA C-α	cAMP-dependent protein kinase catalytic subunit alpha
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PrKX	protein kinase X-linked
qPCR	quantitative polymerase chain reaction
SCG	superior cervical ganglion
SEM	standard error of mean
shRNA	small hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
SIK3	salt-inducible kinase 3
SLC6	solute carrier family 6
SNARE	soluble *N*-ethylmaleimide-sensitive attachment factor receptor
SNP	single nucleotide polymorphism
TH	tyrosine hydroxylase
TMD	transmembrane domain
VMAT2	vesicular monoamine transporter 2
WT	wild-type

LIST OF ORIGINAL PUBLICATIONS

I Vuorenpää A, Jørgensen TN, Newman AH, Madsen KL, Scheinin M & Gether U. Differential internalization rates and postendocytic sorting of the norepinephrine and dopamine transporters are controlled by structural elements in the N termini. The Journal of Biological Chemistry 2016; 291:5634–51.

II Vuorenpää A*, Ammendrup-Johnsen I*, Jørgensen TN & Gether U. A kinome wide screen identifies novel kinases involved in regulation of monoamine transporter function. Neurochemistry International 2016; 98:103-14.

III Vuorenpää A, Newman AH, Scheinin M, Madsen KL & Gether U. The PDZ domain scaffolding protein PICK1 promotes surface availability and function of the norepinephrine transporter. Manuscript.

* Equal contribution.

1 INTRODUCTION

The catecholamine norepinephrine (NE) is an important neurotransmitter in the central and peripheral nervous systems that plays modulatory roles in several fundamental brain functions, including arousal, vigilance, attention, learning and autonomic functions. The norepinephrine transporter (NET) expressed in central noradrenergic neurons and peripheral sympathetic neurons mediates high-affinity reuptake of synaptically released NE and is the primary determinant of synaptic NE levels (Iversen, 1971; Pacholczyk et al., 1991). The significance of NET in noradrenergic homeostasis and normal physiology has been evidenced by studies on NET knock-out (KO) mice and patients with genetic defects in the gene encoding NET. Deletion of the NET gene in mice enhances extracellular NE levels, reduces tissue NE content and alters midbrain dopaminergic homeostasis. These neurochemical changes are accompanied with reduced behavioral despair and increased responsiveness to psychostimulants (Xu, 2000). Mutations of the human NET gene (*SLC6A2*) have been implicated in cardiovascular diseases and in some disorders of mood and cognition (Shannon et al., 2000; Kim et al., 2006). NET is targeted by therapeutic agents used in the management of depression and attention deficit hyperactivity disorder (ADHD), as well as by substances of abuse, such as cocaine and amphetamine.

Given the critical role of NET in noradrenergic homeostasis, cellular mechanisms regulating the plasma membrane availability or functioning of NET are likely to have impacts on physiology and behavior. The amount of functional NET present in the plasma membrane is variable and is tightly controlled to sustain NE homeostasis (Torres et al., 2003a), but the underlying molecular and cellular mechanisms are poorly understood. Several mechanisms have been suggested to regulate NET distribution, including protein kinase C (PKC) whose activation, e.g. by G_q-coupled muscarinic acetylcholine receptors, can increase NET internalization and reduce NE transport capacity (Apparsundaram et al., 1998a; Apparsundaram et al., 1998b; Jayanthi et al., 2004). Better understanding of the processes regulating the functioning and subcellular location of NET could provide a critical framework for deciphering the precise role of NET in neurotransmitter homeostasis and how this might be altered in diseased states.

The present series of studies was designed to characterize different aspects of the cellular regulation of NET distribution and function in neuronal cell models. Since NET is closely related to the corresponding transporter for dopamine, DAT, neuronal cell models were employed to directly compare the cellular regulatory mechanisms of the two transporters. Furthermore, the involvement of the entire ensemble of kinases in the regulation of monoamine transporter function was investigated with a kinome-wide siRNA screen. The research aimed to improve our understanding of the cellular regulation of NET and hopefully also our ability to predict and understand the regulation of noradrenergic signalling in the intact brain and entire organism, both in

physiological conditions and in different disease states, such as in depression, ADHD and anxiety disorders. Increased understanding of the cellular regulation of NET may ultimately be beneficial for the design and development of improved therapeutic agents.

2 REVIEW OF THE LITERATURE

2.1 Fundamental discoveries in noradrenergic neurotransmission

The nervous system consists of functionally and structurally distinct cell types, each of which is engaged in multiple cellular interactions to generate neuronal networks that process information. The concept that individual neuronal cells are the fundamental units of the brain (Ramón y Cajal, 1889) and that neuronal cells communicate with each other via chemical neurotransmission (Elliot, 1904) were first introduced at the turn of 20th century. Moreover, Langley proposed in 1905 that the "receptive substances" were the sites of actions for the chemical mediators released by nerve stimulation. In 1921 Dale and Loewi demonstrated that catecholamines released by sympathetic nerve endings transmit the effects of sympathetic stimulation to the effector organs. Several decades later, NE was identified as the physiological neurotransmitter of sympathetic nerves in mammals (von Euler, 1946) and the actions of NE on postsynaptic sites were suggested to be mediated by two types of adrenergic receptors, α and β (Ahlquist, 1948). Furthermore, Axelrod demonstrated in 1961 that intravenous administration of radioactively labeled NE resulted in selective accumulation of the neurotransmitter in the sympathetic nerve terminals innervating different organs. Thus, Axelrod postulated that following synaptic release, NE is recaptured by pre-synaptic nerve endings and recycled for later release. The early discoveries by Ahlquist, Axelrod and their colleagues, defining the essential processes in noradrenergic neurotransmission, have been the foundation for characterization of the fundamental concept of neurotransmitter actions and reuptake, which have led to the discovery of therapeutic treatments for several neuropsychiatric disorders (for review see e.g. (Iversen, 1971; Ruffolo and Hieble, 1994; Rubin, 2007).

2.2 Noradrenergic pathways in the central nervous system

The noradrenergic pathways in the central nervous system consist of NE synthesizing neurons and their fiber projections (Figure 1). The noradrenergic neurons are clustered in distinct nuclei in the lower brainstem, at the level of the pons and medulla oblongata. Noradrenergic neurons receive input from and send output to almost all parts of the brain and spinal cord (Cedarbaum and Aghajanian, 1978; Aston-Jones et al., 1991; Luppi et al., 1995; Robertson et al., 2013; Szabadi, 2013). Given the extensive anatomical distribution of the noradrenergic projections, it is not surprising that the noradrenergic system of the brain plays modulatory roles in virtually every aspect of brain function. NE has been shown to be involved in the regulation of cognition (Amaral and Sinnamon, 1977; Aston-Jones and Cohen, 2005; Sara, 2009), arousal (Aston-Jones and Cohen, 2005), attention and sleep-wake cycles (Roussel et al., 1967; Hobson et al., 1975; Aston-Jones et al., 1999; Arnsten and Li, 2005; Aston-Jones and Cohen, 2005), stress (Benarroch, 2009), memory formation (Cahill and McGaugh, 1996), emotions, synaptic plasticity (Harley, 2007), sensory processing

(Berridge and Waterhouse, 2003), cardiovascular function (Amaral and Sinnamon, 1977) and neuroendocrine signaling (Simonneaux and Ribelayga, 2003).

2.2.1 Noradrenergic cell bodies

All noradrenergic cell bodies are located in the brainstem, clustered in seven cell groups, A1-A7 in the nomenclature of catecholaminergic cell groups (Hokfelt et al., 1984), based on the original designations described by Dahlström and Fuxe (Dahlstrom and Fuxe, 1964) (Figure 1). Anatomically, NE neurons can be divided into the pontine coerulear group, formed by the locus coeruleus (LC; A6) and subcoeruleus (A4), and to the lateral tegmental group, consisting of the A1, A2, A3, A5 and A7 neuron groups distributed as a series of loosely organized cell groups in the hindbrain (Moore and Bloom, 1979). Although the organization of the noradrenergic nuclei is conserved across different mammalian species, there are some species differences e.g. in the distribution and number of NE neurons within the nuclei (Grzanna and Molliver, 1980; Kemper et al., 1987).

It is well established that noradrenergic neurons of the brain are heterogeneous in terms of their morphology, electrophysiological properties, projectional organization, and molecular characteristics, such as patterns of gene expression (Gonzalez and Smeets, 1991; Grzanna and Fritschy, 1991; Guyenet, 1991; Robertson et al., 2013; Chandler et al., 2014). The largest and best known cluster of noradrenergic neurons in the central nervous system (CNS) is the LC. The LC is located near the lateral floor of the fourth ventricle at the level of the rostal pons and provides the principal source of noradrenergic innervations in the brain (Amaral and Sinnamon, 1977; Foote et al., 1983; Szabadi, 2013). The LC was originally characterized with Nissl staining (Russell, 1955) and later by formaldehyde-induced fluorescence (Dahlstrom and Fuxe, 1964), whereas immunohistochemical visualization of tyrosine hydroxylase (TH) (Pickel et al., 1975a) and dopamine-β-hydroxylase (DβH) (Swanson and Hartman, 1975) are nowadays the most commonly used methods to identify the perikarya of noradrenergic neurons. The total number of noradrenergic neurons in the human LC (unilaterally) is estimated to be 60 000, and in the rat LC (unilaterally) approximately 1600. The LC of rats has been estimated to extend 900 μm rostro-caudally and to occupy a volume of approximately 0.8 mm^3 (Swanson, 1976). The LC can be divided into a dorsal main component (LCd) containing mainly tightly packed fusiform cells, and a contiguous smaller ventral portion (LCv) containing primarily larger multipolar cells (Descarries and Saucier, 1972; Ross et al., 1975; Swanson, 1976). Delimitation of the subcoerulear area varies and in the simplest, it can be defined as the pontine noradrenergic cells that are immediately antero-ventral to the LC based on traditional Nissl staining (Amaral and Sinnamon, 1977). The pontine coerulear noradrenergic neurons are generally characterized by complex neuropil and high content of neuromelanin (Amaral and Sinnamon, 1977).

2.2.2 Efferent projections

The noradrenergic projections stemming from the LC innervate virtually all brain regions (Figure 1) and provide the bulk of NE to the entire neuraxis (Amaral and Sinnamon, 1977; Foote and Morrison, 1987; Grzanna and Fritschy, 1991; Aston-Jones, 1995). Various innervation mapping techniques, including retrograde tracing analysis and autoradiographic studies of receptor expression, have shown that the brain regions receiving noradrenergic inputs from the LC include the olfactory bulb, cerebral cortex, basal forebrain, hippocampus, hypothalamus, thalamus, amyglada, cerebellum, brainstem and spinal cord. The axonal projections originating from the LC can be divided into three major pathways: the ascending pathway (dorsal noradrenergic bundle), the cerebellar pathway and the descending pathway (Moore and Bloom, 1979). The main noradrenergic projection, the dorsal noradrenergic bundle, innervates structures in the midbrain (i.e. periaqueductal grey and dorsal raphe nucleus), thalamus, limbic system (i.e. amygdala, hippocampus, cingulate cortex and parahippocampal gyrus), and all areas of the cerebral cortex (Swanson and Hartman, 1975). The cerebellar pathway projects to the cerebellar nuclei and the cerebellar cortex, and the descending pathway sends projections to the lower brainstem and to the spinal cord (Figure 1).

The density of noradrenergic terminals varies in the target regions and the specific distributions of the projections within some of the target regions remain unclear. For example, in the rat cerebellar cortex, noradrenergic projections from the LC have been shown to target mainly the granular and molecular cell layers (Hokfelt and Fuxe, 1969), whereas some reports identify the Purkinje cells as the main targets of LC noradrenergic innervations in the cerebellar cortex (Hoffer et al., 1971; Schwarz et al., 2015). Due to extensive bifurcation and branching, an individual LC noradrenergic neuron can have ipsi- and bilateral projections to several brain regions (Ungerstedt, 1971). Thus, a single LC neuron can exert simultaneous influences on diverse target regions of the brain (Nakamura and Iwama, 1975), indicating that LC-NE neurons can integrate the activity of functionally different brain regions.

The projections from the lateral tegmental noradrenergic cell group can be divided into ascending and descending pathways. The ascending fiber system (also called the ventral noradrenergic bundle) innervates the midbrain reticular formation, the entire hypothalamus and parts of the limbic cortex. The descending projections from the non-coerulear NE nuclei form two bulbospinal bundles that terminate in the spinal cord (Szabadi, 2013). Thus, many of the brain regions receiving the LC-NE innervation are also targeted by non-coerulear NE neurons (Grzanna and Fritschy, 1991).

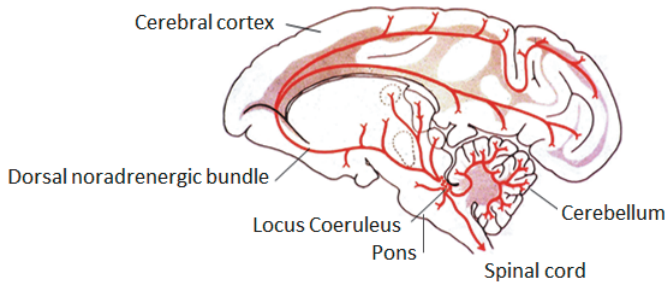

Figure 1. A schematic representation of the LC projections in the sagittal plane of a monkey brain. The illustration shows the noradrenergic A6 cell group located in the pons and its efferent projections throughout the central nervous system. Modified from Aston-Jones, G and Cohen, JD. 2005.

2.2.3 Afferent projections

The activity of brainstem noradrenergic neurons is regulated by afferent projections to their neuronal somata and dendrites. Many neurotransmitter receptors are expressed on the cell membranes of noradrenergic neurons and have been shown to regulate their firing or neurotransmitter release (Aston-Jones et al., 1991). Results of retrograde tracing studies indicate that neurons innervating noradrenergic neurons originate from wide regions of the brain, including the cerebellum, forebrain, hypothalamus, ventrolateral medulla, bed nucleus of the stria terminalis, central nucleus of the amygdala and the spinal cord (Amaral and Sinnamon, 1977; Cedarbaum and Aghajanian, 1978; Clavier and Hinkley, 1978). Thus, noradrenergic neurons have been shown to receive reciprocal inputs from several brain regions (Conrad et al., 1974; Amaral and Sinnamon, 1977). The reciprocal arrangement of the NE systems in the brain was recently supported by the "tracing the relationship between input and output" (TRIO) method using viral-genetic tools, which also supported that LC noradrenergic neurons mostly receive inputs from the same brain regions where they send projections (Schwarz et al., 2015).

2.3 Noradrenergic neurons in the sympathetic nervous system

Outside of the CNS, NE is synthesized and released from postganglionic neurons of the sympathetic nervous system. The peripheral noradrenergic neurons cluster in the sympathetic ganglia located next to the spinal column and contain long axonal fibers that arborize extensively to create dense terminal networks that innervate smooth muscle and gland cells throughout the body. In addition, NE and epinephrine (EPI) are synthesized and released from the chromaffin cells of the adrenal medulla, from where the catecholamines are released into the circulation. The sympathetic noradrenergic neurons transmit the body´s fight-or-flight responses and maintain tissue homeostasis (Brodal, 2004).

The superior cervical ganglia (SCG) are clusters of NE synthesizing and releasing neurons located at the level of the second and third cervical vertebrae. The afferent sympathetic projections originating from SCG neurons innervate organs in the head, such as the pineal, lacrimal, salivary and thyroid glands as well as the blood vessels of the cranial muscles. The SCG projections, like other sympathetic noradrenergic fibers, contain axonal enlargements, ”varicosities” and ”boutons“ that have the structure and function of presynaptic terminals, and conceivably represent presynaptic release and reuptake sites (Geffen and Livett, 1971; Livett, 1973; Matthies et al., 2009) (Figure 2). SCG neurons are commonly used as an experimental model of noradrenergic neurons (Schroeter et al., 2000; Savchenko et al., 2003; Brum et al., 2006; Matthies et al., 2009; Matthies et al., 2010).

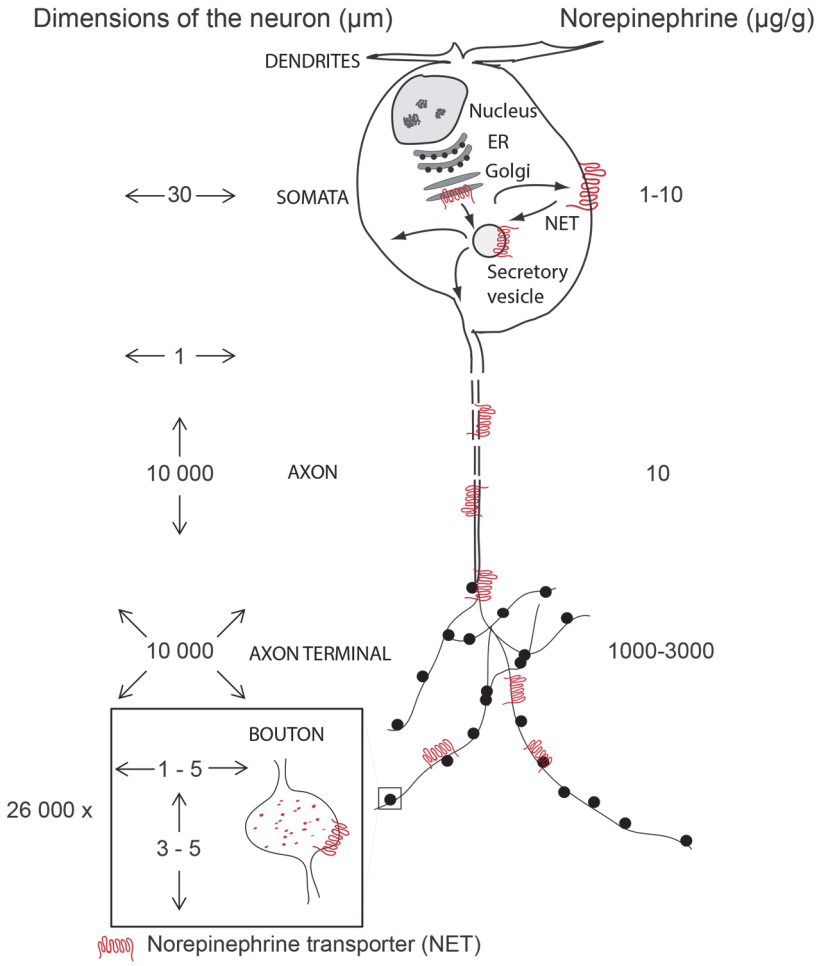

Figure 2. Schematic representation of a typical sympathetic noradrenergic neuron with neuronal dimensions and estimated distribution of NE content. The norepinephrine transporter (NET) protein is synthesized in the neuronal soma and targeted to nerve terminals. Figure modified from (Geffen and Livett, 1971).

2.4 Noradrenergic neurotransmission

Neurotransmission allows the specific pairing of communicating cells in space and time and facilitates rapid responses to environmental stimuli (Mattson and Bruce-Keller, 1999). Noradrenergic neurotransmission follows the classical synaptic transduction paradigm that requires the synthesis of the neurotransmitter, its storage and transport, and its release upon stimulation from the presynaptic nerve terminal. The released neurotransmitter diffuses into the synaptic cleft (40 - 60 nm), interacts with post- and presynaptic receptors, and is subsequently removed from the synaptic cleft to restore the dynamics of neurotransmission. In addition to synaptic neurotransmission, NE is also released at sites that are distant from the synaptic cleft in a process called volume transmission (see chapter 2.4.2). The following sections will provide an outline of the different steps in noradrenergic neurotransmission.

2.4.1 Norepinephrine biosynthesis and storage

In both central and peripheral noradrenergic neurons, NE is synthesized from the amino acid tyrosine through three enzyme-catalyzed chemical reactions (Weiner, 1970; Axelrod, 1971) (Figure 3). Tyrosine, the common precursor of all three catecholamines, dopamine (DA), NE and EPI, is taken up from the circulation into catecholaminergic neurons and hydroxylated to form L-dihydroxyphenylalanine (L-dopa). This rate limiting step is catalyzed by the enzyme TH that is present in the catecholaminergic neurons of the brain, in sympathetic neurons and in the chromaffin cells of the adrenal glands. In the central noradrenergic neurons, TH is diffusely expressed in the perikaryon, whereas in the axonal and dendritic processes, its expression appears more compartmentalized (Pickel et al., 1975b; Pickel et al., 1975a). The activity of TH is controlled by post-translational modifications and through end-product inhibition (Zigmond et al., 1989). The decarboxylation of L-dopa to DA is catalyzed by aromatic L-amino acid decarboxylase (AADC) present in the cytoplasm. DA is then rapidly taken up into synaptic vesicles via the actions of the H^+-dependent vesicular monoamine transporter 2 (VMAT2) (Liu et al., 1992). In the synaptic vesicles of the noradrenergic and adrenergic neurons, DA is further converted to NE by DβH (Kaufman and Friedman, 1965) (Figure 3). In the central adrenergic neurons and in adrenal chromaffin cells, the synaptic vesicles also contain phenylethanolamine N-methyltransferase (PNMT), which methylates NE to EPI. The NE-containing vesicles migrate down the efferent projections towards the axonal terminals (Dahlstrom and Haggendal, 1966; Geffen and Livett, 1971), providing a ready supply of NE available for release. The intravesicular concentration of NE in the presynaptic storage vesicles is estimated to be approximately 0.5 M (Geffen and Livett, 1971). Based on an estimation of the half-life of the synaptic vesicles (approximately 21 days) and the estimated turnover rate of NE (less than 24 h), it has been suggested that the synaptic vesicles may spontaneously leak NE into the cytoplasm and complement their NE content by reuptake and synthesis (Geffen and Livett, 1971).

Subcellular analyses of the proteins required for NE synthesis and storage (TH, DβH and VMAT2) have reported them to be localized in the membranes of both small synaptic vesicles (SSV; ø 40–60 nm) and large dense-core vesicles (LDCV; ø 80–120 nm) (Pickel et al., 1996). Furthermore, amperometric recordings of rat brainstem slice cultures have reported differential quantal sizes of the NE release events, i.e. small and large release spikes, suggesting that NE is stored and released from different vesicle populations (Chiti and Teschemacher, 2007). The frequency of the large release events was found to be lower than the frequency of the small release events, which could result from a limited pool size of the large vesicles and suggest that most NE is stored in small synaptic vesicles (Chiti and Teschemacher, 2007). In sympathetic neurons and in the chromaffin cells of the adrenal medulla, NE is found within LDCVs as well as in small synaptic vesicles (De Potter et al., 1997). The LDCVs are found in the sympathetic cell body, dendrites and nerve terminals, and they undergo regulated exocytosis after stimulation (Kanner and Schuldiner, 1987).

Despite marked fluctuations in the activity of noradrenergic neurons, the tissue levels of NE remain remarkably stable. To a considerable extent, this is due to reuptake of synaptically released NE back into presynaptic neurons by the NE transporter (NET) and reincorporation of the transmitter into storage vesicles (Figure 3). However, long-lasting changes in the activity of noradrenergic neurons have been shown to affect the rate of NE biosynthesis. For example, prolonged stress has been shown to markedly increase the turnover of the NE pool in several tissues (Spector, 1966). Moreover, various drugs can modulate the biosynthesis and levels of endogenous catecholamines. For example, reserpine, an irreversible inhibitor of VMAT2 that was earlier used in the management of high blood pressure and for relief of psychotic symptoms, blocks the ability of the synaptic vesicles to concentrate monoamine neurotransmitters, resulting in profound catecholamine depletion (Henry and Scherman, 1989).

2.4.2 Neuronal firing and NE release

The noradrenergic neurons exhibit two main modes of firing, i.e. tonic firing, lasting seconds, minutes or even hours, and phasic firing, lasting a few hundred milliseconds. The basal, tonic activity of noradrenergic neurons provides the main bulk of synaptic NE in the different target regions (Foote et al., 1980; Williams et al., 1984; Alreja and Aghajanian, 1991). Intracellular recording studies of noradrenergic neurons in rat brainstem slices have identified the frequencies of these spontaneous action potentials to be between 1 and 5 Hz, depending on the age of the animals from which the slices were taken (Williams et al., 1984; Williams and Marshall, 1987).

Electrophysiological recordings from noradrenergic neurons in animals have shown clear changes in noradrenergic neuronal activity in response to environmental stimuli. During normal wakefulness, the noradrenergic neurons demonstrate regular, tonic firing, which is markedly decreased during sleep and is nearly absent during rapid eye movement (REM) sleep (Takahashi et al., 2010). The noradrenergic neurons exhibit phasic activation when animals are processing task-relevant stimuli, which is

associated with high levels of task performance (Aston-Jones et al., 1999; Bouret and Sara, 2004; Aston-Jones and Cohen, 2005; Sara, 2009). The firing rate of noradrenergic neurons can markedly increase (high-tonic firing) in response to stressful or aversive stimuli, which has been associated with poor performance in tasks requiring focused attention and with increased distractibility (Aston-Jones and Cohen, 2005; Gompf et al., 2010). Thus, the relationship of firing rate of noradrenergic neurons and performance in tasks follows an inverted U-shaped curve, where the performance of animals in most tasks is best with an intermediate level of arousal and is worse with too little or too much arousal (Aston-Jones and Cohen, 2005).

When an action potential reaches the nerve terminal, NE is released from the synaptic vesicles into the synaptic cleft in a Ca^{2+} -dependent manner. Typically, neurotransmitters and hormones can be released in response to stimuli by two different modes of exocytosis: by full fusion and in a so called kiss-and-run manner. Full fusion involves complete vesicle collapse and release of the entire vesicle content, whereas kiss-and-run release is a partial release mode, in which a vesicle rapidly forms a fusion pore in the plasma membrane and then retrieves. Thus, the size of the fusion pore limits the release of the contents of the vesicle (Wu et al., 2014). The kinetics of NE release events suggests that NE is mainly released through full fusion of storage vesicles with the presynaptic nerve terminal membrane (Chiti and Teschemacher, 2007). However, the NE release characteristics might vary in different compartments of the cells, i.e. in the somato-dendritic and axonal release sites, and between different noradrenergic cell populations (Chiti and Teschemacher, 2007).

Noradrenergic neurons also release NE from non-synaptic release sites in a process called volume transmission (or quantal NE release) that permits broad spatial and temporal effects of NE (Beaudet and Descarries, 1978; Pickel et al., 1996; Chiti and Teschemacher, 2007). Amperometric and path-clamp studies of quantal NE release from somato-dendritic sites in rat brainstem slices have only detected release events when the firing rate is high (15–20 Hz) (Berridge and Waterhouse, 2003; Huang et al., 2012). Quantal NE release has long latency, low sensitivity to the Na^{+} ion channel blocker tetrodotoxin, and is Ca^{2+} -dependent (Huang et al., 2012). Somato-dendritic NE release has been suggested to produce negative feedback inhibition and to down-regulate neuronal hyperactivity via activation of presynaptic autoreceptors, which consequently inhibits synaptic NE release (Huang et al., 2012). Analogous volume transmission has been described in dopaminergic and serotonergic neurons (Descarries and Mechawar, 2000).

In addition to NE, noradrenergic neurons synthesize, store and release several different neuropeptides, such as neuropeptide Y, galanin and enkephalin, and non-peptide neurotransmitters, such as adenosine triphosphate. Moreover, LC noradrenergic neurons have also been reported to express glutamate (Otto and Nichols, 2011) and to express and to release DA (Smith and Greene, 2012; Kempadoo et al., 2016). The simultaneous release of co-transmitters is suggested to modulate the actions of NE both at pre- and postsynaptic sites (Kupfermann, 1991; Burnstock, 2004).

2.4.3 Pre- and postsynaptic receptors and reuptake

Synaptically released NE enters the synaptic cleft, binds to and activates pre- and postsynaptic α- and β-adrenoceptors located on the plasma membrane of the adjacent cells (Figure 3). The complex responses to receptor activation by NE in target regions are mediated through effects on cell membrane potentials, excitability and intracellular second messenger pathways. Thus, depending on the amount of released NE and the receptor composition in the target region, activation of adrenoceptors may result in either excitatory or inhibitory responses. Molecular cloning has identified 9 adrenoceptor subtypes (α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2 and β_3), which all belong to the family of G-protein coupled receptors (Hieble et al., 1995; Gilsbach and Hein, 2008). All adrenoceptor subtypes are expressed in the CNS, but there are developmental and species differences in the receptor distributions and densities (Palacios et al., 1987; Williams and Marshall, 1987; Booze et al., 1989).

Adrenoceptors couple the binding of NE and their other ligands to the activation of certain heterotrimeric G-proteins, leading to the modulation of intracellular signaling pathways (Rosenbaum et al., 2009). α_1-adrenoceptors mediate their intracellular effect via $G_{q/11}$-proteins leading to increases in intracellular inositol trisphosphate (IP_3) and intracellular Ca^{2+} concentrations, whereas α_2-adrenoceptors are mainly coupled to $G_{i/o}$-proteins that mediate inhibition of adenylyl cyclases. In addition, Gβγ subunits released from the activated inhibitory $G_{i/o}$-proteins activate G-protein coupled inwardly-rectifying potassium channels (GIRKs) and inhibit voltage-dependent calcium channels (Hein, 2006). Activation of β-adrenoceptors leads to activation of adenylyl cyclases through G_s-protein activation (Frielle et al., 1988). In addition, adrenoceptors have been shown to modulate some cellular functions independently of G-proteins. The multifunctional adaptor and scaffold proteins, β-arrestins, have been shown to be involved in agonist-induced adrenoceptor desensitization and signaling (Lefkowitz and Shenoy, 2005). The relevance of distinct intracellular signaling pathways for the physiological functions of adrenoceptors is not fully understood. A single amino acid mutation in the α_{2A}-adrenoceptor (α_{2A}-D79N) selectively disrupts the activation of GIRKs in response to receptor activation in vitro (Surprenant et al., 1992). Introduction of this point mutation into the mouse genome (α_{2A}-D79N mice) did not affect the α_{2A}-adrenoceptor mediated negative feedback of neurotransmitter release (Altman et al., 1999), indicating that α_{2A}-adrenoceptor-mediated control of NE release does not require the activation of GIRK K^+ channels.

Studies on mice carrying deletions in the genes encoding individual adrenoceptor subtypes have been crucial for the knowledge regarding the specific functions of these receptors (MacDonald et al., 1997; Rohrer and Kobilka, 1998; Philipp and Hein, 2004). The G_i-coupled α_2-adrenoceptors are essential feedback regulators of neurotransmitter release. Presynaptic α_2-autoreceptors are located both in the somato-dendritic regions, where they inhibit neuronal firing, and in the noradrenergic nerve terminals, where they inhibit NE release (Norenberg et al., 1997; Starke, 2001; Trendelenburg et al., 2001). In isolated tissue preparations, the α_{2A}-adrenoceptor is the major receptor subtype inhibiting NE release, but also α_{2C}-adrenoceptors and possibly α_{2B}-adrenoceptors contribute to the regulation of neurotransmitter release (Altman et al., 1999; Hein et al., 1999;

Trendelenburg et al., 2003). In addition to their function as inhibitory autoreceptors, α_2-adrenoceptors can also regulate the release of other neurotransmitters in the central and peripheral nervous systems and thus operate as "heteroreceptors". For example, α_{2A}- and α_{2C}-adrenoceptors have been shown to inhibit dopamine release in the basal ganglia (Bucheler et al., 2002) and serotonin release in the mouse hippocampus and cerebral cortex (Scheibner et al., 2001). The importance of α_2-adrenoceptors for normal physiology is evident from the observation that α_{2ABC}-deficient mouse embryos die during mid-gestation (Philipp et al., 2002a; Philipp et al., 2002b). Despite the wide distribution of α_1-adrenoceptors in the brain, the contributions of the α_1-adrenoceptor subtypes to the neuronal functions mediated by NE are still partly unknown. α_1-adrenoceptors have been suggested to be involved in many higher cerebral functions, such as regulation of attention and memory processes (Sirvio and MacDonald, 1999). Moreover, the specific functions of the different β-adrenoceptor subtypes differently expressed in the brain are not fully understood. NE acting at β-receptors has been found to be essential for memory retrieval (Winder et al., 1999; Murchison et al., 2004). The central noradrenergic neurons are also known to make contacts with non-neuronal cells, such as glia cells that express both α- and β-adrenoceptors (Stone and Ariano, 1989; Hertz et al., 2004).

To terminate noradrenergic neurotransmission, NE is removed from the synaptic cleft by reuptake into the presynaptic neuron via NET located in the plasma membrane of the noradrenergic neurons (Kristensen et al., 2011) (Figure 3). This fast termination of synaptic transmission restores NE sensitivity and enables NET to exert accurate control over noradrenergic homeostasis. After reuptake, the NE molecules are either rapidly taken up from the cytoplasm into synaptic storage vesicles by VMAT2 or metabolized in the cytoplasm by the mitochondrial enzyme monoamine oxidase. Also glial cells that express both monoamine oxidase and catechol-O-methyltransferase can metabolize and deactivate NE and other catecholamines (Fillenz, 1990).

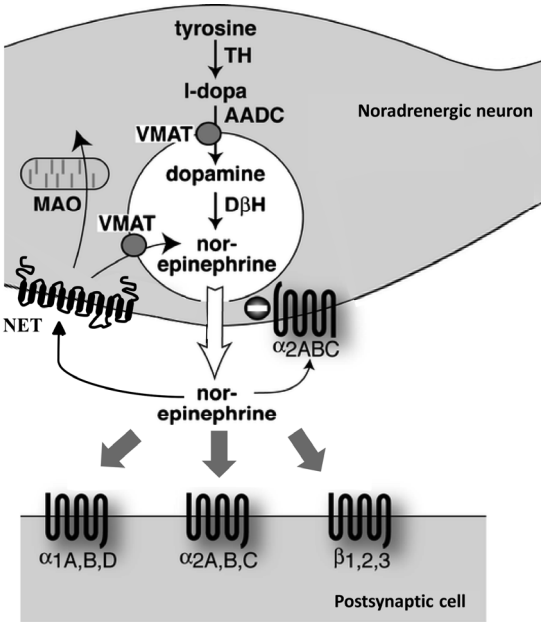

Figure 3. The noradrenergic synapse. NE is synthesized from the aromatic amino acid tyrosine in three sequential steps catalyzed by the enzymes tyrosine hydroxylase (TH), aromatic amino acid decarboxylase (AADC), and dopamine β-hydroxylase (DβH). Following synaptic release, NE activates nine different subtypes of adrenoceptors; α_{1ABD}, α_{2ABC}, β_{1-3}. Presynaptic α_2-adrenoceptors mediate negative feedback of the neurotransmitter release. NE is transported back into presynaptic neurons by the NE transporter (NET) and either restored into synaptic vesicles by a vesicular monoamine transporter (VMAT) or metabolized by mitochondrial enzyme monoamine oxidase (MAO). Figure adopted from Hein, 2006.

2.5 Norepinephrine transporter (NET)

Termination of noradrenergic neurotransmission is critically dependent on the actions of NET (Axelrod and Kopin, 1969; Iversen, 1971; Pacholczyk et al., 1991; Bonisch and Bruss, 1994). NET mediates rapid re-uptake of synaptically released NE into presynaptic nerve terminals, and provides a mechanism to accumulate and conserve the transmitter within the noradrenergic neurons. It is estimated that 80-90 % of released NE is recaptured by NET (Schomig et al., 1989), while the remaining NE is either broken down in the extracellular space, spilled over into the blood circulation or taken up by an extraneuronal monoamine transporter (EMTs), also known as organic cation transporter 3. EMTs are widely expressed in the brain and other tissues and mediate low-level accumulation of NE in most tissues (Iversen, 1965; Trendelenburg, 1978; Grundemann et al., 1998; Wu et al., 1998; Breining et al., 2016).

NET belongs to the solute carrier 6 (SLC 6) family of sodium-coupled symporters, also referred to as the neurotransmitter:sodium symporter (NSS) family or Na^+/Cl^- dependent transporters, which also includes the transporters of DA, serotonin (5-HT), γ-aminobutyric acid (GABA) and glycine. These transporters utilize the transmembrane Na^+ gradient to drive substrate transport across the plasma membrane (Kristensen et al., 2011). Members of this family show a high degree of structural similarity. The overall amino acid sequence identity of human NET and human DAT is greater than 75 %, whereas human serotonin transporter (SERT) and human NET share approximately 60 % sequence identity. SLC 6 family transporters consist of 12 hydrophobic transmembrane domains (TMD), intracellular N- and C-termini and a large extracellular loop connecting TMDs 3 and 4, which contains N-linked glycosylation motifs (Figure 4). This topology has been confirmed by several high-resolution crystal structures including bacterial homologues of the mammalian SLC 6 transporters (Yamashita et al., 2005; Singh et al., 2007; Singh et al., 2008; Penmatsa and Gouaux, 2014), eukaryotic DAT from *Drosophila melanogaster* (Penmatsa et al., 2013a, b, 2015; Wang et al., 2015) and human SERT (Coleman et al., 2016). The following sections will provide a general introduction to the physiological importance, function, structure and regulation of NET.

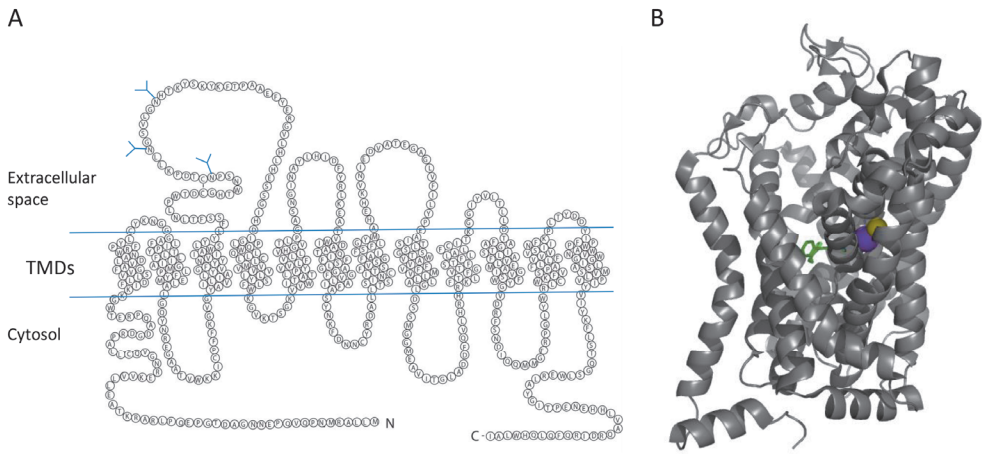

Figure 4. Schematic structure of the human NET and the crystal structure of the *Drosophila melanogaster* DAT in complex with the tricyclic antidepressant drug nortriptyline (Penmatsa et al., 2013a). A, the human NET comprises 617 amino acids organized to 12 hydrophobic membrane spanning segments, intracellularly located N- and C-termini and a large extracellular loop connecting transmembrane domains (TMDs) 3 and 4, which contains three N-linked glycosylation sites. B, the tertiary structure of dDAT in an outward-open conformation with nortriptyline (green) bound to the central binding site formed between TMDs 1, 3, 6 and 8. Two sodium ions are shown in purple and one chloride ion is shown in yellow (modified from Penmatsa et al. 2013b).

2.5.1 Physiological importance of NET

Presynaptically located NET mediates the reuptake and re-storage of the released transmitter NE into presynaptic terminals and therefore plays an important role in fine-tuning NE neurotransmission, and exerts regulated control over NE-mediated behavioral and physiological functions. The significance of NET in normal physiology has been investigated with mice with targeted deletion of the NET gene. NET knock-out (NET KO) mice demonstrate significant neurochemical changes characterized by enhanced extracellular NE levels and reduced tissue NE content, resulting in altered brain and autonomic functions (Xu et al., 2000). In the prefrontal cortex, NE tissue levels are decreased by 70 % and the unperturbed extracellular NE concentrations are approximately 3-fold higher in NET KO mice compared to wild-type (WT) mice (Xu et al., 2000). These observations strongly support the notion that termination of the NE transmission and maintenance of intracellular NE stores are critically dependent on NET function. In behavioral tests, NET KO mice exhibit significantly shortened immobility times both in the forced swim test and the tail suspension test indicating reduced behavioral despair, compared to their WT littermates (Xu et al., 2000). NET KO mice are also less sensitive to stress, as determined by blood corticosterone levels after a forced swim test (Solich et al., 2008) or by chronic restraint or social defeat stress tests (Haenisch et al., 2009). NET KO mice demonstrate also changes in their midbrain dopaminergic homeostasis. Both the tissue and extracellular levels of striatal

dopamine and its metabolites are significantly reduced in NET KO mice compared to WT mice, suggesting down-regulation of presynaptic dopamine activity in NET KO animals (Xu et al., 2000). These effects could be attributable to involvement of NET in the clearance of dopamine (Moron et al., 2002) and/or to enhanced activation of inhibitory α_2-adrenoceptors located on dopaminergic neurons by increased extracellular NE levels in the NET KO animals (Grenhoff and Svensson, 1989; Yavich et al., 1997). Interpretation of the observations derived from NET KO mice should be cautious, since life-long complete deletion of a gene might well give rise to compensatory mechanisms. Indeed, both DAT and SERT have been reported to be upregulated in various brain regions of NET KO mice (Solich et al., 2011). Moreover, the expression of α_1- and β-adrenoceptors have been shown to be decreased (Xu et al., 2000; Dziedzicka-Wasylewska et al., 2006), and the density of α_2-adrenoceptors increased in several regions of the brain and spinal cord of NET KO mice relative to WT mice (Bohn et al., 2000; Gilsbach et al., 2006).

2.5.2 NET gene structure

Human NET (hNET) is encoded by a single gene, *SLC6A2* (solute carrier family 6 member 2), that is located on chromosome 16 in locus 16q12.2 (Pacholczyk et al., 1991; Porzgen et al., 1995). The hNET gene consists of 14 coding exons separated by 13 introns and spans approximately 45 kb. The structure of the mouse NET gene, located on chromosome 8 (homologous with 16q12.2 of the human genome), is similar to the hNET gene (Fritz et al., 1998). Moreover, the organization of the hNET gene is highly homologous to other neurotransmitter transporter genes. However, several NET gene variants arising from alternative mRNA splicing during gene expression have been identified in a human neuroblastoma cell line and in some mammalian tissues (Burton et al., 1998; Porzgen et al., 1998; Kitayama et al., 1999).

2.5.2.1 Alternative splicing

Two alternatively spliced hNET transcripts differing only in the distal residues within the C-termini have been identified from cultured human neuroblastoma SKN-SH-SY5Y cells (Porzgen et al., 1998). The original hNET transcript terminates after exon 14, encoding the last seven amino acids (LQHWLAI) of the hNET C-terminus, whereas the two alternatively spliced transcripts join exon 13 to a new exon (exon 15), skipping exon 14. The alternatively spliced exon 15 encodes for 3 (LSF) or 18 (MKTRQGRRRATNSCQISC) amino acids, resulting in short and long forms of the C-terminal hNET splice variants (hNET-Ex15S and hNET-Ex15L, respectively) (Porzgen et al., 1998). The C-terminal hNET splice variants have been shown to accumulate as immature proteins, and thereby display diminished transport capacity due to inefficient cell surface targeting (Kitayama et al., 2002). In addition, C-terminal splice variants of NET have been identified from rat (Kitayama et al., 1999) and bovine (Lingen et al., 1994; Burton et al., 1998) tissues. Also these splice variants are retained in the

endoplasmic reticulum (ER) when expressed in heterologous host cells, resulting in almost complete loss of transport function (Burton et al., 1998).

2.5.2.2 Single nucleotide polymorphisms

Human genome sequencing efforts have identified approximately 20 non-synonymous single nucleotide variations, called single nucleotide polymorphisms (SNPs), in the promoter, intronic and coding regions of the hNET gene. NET SNPs may affect protein expression, trafficking, substrate transport, ligand selectivity or give rise to total loss of NET function (Hahn and Blakely, 2007). Several studies have investigated the association between *SLC6A2* SNPs and neuropsychiatric as well as cardiovascular diseases (Tellioglu and Robertson, 2001; Hahn and Blakely, 2002).

The first disease-associated transporter variant in the SLC6 family was a non-synonymous SNP of hNET in exon 10, where an alanine residue is replaced with a proline residue (A457P) in the highly conserved TMD 9 (Shannon et al., 2000; Hahn et al., 2003). The A457P mutation results in 98 % loss of transporter function compared to the WT transporter (Hahn et al., 2003); thus, this genetic defect in the NET protein was suggested to contribute to the tachycardia and increased plasma NE levels of a pair of identical twins suffering from a familial form of orthostatic intolerance (Shannon et al., 2000; Robertson et al., 2001; Hahn et al., 2003). The loss of NET-A457P transport activity was reported to result from impaired biosynthetic processing of NET-A457P to the fully glycosylated form and reduction in surface expression to 30 % of normal. Furthermore, hNET-A457P was shown to exert a dominant negative effect when co-expressed together with WT NET (Hahn et al., 2003).

In addition, another SNP in the promoter region of the hNET gene was shown to alter the binding of transcription factors and to lead to loss of *SLC6A2* promoter function, and was suggested to associate with ADHD (Kim et al., 2006). A polymorphism in the non-coding region of the hNET gene has also been suggested to associate with anorexia nervosa (Urwin et al., 2002). Nonetheless, there is no direct evidence that these *SLC6A2* variants are causally related to these psychiatric disorders.

2.5.3 NET localization in vivo

The regional expression of NET *in vivo* has been investigated with radioligand binding techniques employing selective NET inhibitors, such as [^{3}H]-nisoxetine, with NE uptake studies and by immunocytochemical approaches. [^{3}H]-nisoxetine has high affinity for NET (K_d 0.7 nM) and low affinity for both SERT and DAT (K_d 1 mM) (Tejani-Butt et al., 1990; Ordway et al., 1997). Autoradiography studies of [^{3}H]-nisoxetine binding sites in rat and human brain have reported NET expression in noradrenergic cell groups and in brain regions receiving noradrenergic dendritic and axonal projections, such as the cerebellum, medulla-pons region, midbrain, cerebral

cortex and hypothalamus. In postmortem human brains, the amount of [^{3}H]-nisoxetine binding at any particular level of the brainstem varied considerably among individuals (Tejani-Butt et al., 1990; Tejani-Butt and Ordway, 1992; Ordway et al., 1997). [^{3}H]-nisoxetine labeling in the dorsal raphe nucleus, where many serotonergic cell bodies are located, was suggested to reside on the terminals of noradrenergic fibers that are known to innervate this brain region (Donnan et al., 1991; Tejani-Butt, 1992). Low labeling with [^{3}H]-nisoxetine has been detected in the substantia nigra (Ordway et al., 1997). [^{3}H]-nisoxetine binding to NET is virtually eliminated by treatment with noradrenergic neurotoxins, such as 6-hydroxydopamine (Tejani-Butt et al., 1990; Ordway, 1995).

Immunohistochemical studies based on NET antibodies have reported NET protein expression in noradrenergic somata and efferent projections in the same brain regions where NET was observed with autoradiography. In the brainstem noradrenergic somata, NET immunoreactivity has been reported to locate intracellularly and to be absent from the plasma membrane. NET labeling in the axonal projections has appeared non-uniform and enriched in varicosities (Schroeter et al., 2000; Miner et al., 2003). No NET immunoreactivity has been reported in glia cells (Schroeter et al., 2000). In the periphery, NET protein expression is present in sympathetic neurons, adrenal chromaffin cells and in the maternal-facing membranes of the human placenta (Ramamoorthy et al., 1993; Cubells et al., 1995; Ungerer et al., 1996; Schroeter et al., 2000; Backs et al., 2001; Li et al., 2001). In the placenta, NET and other monoamine transporters have been suggested to control the concentrations of circulating monoamines, thus attenuating vasoconstriction of the placental blood vessels and helping to secure stable blood flow to the fetus (Bottalico et al., 2004). NET protein expression has also been detected in cultured neuroendocrine tumor cell lines, such as SKN-SH-SY5Y cells and rat pheochromocytoma (PC12) cells (Bonisch and Bruss, 1994).

The subcellular distribution of NET has been investigated with immunogold techniques and electron microscopy (Miner et al., 2003). In rat prefrontocortical terminals, NET immunoreactivity was unexpectedly observed predominantly in the cytoplasm and only occasionally on the plasma membranes. Intracellularly located NET was mainly associated with small vesicles, likely to represent synaptic vesicles (Miner et al., 2003). Thus, the subcellular localization of NET differs from SERT and DAT that are mainly localized in plasma membranes in the prefrontal cortex and striatum, respectively (Nirenberg et al., 1996; Hersch et al., 1997; Miner et al., 2000). Dual immunogold labeling of NET and DβH in the prefrontal cortex demonstrated co-localization of the two noradrenergic marker proteins in the same axonal profiles, whereas a remarkably small proportion, approximately 10 % of NET immunoreactive terminals, were dually labeled for NET and TH (Miner et al., 2003). The small fraction of NET immunoreactive terminals also containing TH in the rat prefrontal cortex might be ascribed to the experimental procedures (e.g. inadequate sensitivity of TH labeling), or maybe the TH antiserum labeled predominantly DA axons in the cortical areas receiving both NE and DA innervation. *In situ* hybridization studies to visualize NET mRNA have mainly reported NET gene expression in the brainstem, supporting the

notion that synthesis of the transporter protein takes place predominantly in the noradrenergic somata and proximal dendrites (Lorang et al., 1994; Eymin et al., 1995).

The ontogeny of NET expression during embryonic and postnatal development has been investigated in rodents. NET is expressed already in the young embryo (Sieber-Blum and Ren, 2000), and its expression remains high in the brainstem in the early postnatal period but decreases significantly during maturation (Sanders et al., 2005). In contrast, NET expression in forebrain structures increases during maturation, i.e. NET binding sites increase approximately five-fold from postnatal day 10 to day 15, to similar levels as generally found in the adult brain (10 fmol/mg tissue) (Sanders et al., 2005). The developmental alterations in NET expression levels suggest an important role for the transporter in the development of the brain and its noradrenergic functions (Hu et al., 2009); still, NET KO mice are viable and no major abnormalities have been detected in their neuroanatomy.

2.5.4 NET protein structure

NET and the other highly homologous proteins of the *SLC6* gene family share a common structure of 12 transmembrane helical segments with intracellularly located N- and C-termini (Figure 4). The first high resolution crystal structures of a prokaryotic homolog of the SLC6 transporters, a leucine transporter (LeuT) from the hyperthermophilic bacterium *Aquifex aeolicus*, confirmed this overall tertiary structure and identified the primary substrate and ion binding sites between TMDs 1, 3, 6 and 8, approximately halfway across the membrane-spanning region (Yamashita et al., 2005; Singh et al., 2007; Singh et al., 2008; Penmatsa and Gouaux, 2014). Recently, the first high resolution crystal structures of the human SERT (Coleman et al., 2016) and eukaryotic *Drosophila melanogaster* DAT (dDAT) in complex with both substrates and inhibitors (Penmatsa et al., 2013a, b, 2015; Wang et al., 2015) have further increased the understanding of the structure-function relationships of the monoamine transporters. The dDAT crystal structures confirmed that the central binding site of the monoamine transporters is formed by the “hinge-like” regions of TMDs 1 and 6 together with residues of TMDs 3 and 8 (Figure 4). All dDAT substrates and inhibitors with varying sizes and shapes bind to the central binding site, suggesting high plasticity of the region. Furthermore, an X-ray structure of human SERT identified an allosteric site ´above´ the central binding site at the periphery of the extracellular vestibule obstructing ligand dissociation from the central site, providing an explanation how allosteric ligands can reduce the off-rate of inhibitors bound to the central site of SERT (Coleman et al., 2016).

Three residues in the large second extracellular loop of NET have been identified as canonical N-linked glycosylation sites (Pacholczyk et al., 1991) (Figure 4). Two major forms of the NET protein with glycosylation extensions have been identified: immature and mature (migrating at 54 kDa and 80 kDa, respectively) (Melikian et al., 1994; Kippenberger et al., 1999). Metabolic pulse-chase labeling experiments have suggested that the immature NET (54 kDa) is a short-lived metabolic intermediate that is further

processed or degraded in a few hours (Melikian et al., 1996). Only a small fraction of the fully glycosylated NET (80 kDa) was detected at the cell surface in PC12 cells, whereas fully glycosylated NET (80 kDa) was highly enriched at the plasma membrane in HEK 293 and LLC cells, suggesting that NET could be targeted differently in neuronal and epithelial cells (Melikian et al., 1996; Burton et al., 1998; Kippenberger et al., 1999). For a note, also DAT and SERT have been found to undergo heterogenous *N*-glycosylation with regional and developmental variation (Patel et al., 1994; Qian et al., 1995). Mutation of the three canonical *N*-glycosylation sites of NET (NET N184,192,198Q) has been shown to decrease transporter stability and significantly reduce the uptake capacity compared to WT NET. However, the residual surface-expressed NET N184,192,198Q exhibits similar pharmacology to WT NET (Melikian et al., 1996; Nguyen and Amara, 1996). The half-life ($t_{1/2}$) of mature NET, as determined by pulse-chase analysis in transfected HeLa and COS cells, has been estimated to be approximately 24 h (Melikian et al., 1996), whereas the half-lives of DAT and SERT, as determined *in vivo* in rat brain with intraventricular administration of irreversible inhibitors, were estimated to be 2 and 3.4 days, respectively (Vicentic et al., 1999; Kimmel et al., 2000).

2.5.5 Mechanism of transport

NET-mediated substrate transport is saturable and dependent on the Na^+ gradient across the plasma membrane that is maintained by Na^+/K^+ ATPase activity (Kristensen et al., 2011). NET is believed to translocate one positively charged NE molecule with one Na^+ ion and one Cl^- ion (stoichiometry of 1 NE^+: 1 Na^+: 1 Cl^-) (Figure 5). Thus, NE transport by NET is electrogenic with a positive charge (Trendelenburg et al., 1991). Kinetic studies of NE transport suggest that binding of Na^+ and Cl^- is necessary for the substrate to bind to the transporter (Trendelenburg et al., 1991; Bonisch and Bruss, 1994). The transport turnover rate has been variably estimated to range from 1-2.5 transport cycles per second (Gu et al., 1996) to 0.4-0.6 transport cycles per second (Bonisch and Harder, 1986; Galli et al., 1995; Sucic and Bryan-Lluka, 2005).

The translocation of substrates across the plasma membrane has been suggested to function *via* an alternating access model, where the inner and outer gates of NET are predicted to open alternately trough successive conformational changes (Jardetzky, 1966) (Figure 5). According to this classical simple allosteric model for membrane pumps, substrate molecules and ions bind to the central binding pocket in the ´outward open´ conformation, where the extracellular gate is open, the binding sites are exposed to the extracellular environment and the intracellular gate is closed. Binding of the substrate and ions will trigger the closure of the outer gate (´outward-occluded´ conformation), and induce the opening of the intracellular gate (´inward open´ conformation), allowing the substrate and ions to dissociate. Subsequently, the transporter will close the inner gate and return to an ´outward open´ conformation (Kristensen et al., 2011) (Figure 5). The crystal structures of LeuT in different conformations support this model (Penmatsa and Gouaux, 2014), but the precise mechanism of the transport is still debated. In addition to the secondary active transport

of its substrate across the plasma membrane, NET has been reported to have rapid channel-like activity, with the formation of an adequate actual pore through the transporter protein during the transport cycle (Galli et al., 1996). This hypothesis is supported by studies on amphetamine that can induce reversal of NE transport by NET that occurs both in slow carrier-like mode and in rapid channel-like mode (Robertson et al., 2009).

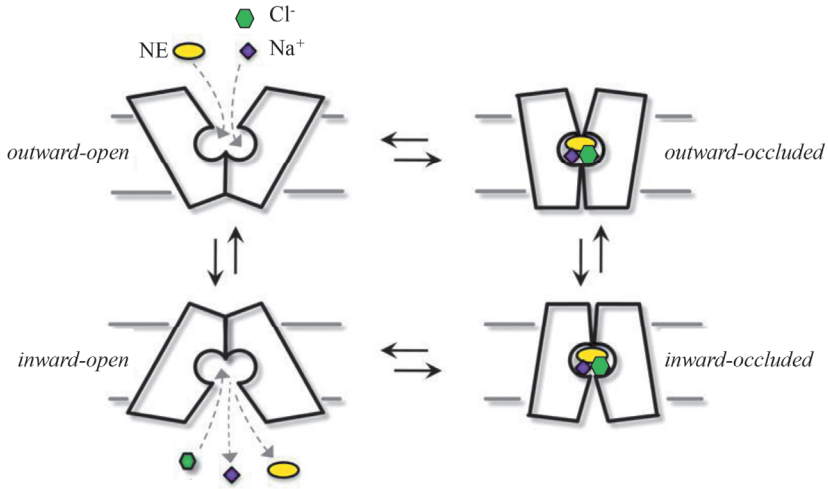

Figure 5. NET-mediated transport of substrate and ions is believed to follow an alternating access model. NE binds together with one sodium ion and one chloride ion to the central binding pocket of the transporter in the outward open conformation initating the transport cycle, which involves closing of the extracellular gate and opening of the internal gate via the outward occluded and inward occluded intermediate conformations. Opening of the intracellular gate exposes the binding site to the cytoplasm and allows dissociation of substrate and ions (modified from Kristensen et al., 2011)

2.5.6 NET substrates and inhibitors

NET transports all three endogenous catecholamines, DA, NE and EPI, but with different affinities (K_m 0.1 µM for DA, 0.5-0.8 µM for NE and 3 µM for EPI) (Buck and Amara, 1994; Giros et al., 1994; Apparsundaram et al., 1997; Gu et al., 2001). The maximal velocity (V_{max}) for DA transport is approximately four-fold higher than for NE (Giros et al., 1994); thus, NET transports DA more efficiently than NE when evaluated by the V_{max}/K_m ratio that is commonly used as an indicator of the catalytic efficiency of the translocation. *In vivo*, NET has been shown to mediate cellular uptake of DA in those brain regions where NET is more concentrated than DAT, such as in the frontal cortex (Moron et al., 2002). NET also transports other monoamines, such as serotonin and many indirectly acting sympathomimetic amines, such as tyramine and *d*-amphetamine.

Besides its physiological function as a regulator of monoamine levels in the brain, NET constitutes an important target for the treatment of several neuropsychiatric disorders,

such as depression and ADHD. Thus, many selective NET inhibitors have been developed in spite of its high structural homology with DAT and other members of the SLC6 protein family and the similar structures of their endogenous substrates (Tatsumi et al., 1997; Eshleman et al., 1999). Widely used tricyclic antidepressant drugs, such as amitryptiline and desipramine, inhibit the action of NET, SERT and DAT with varying affinities, whereas the newer selective NE reuptake inhibitors (NRIs), such as reboxetine and atomoxetine, inhibit the action of NET at nanomolar concentrations (Owens et al., 1997; Burrows et al., 1998; Sacchetti et al., 1999; Hajos et al., 2004). Inhibition of NET and other neurotransmitter transporters and receptors by antidepressants increases the synaptic levels of NE, 5-HT and DA, which is believed to underlie the mechanism of antidepressant actions (Zhao et al., 2008; Zhao et al., 2009). However, the therapeutic antidepressant effect becomes apparent after several weeks of daily treatment, and the molecular mechanisms of actions during long-term exposure to antidepressants are not fully understood.

NET is also targeted by several psychostimulants that elevate synaptic NE levels by different mechanisms of action (Ritz et al., 1990; Pacholczyk et al., 1991; Wall et al., 1995). Cocaine and its derivatives inhibit the reuptake activity of NET and other monoamine transporters by binding competitively to the central binding site and by thus preventing the binding and subsequent uptake of NE (Beuming et al., 2008). Amphetamine and its derivatives also competitively inhibit the binding of the endogenous substrates to NET and to other monoamine transporters, but in addition, they are also substrates of the monoamine transporters and are therefore actively transported into monoaminergic neurons (Robertson et al., 2009). Inside the neuron, amphetamine and its derivatives is further taken up into synaptic vesicles and they translocate NE, as well as DA and 5-HT, from the vesicles into the cytoplasm. Furthermore, amphetamine evokes reverse transport of monoamines out of neurons *via* the monoamine transporters, which further elevates the extracellular levels of monoamines (Zhu et al., 2000). The precise molecular mechanisms of amphetamine - evoked efflux are not fully understood but they have been proposed to involve a channel-like transporter mode or facilitated exchange diffusion (Wall et al., 1995; Sulzer et al., 2005). The main behavioral effects of cocaine, amphetamine and their derivatives, i.e. reinforcement and positive subjective effects are considered to be mediated mainly through DAT, but also NET and SERT appear to be involved (Rocha et al., 1998; Sora et al., 2001). Several psychostimulants acting on monoamine transporters are currently in therapeutic use, i.e. in the management ADHD and narcolepsy (Howell and Kimmel, 2008).

2.6 Cellular regulation of NET

It was originally considered that NET has a static presence in the plasma membrane and that its function in terminating NE-mediated cellular signaling is stable. However, numerous recent studies have shown that NET, like the other members of the SLC 6 family of neurotransmitter transporters, is subject to dynamic regulation by diverse stimuli, including membrane potential changes, substrate exposure and intracellular

second messengers, providing mechanisms for fine-tuning of the NE homeostasis. The next sections describe different aspects of the cellular regulation of NET.

2.6.1 NET gene expression and biosynthetic pathway

NET gene expression in noradrenergic neurons and in neurosecretory cells is regulated by physiological and pharmacological stimuli, such as neurotransmitters, growth factors and drugs, suggesting that changes in environmental demands may result in altered noradrenergic neurotransmission *via* alterations of NET gene expression (Mandela and Ordway, 2006b). For example, prolonged administration of antidepressants targeting NET has been shown to alter NET gene expression (Zhao et al., 2009). The promoter region of the NET gene has multiple transcription start sites that appear to be clustered into two subdomains preceded by a TATA-like sequence motif (Kim et al., 1999). However, the mechanisms of transcriptional regulation of NET are still poorly understood. Some transcription factors, such as the Paired mesoderm homeobox protein 2 (Phox2), have been shown to regulate NET gene expression (Kim et al., 1999; Esler et al., 2006; Fan et al., 2011). In addition, the promoter region of the NET gene is rich in CpG dinucleotides, suggesting that the NET promoter is a target for DNA methylation-related gene silencing (Esler et al., 2006).

Newly synthesized proteins are folded in the ER from where they then are passed forward via the Golgi compartment towards their sites of action, or targeted to ER-associated degradation (Ellgaard and Helenius, 2003) (Figure 6). Both intrinsic sorting signals expressed in the cytoplasmic domains of NET and NET oligomerization are suggested to be important for the ER exit of NET and transit of the transporter through the Golgi compartment to the plasma membrane. Evidence from research on NET splice variants indicates that the NET C-terminal sequence is important for correct targeting and surface expression of the transporter; the two splice variants with exon 15 sequences, hNET-Ex15L and hNET-Ex15S, are unable to efficiently complete their transit through the Golgi compartment and accumulate as immature proteins (Bauman and Blakely, 2002). However, ER retention of immature splice variants might involve additional mechanisms specific to exon 15 sequences. Artificial truncations of the NET C-terminus, including loss of the terminal isoleucine alone (hNET-Δ617), or deletion of the last three hydrophobic amino acids (hNET-ΔLAI) that comprise a type II PSD95/Discs-large/ZO-1 (PDZ)-binding domain (see section 2.6.5.), result in significant increases in protein degradation and diminish the levels of surface NET in neuronal SK-N-MC cells. This phenotype is significantly less severe in HEK 293 cells, suggesting that the biosynthetic processing of NET is dependent on the host cell type (Bauman and Blakely, 2002). Complete deletion of the NET C-terminus (residues 575-617) abolishes NE transport activity, suggesting that efficient maturation, surface targeting and acquisition of transport capacity require the recognition of the C-terminal residues of NET (Bauman and Blakely, 2002). Similarly, C-terminal deletion mutants of DAT and SERT demonstrate the importance of an intact C-terminus for proper surface expression of the monoamine transporters (Torres et al., 2001; Bjerggaard et al., 2004; Larsen et al., 2006). However, in polarized epithelial cells, the basolateral

sorting signal of NET has been shown to be located in the N-terminus, whereas DAT was predominantly located in the apical plasma membrane, and insertion of the NET N-terminus to DAT directed this transporter to the basolateral membrane (Gu et al., 2001). Also the apical localization signal of the GABA transporter 1 (GAT1) appears to reside in the N-terminus of the protein, although the basolateral localization signal for GAT2 was reported to be located in the C-terminus (Perego et al., 1997; Muth et al., 1998; Brown et al., 2004). The biosynthetic processing of the monoamine transporters has been suggested to be regulated also by transporter oligomerization (Kilic and Rudnick, 2000; Hastrup et al., 2001; Scholze et al., 2002; Hahn et al., 2003; Kocabas et al., 2003; Sorkina et al., 2003; Torres et al., 2003b; Sitte et al., 2004). NET has been shown to form homo-oligomers in heterologous cell lines, and oligomerization has been suggested to be important for the transport of the cargo from the ER to the plasma membrane (Hahn et al., 2003).

Several lines of evidence suggest that Golgi-derived NET is mainly transported to the plasma membrane in a regulated manner. Depolarization has been shown to increase NET function and surface expression in a Ca^{2+} -dependent manner (Savchenko et al., 2003; Mandela and Ordway, 2006a). In neurosecretory cells, such as PC12 and chromaffin cells, NET has been suggested to traffic to the plasma membrane in secretory vesicles (equivalent to dense core vesicles) since NET colocalizes with markers of large dense core granules (Kippenberger et al., 1999; Schroeter et al., 2000). Little association of NET has been observed with markers of synaptic vesicles, such as VMAT2 and synaptophysin, in dissociated sympathetic neurons in culture whereas in cortical brain slices, terminal NET was shown to colocalize with markers of synaptic vesicles (Matthies et al., 2009). Also, NET surface expression and function is reduced by suppression of the SNARE (soluble *N*-ethylmaleimide-sensitive attachment factor receptors) protein syntaxin 1A (Sung et al., 2003; Dipace et al., 2007; Sung and Blakely, 2007), suggesting that NET is at least partly located in small synaptic vesicles. Thus, NET might be located in different vesicle types in different host cells.

2.6.2 Lipid rafts

Growing evidence indicates that membrane proteins, including the monoamine transporters, might be segregated into distinct plasma membrane domains with high cholesterol density, suggested to represent lipid rafts. Lipid rafts (also called membrane rafts) are small (ø 10 - 200 nM), sterol- and sphingolipid-enriched regions of the plasma membrane with a tight lipid packing (Adkins et al., 2007; Cremona et al., 2011). Lipid rafts are believed to compartmentalize cellular processes, act as sites of endocytosis and modulate cytoskeletal networks (Brown and London, 1998, 2000; Allen et al., 2007; Otto and Nichols, 2011). Recently, it was shown that the lipid environment might even act as an allosteric modulator of some membrane proteins (Dawaliby et al., 2015). In placental trophoblasts, a fraction of plasma membrane resident NET has been suggested to associate with lipid rafts, and rafts to mediate NET endocytosis in a clahtrin-independent manner (Jayanthi et al., 2004). A fraction of NET was also shown to localize in lipid rafts in SCG neurons and to colocalize with

syntaxin 1A, possibly suggesting involvement of lipid rafts in NET exocytosis in SCG neurons (Matthies et al., 2009). In addition, evidence suggests that other SLC6 family transporters, such DAT, SERT and excitatory amino acid transporter 2 (EAAT2), associate with lipid rafts, even if the precise significance of the associations has not been elucidated (Samuvel et al., 2005; Adkins et al., 2007; Foster et al., 2008; Cremona et al., 2011).

2.6.3 Constitutive internalization of NET

The modulation of monoamine transporters occurs largely through redistribution of the transporter proteins from the cell surface rather than changes in the intrinsic transport activity. Thus, it has been hypothesized that endocytic trafficking is the major means by which SLC6 transporters are functionally regulated (Beckman and Quick, 1998; Blakely et al., 1998). A substantial proportion of neuronal NET resides in intracellular vesicles within the somato-dendritic region and in axon terminals (Leitner et al., 1999; Schoroeter et al., 2000; Miner et al., 2003, 2006), indirectly suggesting that the transporter is dynamically shuffled between intracellular compartments and the plasma membrane. Several SLC6 family transporters, such as DAT, SERT and the glycine transporter, have been shown to undergo constitutive internalization (Loder and Melikian, 2003; Fornes et al., 2008; Eriksen et al., 2009; Eriksen et al., 2010a; Rahbek-Clemmensen et al., 2014b). However, the constitutive trafficking properties of NET have not been investigated in detail.

From the plasma membrane, proteins may be endocytosed in a clathrin-dependent or -independent manner into early endosomes, from where they are sorted either into recycling endosomes or into degradation pathways (Bonifacino and Glick, 2004) (Figure 6). The endosomal compartments are characterized by different Rab proteins, small GTPases that have specific localizations and functions in distinct steps of endocytic trafficking (Maxfield and McGraw, 2004; Schwartz et al., 2008; Stenmark, 2009). Studies on cultured fibroblasts have shown that Rab5 regulates cargo internalization into early endosomes, whereas Rab4 contributes to the vesicular transport from early endosomes to the plasma membrane, marking the “fast recycling” pathway (Gorvel et al., 1991; Bucci et al., 1992). In contrast, Rab11 mediates cargo recycling back to the plasma membrane from the perinuclear endocytic recycling compartment, marking the “slow recycling” pathway (Ullrich et al., 1996; Chen et al., 1998). The lysosomal pathway is characterized by Rab7 which is required for vesicle transport from early endosomes to late endosomes and to lysosomes (Feng et al., 1995; Meresse et al., 1995). Dominant negative mutant forms of numerous Rab proteins have been generated to dissect the specific function of the Rab proteins in membrane trafficking.

The fate of constitutively internalized SLC6 family transporters has not been clearly defined. DAT was suggested to constitutively internalize and recycle back to the plasma membrane in rat PC12 cells, since DAT surface levels and DA transport were shown to be decreased when cells were preincubated at 18 °C (referred to as non-

trafficking conditions) compared to 37 °C (Loder and Melikian, 2003). In addition, monensin (20 µM), a cation ionophore used as recycling inhibitor (Mollenhauer et al., 1990), was shown to time-dependently increase the intracellular accumulation of DAT in porcine aortic endothelial cells stably expressing DAT (Sorkina et al., 2005). However, more recent studies using confocal imaging and biochemical approaches support the notion that DAT, as well as SERT, is primarily targeted to Rab7 -positive late endosomes and subsequently to lysosomal degradation upon both constitutive and stimulated internalization (Daniels and Amara, 1999; Eriksen et al., 2010b; Rahbek-Clemmensen et al., 2014b). In neuroendocrine cells and in sympathetic neurons, intracellularly located NET has been shown to partly associate with markers of both lysosomes (Lamp-1) and recycling endosomes (Rab11) at steady-state conditions (Kippenberger et al., 1999; Matthies et al., 2009). However, the postendocytic sorting of constitutively internalized NET has not been characterized.

The molecular mechanisms and structural elements responsible for different internalization pathways of the SLC6 family transporters are not fully understood, but evidence suggests that residues in the N- and C-termini can influence the trafficking properties of the transporters (Distelmaier et al., 2004). A short sequence in the DAT C-terminus (FREKLAYAIA), which is only partially conserved in NET, was shown to be important for the constitutive and PKC-mediated internalization of DAT (Holton et al., 2005). Mutation of the cognate sequence (584–593) in NET to alanines (NET584–59310A) decreased the internalization rate of the transporter compared to wild-type NET (Holton et al., 2005), suggesting that the C-terminal residues are important also for NET internalization.

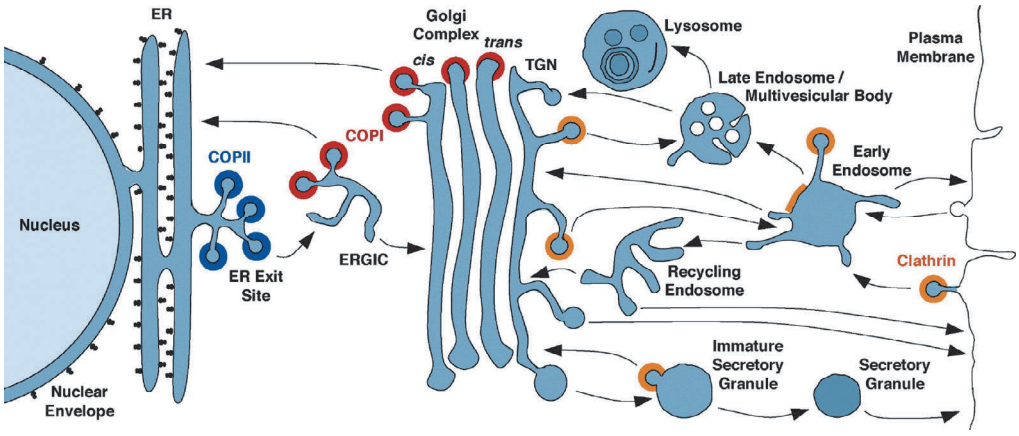

Figure 6. Schematic representation of the intracellular trafficking pathways of cell surface proteins. Newly synthesized membrane proteins are transported through the secretory pathway (endoplasmic reticulum (ER), ER-Golgi intermediate complex (ERGIC), Golgi and trans-Golgi network (TGN)). COPI and COPII protein complexes coat vesicles (shown in red and blue, respectively) transporting proteins between ER, ERGIC and Golgi. Endocytosis of proteins from the plasma membrane is mediated in clathrin-dependent (yellow) and -independent manners, and postendocytic sorting to recycling or lysosomal pathways takes place in early endosomes (Bonifacino and Glick, 2004).

2.6.4 Stimulated internalization of NET

Growing evidence indicates that diverse stimuli, including second messengers generated after receptor activation, can regulate the subcellular distribution and function of monoamine transporters, with possible impact on the dynamics and concentrations of monoamines present in the synaptic cleft (Zahniser and Doolen, 2001; Torres et al., 2003a; Kristensen et al., 2011). In addition, NET substrates and inhibitors have shown to influence NET distribution and function. In cellular expression systems, NE has been shown to concentration-dependently reduce NET function (Zhu and Ordway, 1997), possibly through oxidative stress pathways (Mao et al., 2004; Mao et al., 2005). Moreover, amphetamine has been shown to induce redistribution of NET from the plasma membrane in a time- and concentration-dependent manner (Zhu et al., 2000; Matthies et al., 2010). Amphetamine - mediated reductions of cell surface NET density have been shown to be both Ca^{2+}- and protein kinase dependent in catecholaminergic cell lines and in rat cortical slices (Kantor and Gnegy, 1998; Kantor et al., 2001; Robertson et al., 2009; Matthies et al., 2010). Both PKC and PKA, as well as Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) have been suggested to be involved in the downstream signaling initiated by amphetamine (Robertson et al., 2009). Furthermore, amphetamine -induced downregulation of NET has been suggested to involve interactions of NET and syntaxin 1A (Dipace et al., 2007).

Repeated exposure (21 days) of rats to the tricyclic antidepressant drug desipramine has been shown to decrease [^{3}H]-nisoxetine binding in several brain regions (Bauer and Tejani-Butt, 1992; Hebert et al., 2001), whereas NET mRNA levels were increased in response to repeated exposure to desipramine (Szot et al., 1993). Moreover, 3-day exposure to desipramine was found to reduce NET uptake capacity, binding sites and immunoreactivity in PC12 cells (Zhu and Ordway, 1997; Zhu et al., 1998; Ordway et al., 2005). A single dose of desipramine had no effects on [^{3}H]-nisoxetine binding in the rat brain (Bauer and Tejani-Butt, 1992). Studies investigating chronic exposure to cocaine have resulted in incongruent findings. Continuous exposure to cocaine does not seem to downregulate NET in cultured cells but chronic self-administration of cocaine was found to increase NET binding sites in several brain regions of non-human primates (Zhu et al., 2000; Macey et al., 2003; Beveridge et al., 2005). Consistently with this, increased NET protein levels were reported in postmortem brains of human habitual cocaine users (Mash et al., 2005).

Amino acid sequence analysis of NET, as well as the other SLC6 family transporters, has revealed numerous consensus phosphorylation sites within the intracellular domains as well as in the N- and C-termini. Involvement of PKC in the regulation of monoamine transporter function and surface expression has been amply documented in both native preparations and in heterologous expression systems (Huff et al., 1997; Vaughan et al., 1997; Blakely et al., 1998; Apparsundaram et al., 1998a; Apparsundaram et al., 1998b; Jayanthi et al., 2004; Eriksen et al., 2010b). In a variety of preparations, including synaptosomes, primary cells and different heterologous cell lines, application of the PKC activator, phorbol 12-myristate 13-acetate (PMA), has

been demonstrated to redistribute NET, DAT and SERT from the plasma membrane to intracellular compartments (Vaughan et al., 1997; Ramamoorthy et al., 1998; Apparsundaram et al., 1998a; Apparsundaram et al., 1998b; Jayanthi et al., 2004; Jayanthi et al., 2005). PMA-induced downregulation of NET is accompanied with decreases in the maximal transport velocity of NE (Apparsundaram et al., 1998a; Apparsundaram et al., 1998b; Jayanthi et al., 2004; Jayanthi, 2006). In placental trophoblasts, NET was shown to undergo clahtrin-independent and lipid raft-dependent endocytosis in response to activation of PKC by PMA (Jayanthi et al., 2004). Interestingly, clathrin-mediated and dynamin-dependent internalization is involved in the regulation of DAT and GAT1 trafficking (Deken et al., 2003; Sorkina et al., 2005; Eriksen et al., 2009). In addition, inhibition of CaMKII has been reported to reduce NE uptake in SK-N-SH cells (Apparsundaram et al., 1998a). The possible impact of intracellular levels of cyclic AMP (cAMP) and concomitant activity of cAMP-dependent kinases on NET surface availability has not been clearly defined. Forskolin, a direct activator of adenylyl cyclases, has been suggested to mediate biphasic effects on NE uptake. At low concentrations (1-3 μM), forskolin has been shown to increase NE uptake, while at higher concentrations (10-100 μM) forskolin inhibits NET function (Mandela and Ordway, 2006b). However, cAMP analogues had no effects on NE uptake in human SK-N-SH-SY5Y cells endogenously expressing NET or in COS cells transiently expressing hNET (Apparsundaram et al., 1998a; Bryan-Lluka et al., 2001).

The peptide hormone insulin has been shown to regulate monoamine transporter function and monoamine homeostasis. Insulin has been reported to inhibit NE uptake at nanomolar concentrations in PC12 cells, primary neuronal cultures, synaptosomes and hypothalamic and hippocampal brain slices (Boyd et al., 1985; Boyd et al., 1986; Masters et al., 1987; Figlewicz et al., 1993). Robertson et al. (2010) demonstrated that in mouse hippocampal slices, SCG neuronal boutons (sites of synaptic NE release) and in NET-expressing CHO cells, acute (20 min) insulin treatment (1-1000 nM) decreased NET surface levels in a concentration-dependent manner, and pretreatment with an Akt inhibitor abolished the insulin-mediated downregulation of NET (Robertson et al., 2010). Furthermore, treatment with streptozotocin that induces a type 1-like diabetic hypoinsulinemic state in mice has been reported to increase NET surface expression in hippocampal slices and to increase NE clearance in the dentate gyrus, suggesting that aberrations of insulin signaling have the potential to alter NET function and noradrenergic tone in the brain (Robertson et al., 2010; Siuta et al., 2010). Surprisingly, insulin was reported to have opposite effects on DAT: acute insulin treatment and overexpression of constitutively active phosphatidyl-inositol (PI)-3 kinase were found to increase DA uptake (Carvelli et al., 2002), and insulin-depleted rats exhibit reduced cell surface expression of DAT (Williams et al., 2007).

2.6.5 Scaffolding of NET

Scaffolding proteins play important roles in the assembly of large multi-protein complexes, affecting the localization, trafficking and function of their binding partners.

NET, as well as DAT, has been shown to interact with the scaffolding proteins PICK1 (protein interacting with C kinase 1) and Hic5 (hydrogen peroxide-inducible clone-5) (Torres et al., 2001; Carneiro et al., 2002). The focal adhesion protein Hic-5 was shown to interact with the N-terminus of DAT, and overexpression of DAT together with Hic-5 in HEK 293 cells decreased the function and surface levels of the transporter compared to cells expressing only DAT. In contrast, the interaction of NET and Hic-5 was suggested to be mediated through the C-terminus of NET (Carneiro et al., 2002), but the functional implications of this interaction still remain to be explored.

NET, similarly to DAT, contains at its extreme C-terminus a type II PDZ binding domain that has been found to interact with the PDZ domain of PICK1 in a yeast two-hybrid screen (Torres et al., 2001). For DAT, the interaction with PICK1 was originally proposed to be important for the ER export of the transporter, since overexpression of PICK1 was shown to enhance DA transport, and truncation of the DAT C-terminus abolished the interaction with PICK1 and impaired the surface expression of the transporter in HEK 293 cells (Torres et al., 2001). Recently, the significance of the C-terminal PDZ domain binding sequence for DAT function was studied in knock-in mice expressing DAT mutants with disrupted PDZ binding domains, providing *in vivo* evidence that an intact PDZ motif of DAT is indeed crucial for proper surface expression of the transporter (Rickhag et al., 2013). However, the disrupted PDZ-binding domain did not disturb the maturation and ER-export of DAT but rather its stabilization in the presynaptic plasma membrane (Rickhag et al., 2013). Moreover, in mice with targeted deletion of the PICK1 gene, DAT distribution and protein levels were unaltered (Rickhag et al., 2013), indicating that PICK1 is not critical for synaptic DAT distribution *in vivo.* However, no other PDZ-domain containing proteins have been identified to interact with DAT or NET. For NET, PICK1 was shown to form intracellular clusters when co-expressed with NET in HEK 293 cells (Torres et al. 2001). However, the functional significance of the NET and PICK1 interaction is unknown.

2.7 NET and human diseases

Alterations in the noradrenergic circuitry of the brain have been associated with the pathophysiology of many neuropsychiatric and neurodegenerative disorders, such as depression, schizophrenia, ADHD, Parkinson's disease and Alzheimer's disease (Ressler and Nemeroff, 1999). It is possible that abnormalities in NET function may lead to perturbed synaptic NE levels and increase the risk of developing neurological and psychiatric disorders, as well as diseases of the cardiovascular system (Hahn and Blakely, 2002; Hahn and Blakely, 2007). Patients suffering from major depression have demonstrated reduced expression levels of NET in the brain compared to age-matched normal control subjects as measured by [^{3}H]-nisoxetine binding sites in postmortem brain samples (Klimek et al., 1997). In addition, changes in the levels of NE and its metabolites in cerebrospinal fluid and plasma have been reported in patients with major depression (Lake et al., 1982; Yehuda et al., 1998). Furthermore, several studies have investigated the possible associations of SNPs in the hNET gene to

depression and other psychiatric disorders, but no consistent associations have been identified (Mandela and Ordway, 2006b; Hahn and Blakely, 2007). The role of catecholaminergic neurotransmission in schizophrenia has been an area of intensive research, and increased NE levels in the brain and spinal fluid have been reported in patients with schizophrenia (Sternberg et al., 1982; van Kammen and Antelman, 1984; Breier et al., 1990). Possible associations of NET SNPs with bipolar disorder or schizophrenia have been investigated, but none have so far been identified (Need et al., 2009). Based on the important role of NE for maintaining attention and vigilance, it has been suggested that disturbed NE neurotransmission might contribute to the pathological mechanisms of ADHD (Biederman and Spencer, 1999). Moreover, a polymorphism in the NET promoter region, significantly decreasing promoter function, was suggested to associate with ADHD (Kim et al., 2006).

Despite the lack of direct evidence of the causality of altered NET function in psychiatric disorders, NET constitutes a primary target for commonly prescribed therapeutic agents used in the symptomatic management of depression, ADHD and eating disorders. Commonly prescribed antidepressants include tricyclic antidepressants, such as desipramine and amitriptyline. The NRI drug atomoxetine is commonly used in both children and adults for the treatment of ADHD.

Moreover, loss of brainstem noradrenergic neurons and diminished levels of NET binding sites in the brainstem are early events in major progressive neurodegenerative disorders, such as Parkinson's disease and Alzheimer's disease (Tejani-Butt et al., 1993; Zarow et al., 2003; Szabadi, 2013). In addition, NET could be an interesting target for both diagnosis and therapy of neuroblastoma since 90 % of neuroblastomas have been reported to express high levels of NET (Castleberry, 1997).

3 AIMS OF THE STUDY

The current series of studies was aimed to discover new information about the cellular regulation of NET distribution, trafficking and function. Cultured, dissociated sympathetic neurons were employed as the main test model. The goal was to elucidate the molecular mechanisms that regulate and maintain the function of NET in the plasma membrane of the noradrenergic neurons. It was hoped that such knowledge would be useful for the understanding of the spatio-temporal control of synaptic NE levels in physiological conditions, and to project how the function of NET, and therefore NE homeostasis, might be altered in different disease states.

The specific aims of this study were:

1. To characterize the cell surface distribution, constitutive internalization and postendocytic sorting of NET in dissociated noradrenergic neurons in culture, and to compare these properties to related transporter of dopamine in neuronal cell models.

2. To delineate the possible roles of protein kinases in the regulation of monoamine transporter function and to possible identify novel protein kinases involved in the regulation of function and/or distribution of monoamine transporters; and

3. To elucidate the involvement of the PDZ domain protein PICK1 in the regulation of NET function, and to compare the functional role of PICK1 interaction between NET and DAT.

4 MATERIALS AND METHODS

4.1 Outline of studies

The outlines of the present studies are summarized in Table 1.

Study	Used model system	Treatments	Aims
I	Dissociated SCG neurons in culture CAD cells	Lentivirus-mediated expression of Rab proteins Phorbol 12-myristate 13-acetate (PMA)	Characterize the constitutive internalization and postendocytic sorting of NET
II	HEK 293 cells CAD cells PC12 cells	siRNA mediated knockdown of 573 kinases	Identify kinases involved in regulation of NET, DAT and SERT function
III	Brain slices and synaptosomes from WT and PICK1 KO mice Dissociated SCG neurons in culture CAD cells	ShRNA mediated knockdown of PICK1 Molecular replacement of endogenous PICK1 with recombinant PICK1	Determine the role of the scaffolding protein PICK1 in regulation of NET expression and function

4.2 Molecular biology

4.2.1 Plasmids and DNA cloning

Mammalian pcDNA 3.1 expression vectors containing the cytomegalovirus (CMV) promoter and the human NET cDNA, the synthetic human DAT cDNA and human DAT cDNA tagged with a human influenza virus hemagglutinin (HA) epitope were kindly provided by Dr. Marc G. Caron, Duke University, Dr. Jonathan A. Javich, Columbia University and Dr. Alexander Sorkina, University of Pittsburg, respectively (study I, II and III). Human SERT and HA-tagged SERT in the pcDNA 3.1 vector were generated as previously described (Rasmussen et al., 2009; Rahbek-Clemmensen et al., 2014a) (study II). A pcDNA 3.1 plasmid containing the Flag-tagged β_2-adrenoceptor

cDNA was a kind gift from Dr. Mark von Zastrow, University of California (study II). The enhanced green fluorescent protein (EGFP)-tagged Rab7 and Rab11 constructs (pEGFP-Rab7 and pEGFP-Rab11) were kind gifts from Dr. Katherine W. Roche, NINDS, National Institutes of Health (study I). The plasmid pEGFP-Rab4 was a kind gift from Dr. José A. Esteban, Universidad Autónoma de Madrid (study I). The NET 1-128/DAT construct was a kind gift from Dr. Bruno Giros, Douglas Mental Health University Institute (study I). YFP-PICK1 and YFP-PICK1 A87L were generated as described (Madsen et al., 2008), and the N-terminal glutathione-S-transferase (GST) fusion construct of the rat PICK1 in the pET41a vector (Novagen) was produced as previously described (Thorsen et al., 2010) (study III). The viral vector plasmids pHsSynXW EGFP, PICK1 KD, EGFP-Rab4, -Rab7 -Rab11, - Rab11S25N, -PICK1, -PICK1A87L were generated as previously described (Eriksen et al., 2009) (study II, III). A DAT/NET590-617 chimeric construct was generated by first introducing a unique restriction site, Bsi I, to the coding sequence of NET by PCR, and by then ligating the C-terminal tail of NET into DAT using digestion with Bsi I and Xba (Table 2). Subsequently, the construct was cloned into pcDNA 3.1 (study I). DAT1-59/NET, NET/DAT593-620 and TacNET constructs were generated by a two-step PCR procedure as described in study I (Table 2). All constructs were verified by dideoxynucleotide sequencing (Eurofins Genomics, Ebersberg, Germany).

Table 2. A list of primers used for mutagenesis in studies I and III. Nucleotides that encode an artificial restriction enzyme recognition site (bold black) or overhangs (*italic*) used in the two-step PCR reactions are highlighted.

Construct	Primer name	Sequence
Study I		
DAT/NET590-617	sNET_Cterm_BsiWI	CAGCAGG**CGTACG**GCATCACGCCAGAGAACCAG
DAT/NET590-617	asNET_Cterm (XbaI)	GGCTGATCAGCGAGCTCTAGC
DAT1-59/NET	sNET_body	*GGCCCAGGAC*CGGGAGACCTGGGGCAAG
DAT1-59/NET	asNET_body	CACTATAGAATAGGGCCCTC
DAT1-59/NET	sDAT_Nterm	CCACTGCTTACTGGCTTATCG
DAT1-59/NET	asDAT_Nterm	*GGTCTCCCG*GTCCTGGGCCTCC
NET/DAT593-620	sNET_body	CACTATAGGGAGACCCAAGC
NET/DAT593-620	asNET_body	*CGATAGCGTACGC*CAGTCTCTCCCAAAGAGAGCC
NET/DAT593-620	sDAT_Cterm	*GGAGAGACTG*GCGTACGCTATCGCCCCCGAG
NET/DAT593-620	asDAT_ Cterm	GCTGATCAGCGAGCTCTAGC
Study III		
ShControl	sSh18_remove (AfeI)	AGTAACGGATCCTTTTTCTAGCCCCAAGGGCG
ShControl	asSh18_remove (BamHI)	TCGCCGAGAAGGGACTACTTTTCCTCGCCTG

4.2.2 siRNAs and shRNAs

RNA interference (RNAi) is a biological process employed as a research tool where RNA molecules, namely small interfering RNAs (siRNAs) and small hairpin RNAs (shRNAs), are used to inhibit gene expression via selective mRNA destruction. In the primary siRNA screen (study II), we used a Silencer® human siRNA kinase library (Ambion) targeting 573 human kinases, each with three individual siRNAs (Beck et al., 2010). In the secondary screen, we used the Silencer® Select custom siRNA library

(Ambion) targeting 93 kinases, each with two individual siRNAs, designed using a different algorithm. Separate siRNAs (Ambion) targeting the sequences of mouse/rat salt-inducible kinase 3 (SIK3), protein kinase X-linked (PrKX), and protein kinase A catalytic subunit α (PKA C- α) were used in the final hit validation in CAD and PC12 cells. In addition, all screens included several commercially available negative controls (siRNAs not targeting any annotated genes in the human genome) (Ambion). The dual promoter FUGW-based lentiviral vectors in which the H1 promoter drives the expression of shRNA targeting PICK1 and the ubiquitin promoter drives expression either of GFP (PICK1 KD construct) or an N-terminal GFP-fusion protein of PICK1 resistant to the shPICK1 sequence (GFP-PICK1 rescue construct) (study III) were kindly provided by Robert C. Malenka, Stanford University (Citri et al., 2010).

4.3 Thiazolyl blue tetrazolium bromide assay

Thiazolyl blue tetrazolium bromide (MTT) assay (study II) was used to assess cell metabolic activity and viability 72h post siRNA transfections. HEK 293 and CAD cells were incubated with 0.5 mg/ml MTT for 2 h at 37 °C during which the dye is converted to water-insoluable formazon by mitochondrial dehydrogenases. Subsequently, the blue crystals were solubilized with acidic isopropanol and then intensity of converted dye was measured colorimetrically at 570 nM in a Wallac Victor 2 (Perkin Elmer).

4.4 Primary antibodies

Table 3. A list of primary antibodies used for immunochemical staining (IC), western blot (WB) and ELISA analysis in studies I, II and III.

Antibody	Company	Method and dilution
hNET	Mab Technologies (17-1)	IC 1:1000, WB 1:2500
mNET, rNET	Mab Technologies (05-1)	IC 1:1000, WB 1:2500
hDAT	Millipore (Mab369)	WB 1:1000
hDAT	Santa Cruz (sc-1433)	WB 1:500
hSERT	Santa Cruz (sc-1458)	WB 1:500
mTH	Millipore (Mab318)	IC 1:1000
mPICK1	Novus Biologicals (NBP1-42829B)	IC 1:500, WB 1:500
HA	Covance (HA.11)	ELISA 1:1000
MI (anti-FLAG)	Sigma-Aldrich (F3040)	IC 1µ/ml

4.5 Fluorescence polarization assay

Fluorescence polarization (FP) (study III) allows rapid and quantitative analysis of molecular interactions, such as protein-protein binding events. Here, the affinity of a fluorescently labeled reporter molecule (DAT C-terminal peptide coupled to OregonGreen, DAT C13-Org) to purified recombinant PICK1 was measured by saturation FP experiments. Second, competition FP experiments were performed to

determine the affinity of an unlabeled, competing molecule (NET C-terminal peptide) to the purified PICK1. Thus, the competition FP assay was carried out using a fixed, non-saturating concentration of purified PICK1 (final concentration 4 μM), a fixed concentration of the reporter peptide DAT C13-OregonGreen (final concentration 40 nM) and increasing concentrations of the NET C-terminal peptide (final concentrations from 0.2 nM to 1 mM). The extent of FP (as arbitrary milli-Polarization units, mP) was measured using a POLARstar instrument (BMG Labtech, Ortenberg, Germany) with a 488 nm excitation filter and a 535 nm long-pass emission filter. To determine the IC_{50}-value and to estimate the equilibrium competition binding constant (K_i-value) for the NET C11 peptide (RQFQLQHWLAI), the measured FP was plotted as a function of the concentration of the NET C11 peptide and fitted using a one-site competition binding model in GraphPad Prism™ (GraphPad Software, San Diego, CA).

4.6 Cell lines and primary cells

4.6.1 Cell cultures and transfections

To express proteins of interest for subsequent functional analysis, the vector-based constructs were transfected into mammalian cells (study I, II, III). Human embryonic kidney (HEK 293) cells (ATCC CRL-1573) and HEK 293 -derived cells containing the SV40 T-antigen (HEK 293T cells) (ATCC CRL-3216) were grown in Dulbecco´s modified Eagle´s medium (DMEM) with HEPES and sodium bicarbonate (DMEM 1965), and differentiated CATH.a (CAD) cells (ATCC CRL-11179) were grown in a 1:1 mixture of DMEM 1965 and Ham´s F12 medium (Invitrogen), all supplemented with 10 % fetal bovine serum (FBS) and 0.01 mg/ml gentamicin. Rat PC12 cells (ATCC-CRL 1721) were cultured in DMEM 1965 supplemented with 10 % horse serum and 5 % FBS in the presence of 0.01 mg/ml gentamicin. The construct cDNAs and siRNAs were transfected into the cells using standard Lipofectamine 2000 protocols (Invitrogen), either 48 h (vectors) or 66 h (siRNAs) prior to actual experiments. The production of stable HEK 293 cell lines expressing DAT, HA-DAT, SERT and HA-SERT was performed as described in study II. All cell lines were cultured at 37 ˚C in a humidified atmosphere with 5 % CO_2.

4.6.2 Dissociated sympathetic neurons in culture

In order to investigate the cellular distribution, function and regulation of endogenous NET, dissociated rat sympathetic neurons of the superior cervical ganglia (SCG) were utilized (study I, III). These cells synthesize and secrete catecholamines and neuropeptides from their terminal sites (May et al., 1995; Zhou and Misler, 1995; Koh and Hille, 1997), and express high levels of NET (Savchenko et al., 2003; Matthies et al., 2009). Postnatally derived rat SCG neurons were dissected using a protocol modified from that of Savchenko *et al.* (Savchenko et al., 2003), and cultured on glia

cell monolayer as described in detail in study I. Cultures were grown for 12-16 days in supplemented medium prior to actual experiments.

4.7 Lentivirus production and transduction

Lentiviral vectors derived from the human immunodeficiency virus 1 (HIV-1) were used to deliver genes of interest into dissociated SCG neurons in culture (study I, III). These cells are difficult to transfect and do not readily provide long-term transgene expression. Lentiviral vectors were produced in HEK 293T cells by triple transfecting the cells with essential lentiviral genes: viral structure proteins (pBR_8.91) and vesicular stomatitis virus glycoprotein (pMD.G), together with the gene of interest (EGFP-Rab4, -Rab7, -Rab11, - Rab11S25N, EGFP, PICK1 KD, GFP-PICK1, and GFP-PICK1 A87L expressed in the pHsSynXW vector), to allow the production of lentiviral particles. Media containing lentivirus particles were collected approximately 48 h and 72 h after transfection, filtered and concentrated as described in study I and III. The SCG neuronal cultures were infected with the lentivirus on days 4–5 of *in vitro* culture. The actual experiments were performed 8–14 days after infection.

4.8 Animals

PICK1 knockout (KO) mice (C57BL/6J genetic background) (study III) were kindly provided by Dr. Richard Huganir (Johns Hopkins University). All animal experiments were conducted in accordance with the guidelines of the Danish Animal Experimentation Inspectorate Agency in a fully AAALAC accredited facility, after approval by the local animal welfare committee (Animal Care and Use Committee at the University of Copenhagen).

4.9 Analysis of monoamine transporter function

The central role of monoamine transporters is to mediate the rapid uptake of synaptically released neurotransmitter within and around synapses. The classical radiometric uptake assay is a relatively simple but powerful method to investigate transporter function. Here, the uptake experiments were carried out using 30–60 Ci/mmol [^{3}H]-DA (NET, DAT and chimeras) or [^{3}H]-5-HT (SERT) (PerkinElmer Life Sciences) (study I, II, III). In the uptake assay, whole cells or membrane vesicles expressing monoamine transporters were incubated with radiolabeled substrate for 3-10 minutes. Subsequently, the substrate was removed, the cells or membrane vesicles were washed and solubilized, and the accumulated amount of radioactive substrate was measured by liquid scintillation counting. Saturation kinetics of DA uptake was assessed using a two-fold dilution series of DA (final concentrations: 0.05 to 6.4 µM) with a trace amount of [^{3}H]-DA and CAD cells transiently expressing the transporter of interest (NET, DAT or chimeras). Uptake was initiated by adding serial dilutions of dopamine/ [^{3}H]-dopamine and was allowed to continue for 3 min at room temperature

(RT). Nonspecific uptake was determined in the presence of 100 μM desipramine or nomifensine. Subsequently, cells were lysed, Opti-Phase HiSafe3 scintillation fluid was added and the samples were counted in a Wallac Tri-Lux β- scintillation counter. Uptake data were analyzed with GraphPad Prism 5.0 programs.

4.10 Analysis of NET localization

4.10.1 Immunofluorescent labeling and confocal microscopy

Immunohistochemistry is widely used biological technique that combines anatomical, immunological and biochemical techniques to visualize the distribution and localization of specific antigens in cells or tissue sections by their interaction with specific antibodies tagged with a visible label. Here (study III), adult mice (PICK1 KO and WT littermates) were deeply anaesthetized and transcardially perfused with 4 % paraformaldehyde (PFA) in 0.1 M PBS. Coronal brain sections (40 μm) from the brainstem and cerebral cortex were first incubated with blocking/permeabilization buffer (5 % goat serum (GS), 1 % bovine serum albumin (BSA), 0.3 % Triton X-100 in PBS). Subsequently, mouse anti-NET antibody and chicken anti-PICK1 antibody were added into the same blocking solution and the sections were incubated overnight at 4 °C. Next day, the sections were rinsed (0.25 % BSA, 0.1 % Triton X-100 in PBS) and incubated with goat anti-mouse Alexa Fluor® 568 and goat anti-chicken Alexa Fluor® 488 secondary antibodies for 1 h. Additional rinsing was followed by mounting of coverslips and Prolong Gold antifade reagent.

For the immunocytochemical experiments (study I, III), the SCG neurons grown on glia-coated coverslips or the CAD cells transiently expressing protein(s) of interest on polyornithine-coated coverslips, were fixed and incubated with blocking and permeabilization buffer (5 % GS, 0.2 % saponin in PBS, pH 7.4). Some specimens were incubated with the fluorescent cocaine analogue JHC 1-64 (50 nM) prior fixation to visualize the surface expressed NET. The cells were immunostained using mouse anti-NET antibody, mouse anti-TH antibody or chicken anti-PICK1 antibody diluted in blocking buffer (5 % GS in PBS) for 30 min at RT. Appropriate secondary antibodies were used as above.

Confocal microscopy was performed using a Zeiss confocal laser-scanning microscope (LSM 510) equipped with an oil immersion 63x lens with 1,4 numerical aperture (Carl Zeiss, Oberkochen, Germany). Rhodamine- conjugated JHC 1-64 and Alexa Fluor® 568 dye were excited using 543 nm laser light from a helium-neon (HeNe) laser source, and emitted fluorescent light was filtered using a 585 nm long-pass filter. Alexa Fluor® 488 dye and EGFP were excited using a 488 nm laser line from an argon-krypton laser (argon laser) and the emitted light was filtered using a long-pass 505-530 nm barrier filter. Image processing was performed with Image J (National Institutes of Health).

4.10.2 Surface ELISA

Surface ELISA (enzyme-linked immunosorbent assay) utilizes enzyme-linked antibodies that detect the presence of a substance, such as an antigen, on the surface of a sample, and in response to addition of substrate produces a detectable signal. To evaluate changes in surface expression of NET (study I) upon incubation with JHC 1-64 (0.5 μM), PMA (1 μM) or vehicle (0.01% dimethyl sulfoxide, DMSO) for 1 h at 37 °C, HEK 293 cells transiently expressing Flag-tagged TacNET were seeded on 96-well plates. Accordingly, to evaluate the surface expression of DAT and SERT in response to siRNA transfections (study II), HEK 293 cells stably expressing HA-DAT or HA-SERT were seeded on 96-well plates. The cells were fixed, blocked with 5 % GS in PBS and incubated with 1 μg/ml M1 antibody (TacNET) or mouse anti-HA antibody (HA-DAT and HA-SERT) in 5 % GS in PBS for one hour. Following washes in PBS, a secondary HRP-conjugated antibody was applied, and the enzyme activity was detected by chemiluminescence using Supersignal ELISA femto maximum sensitivity substrate and measured in a Wallac Victor2 (PerkinElmerLife Sciences).

4.10.3 Surface biotinylation

Biotinylation is a process to detect or purify proteins by covalently attaching them with biotin, and then utilizing the extremely high affinity between biotin and streptavidin/avidin. To evaluate the surface expression of NET (study III), CAD cells transiently expressing NET and PICK1 KD or control constructs were washed with cold PBS and surface biotinylated with 1.2 mg/ml disulfide-cleavable biotin for 1 hour on ice. Free biotin reagent was removed by quenching with glycine, cells were lysed in lysis buffer (25 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.2 mM protease inhibitor PMSF (ThermoFisher) and 5 mM *N*-ethylmaleimide), centrifuged and supernatants were collected. Total fractions were reserved from all samples and mixed with an equal volume of 2x loading buffer containing 1 % SDS, 2.5 % β-mercaptoethanol and 100 mM dithiothreitol. To pull down the biotinylated proteins, the samples were incubated overnight with avidin beads at in 4 °C under constant rotation. Subsequently beads were washed and bound proteins were eluted with 2x loading buffer. Proteins were separated by SDS-PAGE and immunoblotted to estimate the relative amounts of NET present in the blots. The transporter band densities were quantified with Image J software.

4.11 Analysis of NET trafficking

4.11.1 JHC 1-64 live cell imaging

Labeling of cultured SCG neurons with the rhodamine-conjugated cocaine analogue JHC 1-64, followed by confocal live imaging, allows an efficient visualization of NET surface expression and internalization, with a high signal/noise ratio (study I, III). SCG

neurons with or without lentiviral transduction were first incubated with JHC 1-64 (50 nM) for 20 min at 4 °C and either immediately imaged to visualize the surface-expressed transporter or incubated for 1 h at 37 °C to allow internalization and then imaged using LSM 510. To assess the effect of activation of PKC on NET internalization, SCG neurons were incubated for 1 h at 37 °C in the presence of 1 µM PMA or vehicle (0.01 % DMSO) after surface labeling of NET with JHC 1-64 at 4 °C. To further assess the postendocytic fate of NET, we visualized lysosomes by incubating non-transduced SCG neurons with Lysotracker Green (50 nM, Invitrogen) during the last 15 min of incubation at 37 °C. To verify the specific binding of JHC 1-64 to NET, some specimens were preincubated with desipramine (100 µM) for 20 min before labeling with JHC 1-64. Likewise, surface expression, internalization and postendocytic sorting of NET, DAT or NET-DAT chimeras were studied in CAD cells transiently expressing protein(s) of interest using JHC 1-64 as described in Paper I.

4.11.2 Antibody feeding assay (internalization assay)

Antibodies recognizing specific cell surface antigens (or epitopes) can internalize with the antigen-presenting proteins, and may be visualized by microscopy with a directly conjugated fluorophore or with a secondary conjugated fluorophore. Antibody feeding experiments (study I, III) were carried out in CAD cells transiently expressing Flag-tagged TacNET together with EGFP-Rab7, EGFP-Rab11, control or PICK1 KD constructs by incubating the cells with mouse anti-Flag antibody (M1 antibody) (1 µg/ml) in DMEM at 4 °C for 30 min to label surface-expressed TacNET. Subsequently, cells were washed and incubated in pre-warmed medium for 1 h at 37 °C to allow internalization (or at 4 °C as a non-trafficking surface control). Internalization was terminated by washing the cells with ice cold PBS followed by fixation in 4 % PFA. Subsequently, the cells were permeabilized in blocking/permeabilization buffer, incubated with anti-mouse Alexa Fluor® 568 secondary antibody, washed, mounted with ProLong Gold Antifade reagent and visualized by confocal microscopy.

4.11.3 Reversible biotinylation assay (internalization assay)

To investigate the extent of transporter internalization in CAD cells transiently transfected with equal amounts of NET, DAT or NET-DAT chimeras, the cells were surface biotinylated with 1.2 mg/ml disulfide-cleavable biotin for 1 hour on ice (study I). Quenching with glycine was followed by transporter endocytosis (1 h at 37 °C) with no treatment, with leupeptin (100 µg/ml) or with leupeptin (100 µg/ml) and monensin (25 µM). Control plates were kept on ice to prevent internalization. Subsequently, the biotin was dissociated from the cell surface resident proteins by incubating the cells with cold sodium-2-mercaptoethanesulfonate (MesNa, 100 mM). To determine total biotinylation, a sample of biotinylated cells was not subjected to reduction with MesNa. After washing steps, the cells were lysed in lysis buffer and the samples were handled identically to surface biotinylated samples (see chapter 4.8.3).

4.12 Quantification of mRNA and protein levels

4.12.1 qPCR analysis

Quantitative real-time PCR (qPCR) is a sensitive method to monitor the amplification of a target DNA molecule during the polymerase chain reaction (PCR). Either specific or nonspecific fluorescent reporters may be employed. Here (study II, III), RNA from cell lysates was extracted using the Nucleospin® RNA kit (Macherey-Nagel), and subsequently the isolated RNA samples (0.5 - 1 µg) were reverse transcribed to cDNA using AffinityScript^{TM}QPCR cDNA kit (Stratagene) (study II) or the Improm-II™ Reverse Transcription System kit (Promega) (study III). After synthesis, the cDNA was diluted ten-fold with RNAse-free H_2O and 2 µl was used in a 20 µl quantitative qPCR reaction containing SYBR Premix Ex Taq (TaKaRa) and 2.0 pmol of each primer. qPCR was performed using the Mx3000P instrument (Stratagene), with cycle threshold (Ct) values obtained using Stratagene Mx3000P software. TATA-binding protein (TBP) was used as a reference gene, and the data were normalized to the control sample.

4.12.2 Western blot analysis

Western blot is an analytical technique used to detect proteins in cell or tissue homogenates using antibodies targeting specific proteins. First, HEK 293 or CAD cells were transiently transfected with cDNAs encoding the protein of interest (DAT, SERT, NET or Flag-β2AR) together with siRNAs (against SIK3, PKA C-α, PrKX or control) or ShRNAs (against PICK1 or control) (study II, III), whereas SCG neurons in culture were transduced with lentivirus encoding the protein of interest (GFP, PICK1 KD, PICK1 WT rescue or PICK1 A87L) (study III). Treatment of neuronal lysates with peptide-*N*-glycosidase F (PNGase F) was carried out according to the manufacturer's instructions (study III). Second, cells were lysed in cold lysis buffer, the lysates were centrifuged at 16 000 g for 20 min at 4 °C, and the supernatants were collected. Equal amounts of lysates were incubated with SDS loading buffer, separated by SDS-PAGE and immunoblotted using primary antibodies listed in Table 3.

To estimate the relative amounts of NET in the brainstem and frontal cortex of adult WT and PICK1 KO mice (study I), the mice were decapitated, the brains were removed from the cranial cavity, chilled and sectioned. Samples of the frontal cortex and brainstem noradrenergic nuclei were dissected from the 1 mm thick coronal sections. The tissue samples were homogenized in lysis buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 % Triton-X, 0.5 % NP-40, 1 mM EDTA and protease inhibitors, and mechanically disrupted with a Teflon pestle (800 rpm, 10 x 5 s). Samples were centrifuged at 16 000 g for 20 min at 4 °C, and the supernatants were collected and processed as described above.

4.13 Data analysis

Quantification of JHC 1-64 labeled transporter internalization in SCG neurons and CAD cells (study I) was performed setting fixed minimum threshold pixel intensity in the 8-bit pictures and defining the ratio of total integrated density of internalized, JHC 1-64 labeled transporter and the total integrated density of the entire labeled transporter pool. 20-29 SCG neurons and 62-75 CAD cells from at least three independent experiments were used for quantification under each condition. Statistical analyses were carried out with one-way ANOVA, Bonferroni´s multiple-comparison test using GraphPad Prism. *P* values smaller than 0.05 were considered to be statistically significant.

Quantification of colocalization (study I) was performed using Van Steensel´s cross correlation function in the JaCoP (Just Another Colocalization Plugin) for Image J. Van Steensel´s cross correlation function reports the Pearson cross-correlation as a function of the relative movement of the two channels with respect to each other. Structures with complete colocalization results in a cross-correaltion function of 1 at *Dx*=0 and exclusive structures demonstrate cross-correlation function of 0 or close to 0 at *Dx*=0. Image acquisition and quantification under each condition was performed blinded and consisted of three to five independent experiments each containing 10-25 cells.

^{3}H-dopamine uptake experiments of transduced SCG neurons (n=3-5) and internalization assays of NET, DAT and NET-DAT chimeras in CAD cells (reversible biotinylation, n=3-5) (study I) were analyzed using one-way ANOVA, Bonferroni´s multiple-comparison test (GraphPad Prism). Surface ELISA (n=6) in HEK 293 cells were analyzed using one-way ANOVA, Dunn´s multiple comparison (GraphPad Prism). *P* values smaller than 0.05 were considered to be statistically significant.

The results of study II were analyzed with standard methods using GraphPad Prism. Secondary siRNA screen hits were analyzed using Student´s *t*-test and for simplicity not corrected for multiple testing. In addition, the effect of siRNA-mediated silencing of kinases on transporter function and expression were analyzed using Student´s *t*-test. *P* values smaller than 0.05 were considered to be statistically significant.

The statistical analysis of NET expression and function in *ex vivo* preparations derived from WT and PICK1 KO mice (study III) were performed using Student´s *t*-test. Accordingly, the effect of shRNA-mediated silencing of PICK on NET and DAT expression, function and trafficking in *in vitro* models were analyzed using Student´s *t*-test. One-way ANOVA, Bonferroni´s multiple comparison test was employed when the effect of PICK1 silencing and molecular replacement with recombinant PICK1 (WT or A87L mutant) on NET function, expression and trafficking was investigated in neuronal cell models. *P* values smaller than 0.05 were considered to be statistically significant.

5 RESULTS

5.1 NET trafficking

5.1.1 Motivation

It is believed that alterations in NET abundance in the plasma membrane modulate noradrenergic signaling by affecting both neurotransmitter clearance and the refilling of presynaptic storage vesicles. Endocytic trafficking has been suggested to be a major means by which NET and other SLC6 transporters are functionally regulated (Kristensen et al., 2011). This is supported by the notion that most NET is located intracellularly in noradrenergic neurons (Savchenko et al., 2003; Sung et al., 2003; Matthies et al., 2009), suggesting that NET is dynamically transported between intracellular compartments and the plasma membrane. A major goal of the present study I was to investigate the internalization and postendocytic properties of NET in noradrenergic neurons by employing several approaches, including the use of the fluorescent cocaine analogue JHC 1-64.

5.1.2 Fluorescent cocaine analogue JHC 1-64

The fluorescent cocaine analogue JHC 1-64 contains a rhodamine moiety, extending from the tropane nitrogen of the parent compound 2β-carbomethoxy- 3β-(3,4-dichlorophenyl) tropane with an ethylamino linker (Cha et al., 2005). JHC 1-64 displays high apparent affinity for NET (Cha et al., 2005) and slow dissociation from the transporter as described in study I. Confocal imaging of live SCG neurons labeled with JHC 1-64 revealed a strong fluorescent signal corresponding to the plasma membrane of the somata, projections and presynaptic boutons (Fig. 7A). In general, labeling with JHC 1-64 demonstrated rather uniform distribution of the fluorescence in the plasma membrane, with strongest intensity in neuronal projections and boutons. Consistent with specific visualization of NET, no labeling was observed in the glial cell layer or in cultures preincubated with the NET inhibitor desipramine (100 μM). Co-staining of JHC 1-64-labeled SCG neurons with antibodies directed against noradrenergic cell markers, NET and TH, demonstrated clear JHC 1-64 plasma membrane labeling of neurons positive for NET and TH immunoreactivity (study I). Taken together, it was shown that JHC 1-64 may be employed as a tool to specifically label surface-resident NET in SCG neuronal cultures. Furthermore, JHC 1-64 labeling demonstrated, to our knowledge for the first time, that NET is also present on the plasma membrane of the noradrenergic somata.

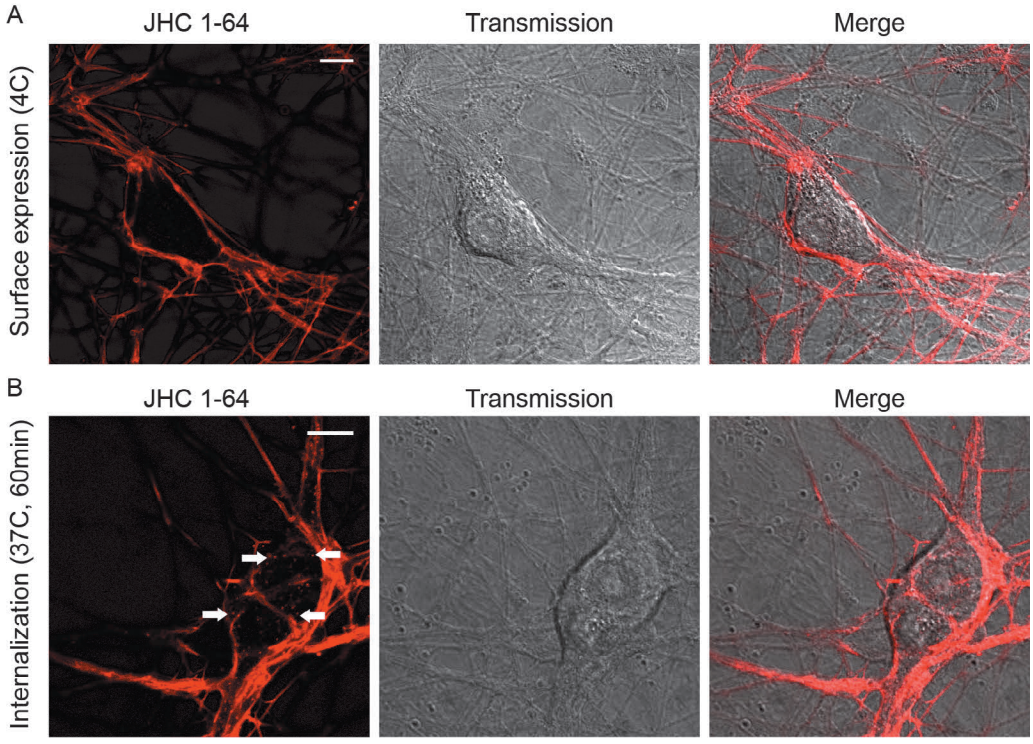

Figure 7. Visualization of NET surface distribution and internalization in live, cultured sympathetic neurons with the fluorescent cocaine analogue JHC 1-64. Confocal images of representative SCG neurons incubated with 50 nM JHC 1-64 for 20 min at 4 °C that are either imaged immediately (A) or subsequently incubated at 37 °C for 1 h allowing internalization and then imaged (B). Scale bars 10 µm. Arrows indicate intracellular fluorescent puncta representing internalized NET.

To exclude the possibility that binding of JHC 1-64 to the transporter would affect the surface expression or trafficking properties of NET, we generated a TacNET fusion construct of the single transmembrane segment protein Tac containing an extracellular Flag recognition sequence and NET. TacNET exhibited functional activity, although its maximum uptake capacity was reduced as compared to wild-type NET, and the fluorescent signal of the M1-antibody labeled TacNET was clearly detected on the plasma membrane in TacNET expressing cells. Importantly, an ELISA-based assay demonstrated that activation of PKC and other kinases by PMA (1 µM) caused a significant reduction of TacNET surface expression as expected (Apparsundaram et al., 1998b; Jayanthi et al., 2004), but incubation of the cells with a saturating concentration of JHC 1-64 (0.5 µM) for 1 h at 37 °C did not cause any change in TacNET surface expression compared to vehicle (0.01 % DMSO). Furthermore, antibody feeding experiments of CAD cells expressing TacNET together with either EGFP-Rab7 or EGFP-Rab11 demonstrated a similar postendocytic fate of the transporter as observed with JHC 1-64 -labeled NET (see section 5.1.3). Together, these results indicate that JHC 1-64 does not by itself affect NET surface expression or trafficking.

5.1.3 Constitutive internalization and postendocytic fate of NET

Surface-expressed NET in the SCG neurons was labeled with JHC 1-64 at 4 °C, followed by internalization at 37 °C for 1 h. Internalized, JHC 1-64 -labeled NET was visible as distinct intracellular vesicular structures in the somata and their proximal extensions as well as in the cytoplasm of the boutons (Fig. 7B). These structures were not observed in cells kept at a temperature (4 °C) not permissive for trafficking (Fig. 7A). Indeed, quantification of confocal sections of live SCG neurons showed significantly higher internalization of JHC 1-64 -labeled NET upon incubation at 37 °C (constitutive internalization) compared to the control cells kept at 4 °C. NET internalization was further increased by activation of PKC and other kinases by PMA (1 μM).

To elucidate the postendocytic fate of internalized JHC 1-64 -labeled NET, cultured SCG neurons were transduced with lentivirus encoding different endosomal markers tagged with EGFP. Internalized JHC 1-64 -labeled NET demonstrated strong overlap with the signal from EGFP-Rab11, which mediates recycling of cargo back to the plasma membrane from pericentriolar recycling endosomes (Ullrich et al., 1996) and marks the “long loop” recycling pathway, both in the somata and proximal extensions, as well as in the boutons. Quantification of the colocalization of JHC 1-64 -labeled NET with the endosomal markers using Van Steensel´s cross correlation analysis confirmed the strong overlap between internalized JHC 1-64 -labeled NET and EGFP-Rab11 -positive endosomes, and little overlap between internalized JHC 1-64 -labeled NET and EGFP-Rab7, marker of the late endosomes, in the somata and boutons. Moderate association of internalized JHC 1-64 -labeled NET was observed with EGFP-Rab4 -positive “fast recycling” endosomes in the somata, but this association appeared stronger in the boutons compared to the somata and proximal extensions. An antibody-based internalization assay in CAD cells expressing TacNET together with either EGFP-Rab7 or EGFP-Rab11 likewise demonstrated strong overlap of the constitutively internalized M1-antibody labeled TacNET with EGFP-Rab11 and showed little colocalization with EGFP-Rab7.

Constitutive internalization of NET and sorting to recycling was further supported by results from a biochemical internalization assay (reversible biotinylation) with pharmacological manipulation of the degradation and recycling pathways. The results obtained with NET expressing CAD cells strongly support extensive constitutive internalization of NET by revealing significantly stronger NET immunoreactivity and thus intracellular NET levels for cells incubated at 37 °C for 1 h as compared to cells kept at 4 °C (Fig. 8A). Inhibition of lysosomal degradation by the lysosomal protease inhibitor leupeptin during the internalization period did not cause any further increase in the amount of NET immunoreactivity (Fig. 8A). However, additional inhibition of recycling by monensin, a cation ionophore blocking recycling (Mollenhauer et al., 1990), caused a significant increase in intracellular NET (Fig. 8A), supporting our results from the imaging experiments that indicated that NET is primarily recycled and not targeted to degradation upon internalization.

We went on to mitigate the function of the Rab11 compartment by overexpressing the dominant negative Rab11 mutant, Rab11 S25N, in SCG neurons. The overexpression of EGFP-Rab11 S25N resulted in a significant decrease in NET transport capacity compared to neurons only overexpressing EGFP, as illustrated by reduced [^{3}H]-DA uptake. Overexpression of EFGP-Rab11 or EGFP-Rab7 did not alter NET transport capacity. A role of the Rab11 pathway in regulation of NET function was further supported by experiments in transfected CAD cells where co-expression of NET with Rab11 S25N, caused a significant decrease in [^{3}H]-DA uptake compared to cells co-expressing NET and wild-type Rab11. Co-expression of NET with dominant negative Rab4 (Rab4 S25N), however, did not cause any change in the NET uptake rate compared to cells expressing wild-type Rab4. Altogether, our results suggest that constitutively internalized NET is to a major extent sorted to the Rab11 recycling compartment and that impairment of the Rab11 pathway results in decreased NET function.

5.1.4 Distinct trafficking properties of NET and DAT

To directly compare NET trafficking properties with DAT, previously shown to be sorted largely to lysosomal degradation (Daniels and Amara, 1999; Eriksen et al., 2010a; Rahbek-Clemmensen et al., 2014b), we employed the JHC 1-64 internalization assay to assess the postendocytic fate of the transporters in CAD cells transiently transfected with NET or DAT together with either EGFP-Rab7 or EGFP-Rab11. Consistently with the results from cultured SCG neurons, JHC 1-64 -labeled NET colocalized prominently with EGFP-Rab11 and only to a limited extent with EGFP-Rab7. In agreement with previous findings for DAT (Eriksen et al., 2010a), JHC 1-64 -labeled DAT appeared to be more associated with EGFP-Rab7 than EGFP-Rab11. Also the results of biotinylation experiments supported that DAT is to a large degree sorted to degradation: inhibition of lysosomal degradation (with leupeptin) showed significantly increased intracellular accumulation of DAT, whereas additional inhibition of recycling (with leupetin and monensin) did not further increase the DAT immunoreactive signal (Fig. 8B).

Differences in the apparent extent of NET and DAT internalization were evident in the reversible biotinylation experiments. DAT demonstrated a significantly lesser extent of internalization compared to NET as determined with accumulated immunoreactivity during the 1 h internalization period at 37 °C, when recycling and degradation were blocked with leupeptin and monensin (12 ± 1 % versus 41 ± 2 %, respectively, n = 3-4) (Fig. 8A, B). Moreover, the confocal images demonstrated that internalized JHC 1-64 -labeled NET vesicles seemed to have a more perinuclear localization compared to internalized JHC 1-64 -labeled DAT vesicles, which were seen as smaller, more peripherally located puncta (Fig. 9A). Quantification of the distance of the brightest vesicles to the plasma membrane revealed that JHC 1-64/DAT vesicles were significantly closer to the plasma membrane compared with NET/JHC 1-64 vesicles, further supporting differential trafficking properties of the two transporters (Fig. 9C).

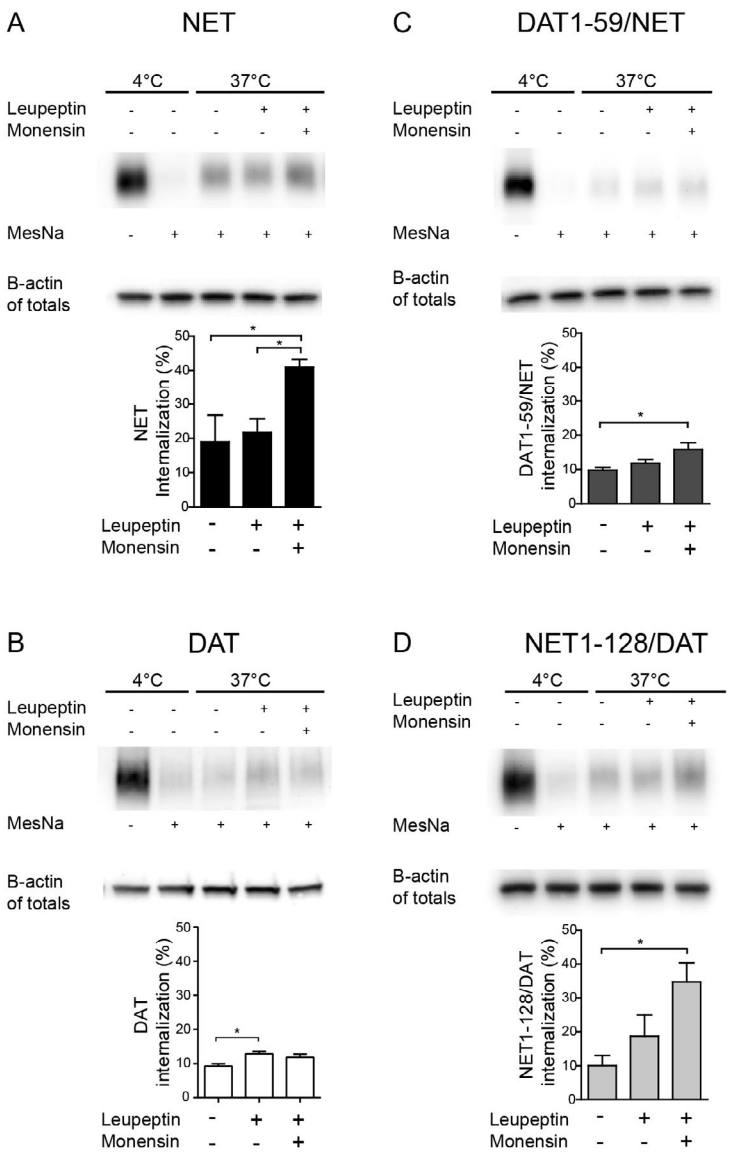

Figure 8. Assessing internalization and postendocytic sorting of NET, DAT and DAT/NET chimeras by reversible biotinylation experiments. A, B) Inhibition of lysosomal degradation (with leupeptin 100 µg/ml) during the internalization period caused significant increase in DAT but not NET intracellular accumulation, whereas additional inhibition of recycling (with leupeptin 100 µg/ml + monensin 25 µM) caused a significant increase in the amount of NET but not DAT immunoreactivity. C, D) DAT(1–59)/NET internalization properties are more similar to those seen for wild-type DAT, whereas NET(1–128)/DAT internalization properties are more similar to those seen for wild-type NET. MesNa, sodium-2-mercaptoethanol sulfonate.

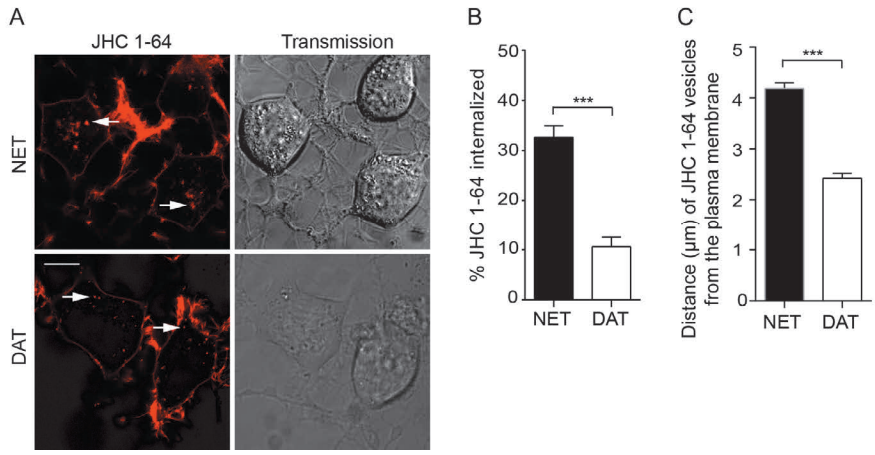

Figure 9. NET displays more constitutive internalization than DAT, and internalized NET vesicles have a more perinuclear localization than internalized DAT vesicles. A, Representative confocal live cell images of JHC 1-64 -labeled NET (top left) and DAT (bottom left) in transfected CAD cells after 1 h at 37 °C. Scales bar 10 µm. Arrows indicate intracellular fluorescent puncta representing internalized transporter. B, Quantification of the internalization of JHC 1-64 -labeled transporters. C. Quantification of the JHC 1-64 vesicle distances from the plasma membrane.

5.1.5 Role of N-termini for NET and DAT trafficking properties

Chimeras between NET and DAT where the N- and C-termini were exchanged between the two proteins were constructed to assess whether specific intracellular domains of NET or DAT encoded information sufficient to direct the internalized transporter to a specific endosomal compartment. All chimeras were readily expressed in the plasma membrane, exhibited normal functional activity, and could be labeled with JHC 1-64 (study I). Confocal images demonstrated that introducing the DAT N-terminus into NET (DAT 1-59/NET) profoundly decreased colocalization with EGFP-Rab11 without altering the colocalization with EGFP-Rab7. Correspondingly, introducing the NET N-terminus into DAT (NET1-128/DAT) markedly increased colocalization with EGFP-Rab11 without altering the colocalization with Rab7. Introducing the DAT C-terminus into NET (NET/DAT593-620) or the NET C-terminus into DAT (DAT/NET 590-617) did not alter the postendocytic fate of the transporters. Results from the reversible biotinylation assay supported the notion that the postendocytic fate of DAT1-59/NET was more similar to that seen for wild-type DAT, i.e. primarily sorting to degradation (Fig. 8B, C) and for NET1-128/DAT we observed a postendocytic fate more similar to that seen for wild-type NET, i.e. sorting to recycling (Fig. 8A, D). In addition, the extent of internalization, as determined with recycling and degradation blocked by leupeptin and monensin, was greater for wild-type NET compared to DAT1-59/NET (41 ± 2 % versus 16 ± 2 %, respectively, mean ± SEM, n = 3-4), and it was also greater for NET1-128/DAT compared to wild-type DAT (32 ± 8 % versus 12 ± 1 %, respectively, mean ± SEM, n = 3-4) (Fig. 8). Together, these results indicate that NET displays a markedly greater extent of

constitutive internalization than DAT and is to a large extent sorted to recycling whereas DAT is mainly sorted to degradation. These differential trafficking properties are determined in part by non-conserved structural elements in the intracellular N-termini of the two transporter proteins.

5.2 Kinome-wide RNAi screen

5.2.1 Motivation

Protein kinases mediate many important modifications of target protein function, localization and interactions with other proteins, and thus play crucial roles in many cellular processes. Only a limited number of protein kinases regulating the function of monoamine transporters have been identified so far (Zahniser and Doolen, 2001; Torres et al., 2003a; Kristensen et al., 2011), although monoamine transporters contain several consensus sites for protein kinase phosphorylation. PKC is the most extensively investigated kinase and activation of PKC by PMA has been shown to downregulate DAT, NET and SERT in both native preparations and in heterologous expression systems (Huff et al., 1997; Vaughan et al., 1997; Blakely et al., 1998; Apparsundaram et al., 1998a; Apparsundaram et al., 1998b; Jayanthi et al., 2004). However, little is known about the entire ensemble of kinases and kinase pathways in the control of monoamine transporter function. Here (study II), whole human kinome RNA interference (RNAi) screen was performed as an unbiased and comprehensive analysis to identify novel kinases involved in regulation of monoamine transporter function and surface expression under non-perturbed steady-state conditions.

5.2.2 Primary, secondary and tertiary siRNA screens

A primary screen performed with siRNAs against 573 human kinases in HEK 293 cells stably expressing DAT or SERT revealed 93 kinases that caused ≥ 30 % up- or down-regulation in the function of at least one of the two transporters for at least two out of the three siRNAs. The identified hits putatively regulating DAT and/or SERT function seemed to operate in various signaling pathways, supporting a notion that monoamine transporters are regulated by complex cellular signaling networks. For example, several members of the cAMP-dependent protein kinases, PI3K/Akt- and MEK/ERK pathway kinases, MAP kinases, receptor and non-receptor tyrosine kinases as well as cyclin-dependent kinases were identified to regulate the function of SERT and DAT.

All 93 hits were validated with a new set of siRNAs in a secondary screen assessing effects on DAT and SERT uptake capacity and surface expression in HEK 293 cells (Figure 10). The siRNAs with at effects at $p < 0.1$ (one-sample t-test, $n = 3$) were ranked according to effect size and a hit was defined as a kinase with >15 % (uptake) or > 10 % (ELISA) up- or down-regulation for one or both of the two siRNAs (Figure 10). Hits for which the siRNA caused significant cell death ($p < 0.05$, >10 %) were

excluded. The secondary screen revealed 26 kinases that potentially modulated the function and/or the surface expression of DAT and/or SERT. The uptake assays suggested that a higher number of kinases functioning in various signaling pathways regulate DAT function compared to SERT. Knock-down of some of the most prominent hits, such as SIK3 and PrKX, caused parallel increases or decreases, respectively, in transporter function and surface expression. We went on with 11 of the candidate kinases from the secondary screen with reported expression in neuronal tissue to validate the effects of depletion of these kinases in HEK 293 cells transiently expressing DAT, SERT or NET. Subsequently three kinases were selected for further validation in CAD cells and PC12 cells.

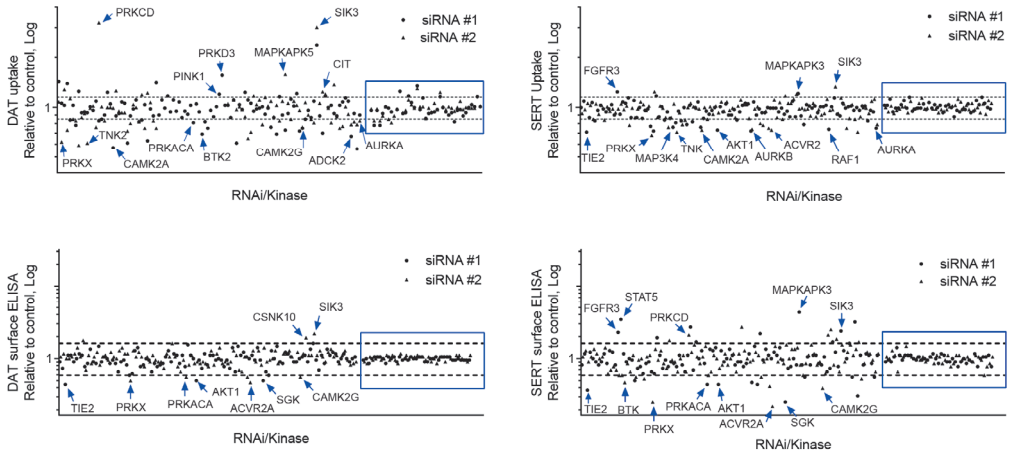

Figure 10. Secondary siRNA screen where 93 kinases identified in the primary screen were silenced with two independent siRNAs and the effects on DAT and SERT function and surface distribution were analyzed in HEK 293 cells stably expressing DAT, SERT, HA-DAT or HA-SERT. Data are means (n=3) normalized to several negative control present in each plate that are visualized in the blue boxes.

5.2.3 Validation the effect of SIK3 and PrKX knock-down on monoaminetransporter function in catecholaminergic cell lines

Knock-down of the most prominent hits from the primary and secondary siRNA screens, SIK3 (also known as QSK or KIAA 0999) and PrKX, were further investigated in the catecholaminergic CAD and PC12 cell lines. Surprisingly, knock-down of SIK3 in CAD and PC12 cells resulted in an opposite effect to that observed in HEK 293 cells, i.e. significant decreases in DAT and SERT function. Furthermore, knock-down of SIK3 caused marked transcriptional upregulation of DAT mRNA and protein levels in HEK 293 cells and significant downregulation in CAD cells. This unexpected effect of SIK3 depletion might be explained by the ability of SIK3 to regulate the CMV promoter under which the transporter was expressed in the different cell lines. CMV promoter contains several cAMP regulatory elements (CREs) and SIK has been described to modulate the transcriptional expression of the CRE regulated genes in a cell-specific manner (Takemori and Okamoto, 2008; Taub et al., 2010). We

were not able to obtain sufficient SIK3 depletion in an endogenous cell system in order to be able to investigate whether SIK3-dependent gene expression is important for the regulation of endogenous monoamine transporter function. Thus, it remained unclear whether SIK3 was a false positive hit in the kinome screen due to kinase-mediated transcriptional regulation of the CMV promoter present in the expression vector.

Knock-down of PrKX in CAD cells transiently expressing DAT, SERT or NET significantly reduced the uptake capacity of DAT and NET, and caused a statistically non-significant trend towards decreased SERT function. Our observation that silencing PrKX also decreased the surface expression of DAT and SERT, without affecting total protein levels, suggests that the reduced transporter function upon PrKX depletion, at least partly, is due to redistribution of the transporter molecules away from the plasma membrane. Together, the whole kinome siRNA screen provided insight into kinome regulation of the monoamine transporters and identified PrKX as a possible regulator of monoamine transporter function.

5.3 Regulation of NET function by scaffolding protein PICK1

5.3.1 Motivation

The activity and availability of neurotransmitter transporters at the cell membrane is important for the maintenance of neurotransmitter homeostasis, and is regulated by multiple mechanisms, including the activity of kinases and other interacting proteins. This control occurs largely through redistribution of the transporter proteins from the cell surface rather than changes in their intrinsic transport activity (Torres et al., 2003a). NET possesses a type II PDZ binding sequence at its distal C-terminus that has been shown to interact with scaffolding protein PICK1 (Torres et al., 2001), but the functional relevance of this interaction is unknown. In the present study III we investigated the role of PICK1 in relation to NET surface expression, function and trafficking to elucidate the physiological significance of the interaction of PICK1 and NET.

5.3.2 NET expression and function in PICK1 KO mice

Immunohistochemical labeling of PICK1 and NET in brain slices derived from PICK1 WT (control) mice demonstrated punctate PICK1 immunoreactivity in the brainstem noradrenergic neuronal somata that were also positive for NET immunoreactivity (Fig. 11A). PICK1 immunoreactivity was not detected in sections derived from PICK1 KO mice (Fig.11A). In the cortical sections, PICK1 immunoreactivity was prominent in the cortical cell bodies and some PICK1 clusters appeared to associate with NET immunopositive noradrenergic terminals. Genetic deletion of PICK1 significantly attenuated NET protein levels in both brainstem and frontocortical tissue lysates compared to PICK1 WT littermates (Fig. 11B). The loss of NET expression in the

noradrenergic terminals was accompanied by significantly decreased [^{3}H]-DA uptake capacity in frontocortical synaptosomes (Fig. 11C). Together, the results support that PICK1 is expressed in central noradrenergic neurons and demonstrate that PICK1 KO mice display attenuated expression and function of NET.

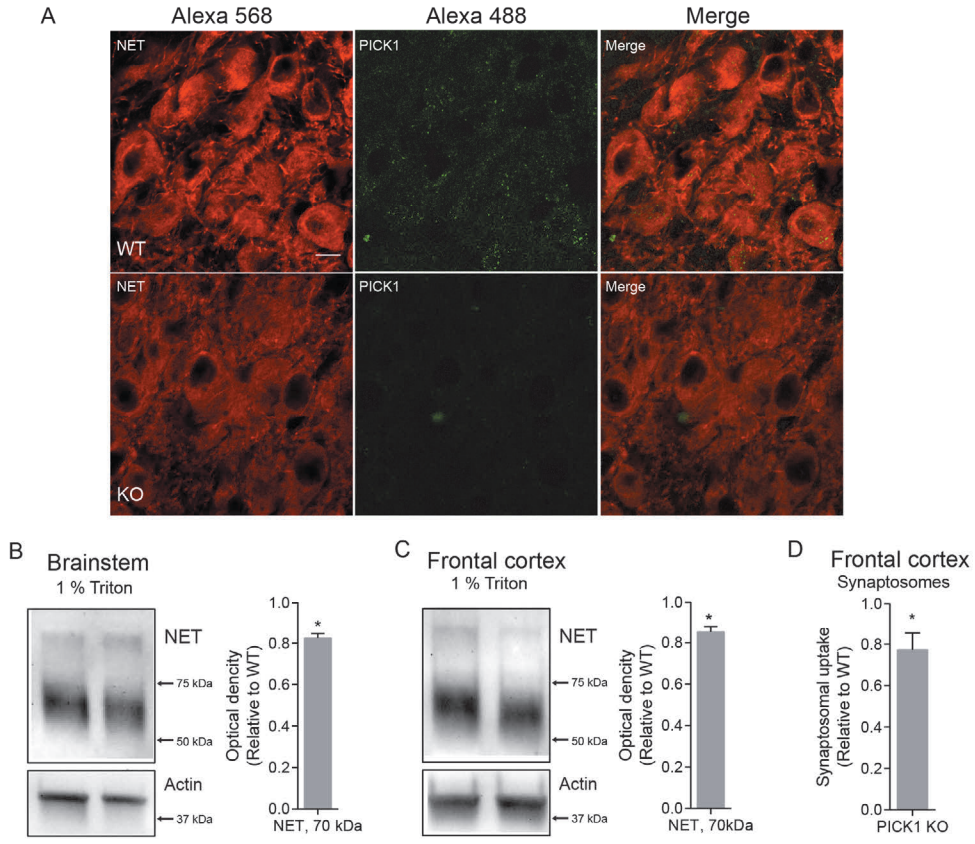

Figure 11. Assessment of NET expression and function in brains of WT and PICK1 KO mice. A, Visualization of NET (red) and PICK1 (green) expression in coronal brainstem slices. Scale bar 10 µm. B, C, Immunoblotting of brainstem and frontocortical brain lysates. D, [^{3}H]-DA uptake capacity of frontocortical synaptosomes. * $p < 0.05$. Error bars indicate SEM, n = 4-6.

5.3.3 PICK1 mediated plasma membrane stabilization of NET

We went on to assess the role of PICK1 in relation to the expression and function of NET in dissociated, cultured sympathetic neurons. PICK1 immunoreactivity demonstrated punctate expression in the noradrenergic neuronal somata, and clustered labeling in the presynaptic boutons. No evident overlap of PICK1 puncta with NET immunoreactivity was detected in the neuronal somata but partial association of PICK1 with NET was evident in the neuronal boutons (study III). Next, we utilized previously

validated shRNA targeting PICK1 (Citri et al., 2010) and infected sympathetic neurons for 8-12 days with dual promoter lentiviral vectors to express either shRNA against PICK1 and GFP (PICK1 KD), or GFP alone (control). Furthermore, we also utilized dual promoter lentiviral rescue/molecular replacement constructs (Citri et al., 2010), where the H1 promoter drives the expression of anti-PICK1 shRNA and the ubiquitin promoter drives the expression of GFP-PICK1 made resistant to shRNA (GFP-PICK1 WT rescue) or GFP-PICK1 A87L (GFP-PICK1 A87L), a PICK1 PDZ domain mutant incapable of binding to its interacting partners (Madsen et al., 2005). shRNA-mediated depletion of PICK1 reduced both total and surface expressed NET compared to neurons transduced with the control vector. Consistently with this, silencing of PICK1 resulted in significantly decreased [^{3}H]-DA transport capacity compared to neurons transduced with the control vector. The specificity of PICK1 knock-down was supported by rescue of NET uptake with recombinant shRNA-insensitive GFP-PICK1. The results demonstrated that PICK1 associates with NET in the neuronal boutons likely representing presynaptic NE release sites, and that silencing of PICK1 attenuates NET expression and function in sympathetic neurons.

Next, we investigated the role of PICK1 depletion on NET trafficking and the importance of the PICK1 PDZ domain on NET function and/or trafficking. Consistently with the previous results, the JHC 1-64 internalization assay demonstrated marked constitutive internalization of NET in control- transduced SCG neurons. However, SCG neurons with PICK1 KD or replacement with GFP-PICK1 A87L demonstrated significantly increased constitutive intracellular accumulation of NET that could be rescued with recombinant shRNA-insensitive GFP-PICK1. Together with the notion that knock-down of PICK1 markedly reduced expression and function of NET, these results substantiated the conclusion that PICK1 promotes NET function by stabilizing the transporter on the cell surface in a PDZ-dependent manner.

To test whether the C-terminus of NET indeed can bind PICK1 with high affinity, fluorescent polarization assays were performed. Competition binding experiments showed that a NET peptide corresponding to the last 11 C-terminal residues potently inhibited binding of the reporter peptide (an Oregon Green-labeled DAT C-terminal peptide) to purified PICK1 in solution, with an estimated affinity of 4.1 μM. Substitution of the distal isoleucine with aspartic acid (NET C11 I617D), thus disrupting the NET PDZ-binding domain, completely abolished the binding to PICK1.

5.3.4 Differential regulation of NET and DAT by PICK1

In addition to NET, PICK1 is known to interact with DAT (Torres et al., 2001), even if the physiological significance of this interactions is still poorly understood (Torres et al., 2001; Bjerggaard et al., 2004; Rickhag et al., 2013). To further validate the role of PICK1 in regulation of NET and to directly compare the functional roles of PICK1 in relation to NET and DAT, CAD cells that endogenously express PICK1 were employed. CAD cells were transiently transfected to express low levels of NET or DAT together with control or PICK1 KD shRNA constructs. Silencing of

endogenous PICK1 resulted in decreased total as well as plasma membrane protein levels of NET as determined by Western blotting, JHC 1-64 surface labeling and surface biotinylation. However, DAT total and plasma membrane protein levels were unaltered. Saturation uptake experiments with [^{3}H]-DA revealed significantly reduced maximal transport velocity (V_{max}) of NET without a change in the apparent substrate affinity (K_d) upon PICK1 knock-down. Furthermore, JHC 1-64 internalization and antibody-based internalization experiments revealed significantly greater intracellular accumulation of NET in PICK1 KD CAD cells compared to control CAD cells, whereas the extent of constitutive DAT internalization was not affected by PICK1 KD.

6 DISCUSSION

6.1 Surface distribution and trafficking of NET

The anatomical and subcellular distributions of NET in the brain and periphery reflect its function to mediate neuronal re-uptake of NE after synaptic release. NET demonstrated uniform plasma membrane distribution in the somata, projections and presynaptic boutons of cultured, live SCG neurons, with strongest surface expression seen on the plasma membrane of the neuronal projections and the boutons. Immunocytochemical labeling of mouse brainstem sections and dissociated sympathetic neurons in culture with NET antibodies supported previous observations that a considerable fraction of NET is present in intracellular compartments, both in the neuronal somata and in terminals (Miner et al., 2003; Savchenko et al., 2003; Sung et al., 2003; Matthies et al., 2009). This indicates that the transporter is dynamically shuffled between intracellular compartments and the plasma membrane. Indeed, the study I demonstrated that NET undergoes rapid constitutive internalization in dissociated sympathetic neurons in culture. NET was shown to be actively sequestered from the plasma membrane, not only in terminal sites but also in the somatic compartment. SCG neurons transduced with different endosomal markers demonstrated that NET is mainly sorted to Rab11 -positive recycling endosomes and little association was observed with markers of late endosomes and lysosomes, suggesting that only a limited fraction of internalized NET is targeted to proteolytic degradation. We observed some colocalization of internalized JHC 1-64 -labeled NET with Rab4 in the boutons of SCG neurons, indicating that NET also to some extent might recycle *via* the Rab4 -dependent “fast recycling” pathway. The importance of postendocytic pathways for proper protein function was evidenced by the observation that impairment of the Rab11 pathway resulted in decreased NET function. However, these observations rely upon overexpression or dominant-negative protein expression of the regulatory proteins in cultured neurons. Matthies et al. showed by the use of a NET endodomain antibody that amphetamine can induce increased association of NET with both Rab4 and Rab11 in SCG neurons, indicating that amphetamine might increase NET internalization and sorting to both fast and slow recycling pathways (Matthies et al., 2010). Moreover, *in vivo* translation of the findings, by *e.g.* observation of NET trafficking properties in brain slice preparations *ex vivo*, remains to be seen. It has been reported that in spite of constitutive internalization of DAT in dissociated midbrain dopaminergic neurons (Eriksen et al., 2009), surprisingly low level of endocytic trafficking of DAT is detected in somatodendritic and terminals areas of dopaminergic neurons *in vivo* (Block et al., 2015). Furthermore, the association of monoamine transporter trafficking to neuropsychiatric diseases states or chronic drug use is not currently known.

6.2 Differential trafficking itineraries of NET and DAT

Following internalization, the closely related SLC6 transporters, SERT and DAT, have been shown to be sorted mainly to lysosomes for degradation (Eriksen et al., 2010b; Rahbek-Clemmensen et al., 2014b). Comparison of the trafficking properties of NET and DAT in transfected CAD cells demonstrated that the extent of constitutive internalization is significantly greater for NET compared to DAT, and whereas NET was largely sorted into Rab11 -positive recycling endosomes, DAT demonstrated sorting to degradation, as reported before. To elucidate the structural domains underlying the differential postendocytic sorting of NET and DAT, we constructed several chimeras of NET and DAT to identify intracellular domains governing postendocytic sorting and/or internalization rates. Results from confocal imaging and biochemical assays suggest that the N-termini of the transporters play critical roles in determining the internalization rate and postendocytic fate of each transporter. Accordingly, a NET N-terminus was also demonstrated to be important for the differential targeting of NET and DAT in polarized epithelial cells (Gu et al., 2001), but to what extent it directly relates to our observations remains to be investigated. NET and DAT are highly homologous but most divergent in their N-terminal sequences that also include several sites for posttranslational modifications, such as protein phosphorylation, ubiquitination or sumoylation (Kristensen et al., 2011). Whereas N-terminal ubiquitination of DAT can regulate PMA-induced DAT internalization (Sorkina et al., 2009), it appeared not to affect the constitutive internalization and sorting of DAT (Eriksen et al., 2010). Moreover, the differential trafficking of NET and DAT might also be controlled by protein-protein interactions as seen with other homologous membrane proteins. For example, the dopamine D2 and D1 receptors as well as δ- and μ- opioid receptors are differentially sorted between degradative and recycling pathways following agonist-induced endocytosis and the sorting of D2 receptors to degradation is critically dependent on binding of the receptor to G protein-coupled-associated sorting protein (Tanowitz et al., 2003; Whistler et al., 2002, Bartlett et al., 2005; Dentin et al., 2007; Vargas et al., 2004). Whereas the distinct trafficking properties of the two G-protein coupled receptors are believed to enable differential physiological adaptation to agonist exposure, for the homologous NET and DAT the distinct trafficking properties may reflect different needs to adjust transporter availability. It is possible that tighter control of NET surface availability may be required for example to ensure proper physiological responsiveness of the sympathetic nervous system.

6.3 Protein kinases mediate regulation of monoamine transporter function and surface distribution

The kinome-wide siRNA screen suggested that kinases affecting DAT, SERT and NET surface distribution and function seemed to function in several signaling pathways. This supports the current understanding that these monoamine transporters are parts of multi-protein networks and subject to dynamic regulation. For example, several members of the cAMP-dependent protein kinase family, in the PI3K/Akt- and

MEK/ERK signaling pathways, scored as positive hits in the RNAi screen. Individual kinases of these pathways have previously been associated with the regulation of monoamine transporters (Moron et al., 2003; Samuvel et al., 2005; Fog et al., 2006; Speed et al., 2010). To our surprise, PKC isoforms were not among the prominent hits in the primary and secondary RNAi screens although the phorbol ester PMA repetitively has been shown to cause rapid redistribution of DAT and SERT away from the cell surface (Huff et al., 1997; Vaughan et al., 1997; Ramamoorthy et al., 1998; Jayanthi et al., 2004). This could indicate that a single PKC isoform is not alone crucial for maintaining basal surface expression of DAT and SERT. Thus, PMA-mediated effects might involve several PKC isoforms with functional redundancy, and/or other kinases than PKC that are similarly activated by PMA. It was previously observed in rodent trophoblasts that endogenous NET undergoes endocytosis in response to PMA (Jayanthi et al., 2004), and earlier studies have reported PMA-induced internalization of NET in transfected cells (Apparsundaram et al., 1998a; Apparsundaram et al., 1998b). In the present study, application of PMA (1 µM) during the internalization period enhanced intracellular accumulation of NET in dissociated sympathetic neurons, demonstrating that also neuronal NET can be subject to regulation by PKC and/or other kinases activated by phorbol esters.

SIK3 had prominent effects on the transcription of the transfected transporter constructs in a host cell -dependent manner. In HEK 293 cells and CAD cells, depletion of SIK3 caused marked increases and decreases, respectively, in monoamine transporter expression. These effects were likely produced by SIK3-mediated regulation of the CMV promoter that contains several CREs (cAMP Regulatory Element) in a tissue-specific manner (Takemori and Okamoto, 2008; Taub et al., 2010).

One of the most prominent hits in the RNAi screen was cAMP-dependent protein kinase PrKX that is highly expressed in the developing and adult brain (Klink et al., 1995). Depletion of PrKX caused simultaneous decreases in the surface expression and function of DAT without changing transporter protein levels, suggesting that PrKX stabilizes the transporter at the cell surface. The precise physiological roles of PrKX are still poorly understood but it is shown to be more sensitive to cAMP than PKA (Zimmermann et al., 1999). Thus, cAMP-dependent protein kinase effects on monoamine transporters may be partly undertaken by PrKX rather than PKA.

6.4 PICK1 mediated regulation of NET function

To date, there has been limited knowledge of the cytoplasmic domains of SLC6 family transporters and their functions driving transporter trafficking between compartments within the biosynthetic and endocytic pathways. The three distal C-terminal residues of human NET and DAT constitute classical class II PDZ motifs (φ-X-φ where φ is any hydrophobic amino acid and X is any amino acid) that are known to interact with the PDZ domain of PICK1 (Torres et al., 2001), but the physiological relevance of these interactions has remained unknown (Bjerggaard et al., 2004; Madsen et al., 2005;

Madsen et al., 2012; Rickhag et al., 2013). In the present study, genetic deletion of PICK1 significantly reduced NET expression levels in both brainstem and frontocortical brain tissue, and the loss of NET protein levels in noradrenergic terminals was accompanied by significant reductions in NET uptake capacity in frontocortical synaptosomes. These results are in contrast to the findings for DAT in PICK1 KO mice, where the expression and function of DAT have been reported to be unaltered (Rickhag et al., 2013). Nonetheless, an intact PDZ motif in DAT is indeed crucial for proper surface expression of the transporter (Rickhag et al., 2013), but the PDZ domain binding protein remains to be resolved. Lifelong knock-down of PICK1 in mice could activate compensatory mechanisms and therefore PICK1 was also acutely silenced in cultured sympathetic neurons and CAD cells to investigate the consequences for NET distribution and function. PICK1 KD markedly reduced overall and surface expression of NET with no accumulation of immature forms of the transporter in the sympathetic neurons or CAD cells. These findings argue against a role for PICK1 in the maturation or ER export of NET and rather support a role for PICK1 in forward trafficking of NET. Indeed, PICK1 KD and expression of the PICK1 PDZ domain mutant (PICK1 A87L) increased NET internalization and decreased uptake capacity in the sympathetic neurons indicating that the scaffolding protein PICK1 stabilizes the transporter in the plasma membrane. The differential role of PICK1 for NET and DAT distribution and function was also observed in CAD cells transiently expressing NET or DAT. Silencing of endogenous PICK1 decreased total and surface expression of NET but did not affect DAT expression. NET uptake rates were also decreased in PICK1 KD cells compared to WT cells while the K_m values for transport were indistinguishable, indicating that the fraction of NET remaining on the cell surface functioned normally. If PICK1 can act as an anchor to stabilize a PDZ domain binding partner on the cell surface, as indicated for the acid-sensing ion channel 1a (ASIC1a), Neph1 and the presynaptic G protein-coupled metabotropic glutamate receptor 7 (Suh et al., 2008; Hu et al., 2010; Hohne et al., 2011), it would be predicted that the protein will have the highest impact on a binding partner undergoing rapid forward trafficking. Consequently, it is possible that PICK1 might have had no measurable effect on DAT compared to NET simply due to the slower plasma membrane turnover kinetics of DAT.

Previous studies have established that in addition to surface stabilization of its binding partners, PICK1 can coordinate and regulate the subcellular localization of its interacting partners through divergent mechanisms (Iwakura et al., 2001; Hanley and Henley, 2005; Lin and Huganir, 2007; Rocca et al., 2008). Considering the differential postendocytic sorting of NET and DAT, it can be hypothesized that PICK1 could promote surface expression of NET by facilitating NET recycling. However, this is in conflict with previous evidence that PICK1 can inhibit recycling of binding partners sorted to the Rab11-positive compartment and thereby reduce surface expression of the binding partner (Madsen et al., 2012). It is possible that the surface stabilizing effect of PICK1 in case of NET simply overrules any other putative effects on the recycling pathway. Otherwise, we have no explanation for this apparent discrepancy, but it further underscores the remarkably versatile and context-dependent functions of PICK1 that conceivably derive from a complex interplay between the properties of the

binding partners, the cellular environment and PICK1 itself. Altogether, we show here the first evidence that PICK1 function is required for maintaining NET expression and function in noradrenergic neurons, possibly revealing a previously unappreciated role of PICK1 in monoamine homeostasis. These findings moreover substantiate that the strikingly different trafficking patterns of NET and DAT are differentially regulated by PICK1.

6.5 Methodological considerations

6.5.1 Cell models

Heterologous cellular models, where recombinant proteins of interest are stably or transiently overexpressed in different host cells, are commonly utilized to investigate complex biological processes in an isolated system. Compared to cells naturally expressing the protein of interest, heterologous cellular models can have some disadvantages. For example, the repertoire of regulatory proteins might differ in the host cell line from that of the "natural" enviroment, or the transcriptional regulation of the promoters present in the expression vectors can mislead the interpretations, as was seen in the present study (study II). In the current series of studies, sympathetic neurons derived from postnatal rat superior cervical ganglia were chosen as a primary cell culture model to investigate the function and regulation of NET. The motivation to employ an endogenous neuronal cell model was derived from previous reports indicating that the biosynthetic processing, distribution and trafficking of NET are dependent on the host cell type, *i.e.* the cellular regulation of NET appears to be distinct in fibroblast or epithelial cell models compared to neuronal cell environments (Melikian et al., 1996; Burton et al., 1998; Kippenberger et al., 1999; Bauman and Blakely, 2002). Thus, primary noradrenergic neurons expressing high levels of NET were considered to provide a more physiological environment than *e.g.* transfected fibroblasts to investigate the cellular regulation of NET. SCG neurons are known to synthesize catecholamines and neuropeptides and to secrete them from their terminal sites (May et al., 1995; Zhou and Misler, 1995; Koh and Hille, 1997) and to express proteins important for NET cellular regulation (Geffen and Livett, 1971; Livett, 1973; Savchenko et al., 2003; Matthies et al., 2009). In addition, SCG neurons are well suited for trafficking studies since the neurons are characterized by large round somata and a very extensive network of neuronal projections with multiple "boutons" likely representing presynaptic NE release sites (Matthies et al., 2009).

Still, caution should be applied when interpreting results derived from cultured postnatal rat SCG neurons because of possible differences in many cellular processes *e.g.* compared to noradrenergic neurons in the brainstem of an adult animal. In addition, species differences between rat and human in their noradrenergic neuronal systems may flaw the interpretation (Sanders et al., 2005). The cellular model closest to human physiology would be primary human noradrenergic neurons. Whereas some human cell types, such as blood cells or adipocytes, are available at relative ease, it is

not since recently that human neuronal cell models can be generated. This is now due to breakthroughs in the reprogramming or direct conversion of adult somatic cells to generate neuronal cells (Takahashi and Yamanaka, 2006; Mertens et al., 2016). Furthermore, to recapture the natural environment of real nerve cells in a model system, acute brain slices and co-cultures of different neuronal and non-neuronal cell types might provide means to investigate NET regulation in more physiological environments where input signals and cross-talk of cells are present.

6.5.2 JHC 1-64

Recent studies have provided insights into the molecular mechanisms involved in the regulation of Na^+/Cl^--dependent neurotransmitter transporters. Their findings have emphasized that altered membrane trafficking of these transporters is an important way to regulate their function (Zahniser and Doolen, 2001). It has proven difficult to directly monitor NET trafficking due to failed efforts to develop highly specific antibodies directed to the extracellular domains of NET. Furthermore, even small modifications of the extracellular loops that were tolerated by DAT and SERT have been found to change NET expression and function and thus, extracellular epitope tagging and large fusion protein approaches to study NET trafficking have not proven successful. In the current series of studies, we employed the novel fluorescent cocaine analogue JHC 1-64, which provides a tool to directly visualize and quantify the surface distribution and internalization of endogenous NET even in single boutons of live neurons. We aimed to carefully validate the use of JHC 1-64 in study I, and therefore documented that the binding of JHC 1-64 was entirely blocked by pre-incubation of the cultures with NET inhibitor desipramine, the dissociation of JHC 1-64 from the transporter was slow and excluded that the compound itself would interfere with transporter surface expression or trafficking properties. Rapid live cell imaging of JHC 1-64 -labeled SCG neurons demonstrated for the first time that NET is clearly present on the plasma membrane of SCG cell somata. Notably, NET expression in the plasma membrane of the noradrenergic somata has not been reported in previous immunolabeling studies utilizing NET antibodies (Sung et al., 2003; Matthies et al., 2009; Matthies et al., 2010). These studies and our investigations using fixation and permeabilization procedures revealed punctate NET expression in the projections and varicosities (Miner et al., 2003; Savchenko et al., 2003; Sung et al., 2003; Matthies et al., 2009) and considerable intracellular immunostaining corresponding to both somata and boutons. The presence of NET on the neuronal somata observed in study I could indicate the importance of volume transmission also for NE actions.

6.5.3 RNAi screen

Genetic screens have become powerful methods to explore biological processes without a pre-formulated exact hypothesis, and have become standard experimental tools during the last decade. A clearly defined assay that is specific and relevant for the biological process being investigated is crucial for a successful screen. Often, the ease

of an assay is inversely proportional to its specificity and one needs to find the best compromise between assay feasibility (*e.g.* cell model, time points and technologies) and its information value. In the present RNAi screen in study II, monoclonal HEK 293 cell lines stably expressing DAT or SERT were chosen as model systems and the [^{3}H]-DA/[^{3}H]-5-HT uptake assays as the primary functional readouts, since both the cell lines and the assays have demonstrated robust performance (Rasmussen et al., 2009). In this end-point assay setup, the functional activity of the transporter involved several independent processes, such as transcription and translation of the transporter, biosynthetic processing and targeting of the transporter to the correct subcellular compartment as well as regulation of intrinsic transport activity and trafficking of the mature transporter. All of these sub-processes may be modified by the siRNA - mediated silencing of the targeted protein kinases. Automated assay set-up and image analysis might be useful to make it possible to include several readouts in the assay. Positive and negative controls were used to optimize the assay set-up in order to achieve a reproducible and robust primary assay with a high signal and low noise. Thus, we selected the cell clones where PMA and the PI3K inhibitor LY294200, which is known to down-regulate transport activity of DAT and SERT (Lin et al., 2003; Qian et al., 1997), gave the best response window, *i.e.* the largest relative reduction in uptake and the best reproducibility of the response. These positive controls caused 20-30 % changes in the uptake capacity of the transporters at steady state, indicating that the assay might only be able to identify strong hits.

The primary screen resulted in a list of hits that scored as positive in the primary assay (>30 % up- or down-regulation in the function of at least one of the two transporters relative to plate mean). The majority of the identified hits (93) in the primary screen regulated the function of DAT, whereas notably fewer kinases altered the function of SERT. Moreover, only a small fraction of the identified hits was found to affect the function of both DAT and SERT. This surprising result might indicate different kinase-mediated regulation of the two transporters, or might also at least partly be due to variation in the sensitivity of the two cell lines and/or the reproducibility of the primary assay.

Secondary screens typically aim to eliminate false positive hits caused *e.g.* by the siRNA off-target effects or by experimental errors, and to identify hits that are relevant for the biological process investigated. Here, the 93 possible targets identified in the primary screen were analyzed both for uptake and with surface ELISA with a new set of siRNAs to increase confidence in the hits. In addition, we tested for off-target effects by monitoring cell viability. Additional tests for non-specific effects, *e.g.* effects on transcriptional regulation of the plasmid, were not included at this secondary screening phase. The secondary screen revealed 26 kinases that potentially modulated the function and/or the surface expression of DAT and/or SERT (>15 % (uptake) or > 10 % (ELISA) up- or down-regulation for one or both of the two siRNAs). Knock-down of most of the hits only affected transporter function with no effect on the transporter surface expression. This most likely represents the lower sensitivity of the ELISA assay rather than the possibility that kinases would more frequently affect transporter intrinsic activity than transporter trafficking. Three selected hits (SIK3,

PrKX, PKA C-α) were then further validated by monitoring the effects of kinase knock-down on transporter expression and function in neuronal CAD and PC12 cell lines transiently expressing DAT, SERT or NET. Despite several attempts, we did not manage to get sufficient knock-down of these kinases in primary neurons with the use of lentivirus-mediated delivery of commercial shRNA vectors.

7 CONCLUSIONS

These studies on the cellular regulation of NET surface distribution, trafficking and function allow the following conclusion:

1. NET expression was clearly observed in the plasma membrane of the neuronal somata, projections and boutons. NET demonstrated extensive constitutive internalization and sorting mainly to Rab 11- positive recycling endosomes. NET displayed significantly greater extent of constitutive internalization compared to DAT in neuronal cell models, and whereas NET was largely sorted for recycling, DAT was mainly sorted for degradation. The differential trafficking of NET and DAT was determined by non-conserved structural elements in the intracellular N-termini of the transporter proteins.

2. The kinome-wide RNAi screen suggested that the function of monoamine transporters is subject to regulation by protein kinases operating in several signaling pathways, such as in PI3K/Akt- and MEK/ERK pathways and cAMP-dependent protein kinase pathways. PrkX was identified as a novel kinase involved in the regulation of the surface expression and function of DAT in neuronal cell models.

3. The PDZ domain scaffolding protein PICK1 promoted NET function in noradrenergic neurons by stabilizing NET in the plasma membrane. In neuronal cell models, it was shown that PICK1 is required to maintain NET, but not DAT, expression and function.

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