Cellular characterization of the outgrowth and connectivity of the brain serotonin system

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Cellular characterization of the outgrowth and connectivity of the brain serotonin system

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Chapter

1

General introduction

General introduction

The mammalian brain is by far the most complex system in the body. The human brain consists of approximately 100 billion nerve cells (neurons), which form a highly complex wiring scheme. How this complex wiring is achieved is still poorly understood. The brain contains several clusters of cells which contain a certain neurotransmitter, such as dopaminergic cells in the substantia nigra or acetylcholinergic cells in ventral and dorsal striatum.

Another example of such clusters are the raphe nuclei in the midbrain that secrete serotonin (5-hydroxytryptamine, 5-HT). 5-HT has diverse modes of action in the central nervous system and is involved in brain development as will be described in this introduction. There are a few remarkable characteristics of the 5-HT system. First of all, the 5-HT system consists of only a few neurons (~1000 in rodents, ~0.5 million in humans) compared to the total number of neurons. Nevertheless, these neurons send projections to virtually every other brain area. It is estimated that each 5-HT neuron makes more than 1×10^5 connections. Secondly, 5-HT is predominantly released extrasynaptically (so called volume or paracrine transmission), which implies that 5-HT acts on a slower and longer-lasting time scale compared to classical neurotransmitters like glutamate. Thirdly, emerging evidence shows that 5-HT has a role during brain development. Finally, the 5-HT system is involved in certain behavioural and psychopathological processes including anxiety, depression, aggression and obsessive-compulsive disorder (OCD).

In this chapter the main aspects of the 5-HT system will be introduced and the diverse modes of action which 5-HT has are described. First, there will be a description about the anatomical organization and development of the 5-HT system. Subsequently, the role of 5-HT in brain development will be introduced and the relation between the 5-HT system and behavioural and psychopathological processes will be discussed. Finally, the aim and scope of this thesis will be outlined.

Anatomical organization of 5-HT cell clusters

After the initial discovery of 5-HT (see box I), it took about 20 years before the cellular distribution of 5-HT was described by Dahlstrom and Fuxe using the Falck-Hillarp technique. They showed that 5-HT cell bodies are clustered in nine cell groups close to the midline in the rhombencephalon, called the raphe nuclei. The nine nuclei are divided in two groups, the rostral and caudal group. The caudal group consists of clusters B1 to B5 and send projections predominantly in the midbrain itself and towards the spinal cord. On the other hand, the rostral

cell group consists of clusters B6 to B9 and sends projections to the forebrain. The largest clusters of cells are the dorsal raphe nucleus (DRN) and the median raphe nucleus (MRN) (B7 and B8 respectively). These raphe nuclei also contain several types of non-5-HT neurons, such as GABA-ergic and noradrenergic neurons. The 5-HT neurons densely innervate virtually every brain area, with the most dense 5-HT innervations in the cortex and limbic structures including the hippocampus and basal ganglia.

Box I **The discovery of 5-HT**

In the 1930's Dr. Vittorio Erspamer tried to extract chemical compounds from enterochromaffin cells capable of causing smooth muscle contraction in the gut. In an extract of rabbit gastric mucosa, he identified a substance that could cause smooth muscle contraction. He called this substance enteramine, since it was an amino acid derivative (**amine)** found in **entero**chromaffin cells.

In the 1940's another group identified a substance which had vasoconstrictive activity in serum. This serum influenced the tone, hence the name serotonin. When analyzing both substances, it was found that enteramine and serotonin were the same substance.

The first indication for a role of 5-HT in the brain came from Woolley, who showed that 5-HT and the hallucinogenic compound lysergic acid diethylamide (LSD) had comparable actions in the cortex of the cat. This eventually led to the discovery of 5-HT in the vertebrate brain like man and dog.

Dahlstrom and Fuxe were the first to describe the distribution of 5-HT cell bodies in the brain by histochemical detection of the 5-HT derivative indoleamine. The description of 5-HT projections from the 5-HT cell bodies towards the telencephalon and the diencephalon followed one year later. It was found that in the brain stem a relatively small population of 5-HT neurons was present, with the largest clusters in the dorsal and median raphe nuclei.

The development of the 5-HT system

In the mouse brain, 5-HT neurons are generated around embryonic day (E) 10, and these cells are among the first neurons expressing a specific neurotransmitter. In the neural tube, 5-HT precursor cells are formed by the combined action of sonic Hedgehog, Fgf4 and Fgf8. The proper position of these 5-HT precursor cells is defined by the midbrain-hindbrain organizing centre (MHO), Shh signaling and the transcription factor Nkx2.2 (see figure 1).

In a knockout (KO) mouse for Nkx2.2, no 5-HT neurons are present in the midbrain raphe nuclei, except in the DRN, showing that Nkx2.2 is essential for 5-HT neuron differentiation (Briscoe et al., 1999). Another transcription factor that is involved in the initial development of 5-HT neurons is GATA3 (van Doorninck et al., 1999). After 5-HT precursor cells are formed, other

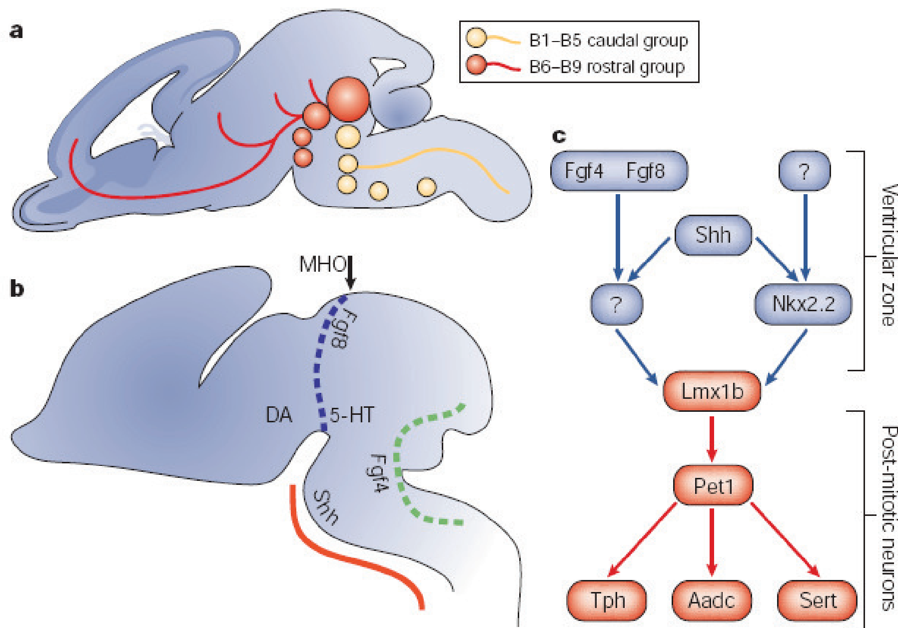

Figure 1. Genetic factors involved in determining the 5-HT system. At the MHO, Fgf4 and Fgf8 are involved in the specification of the 5-HT precursor neurons. Image adapted from Gaspar et al., 2003.

factors are required to establish mature 5-HT neurons. Among these factors are two transcription factors, LIM homeobox transcription factor 1 (Lmx1) and PC12 Ets factor (Pet1).

Lmx1b expression starts at E10.75 in the ventral part of the hindbrain including the floor plate and from E13.5 Lmx1b is present in the raphe nuclei (Ding et al., 2003). Lmx1b KO mice die within 24 hours after birth (Chen et al., 1998). Lmx1b-deficient mice lack all 5-HT neurons, even when Lmx1b was only deleted in 5-HT neurons, (Ding et al., 2003; Zhao et al., 2006). These studies show that Lmx1b is not required for the initial generation of 5-HT neurons, but is necessary for the differentiation and survival of 5-HT neurons during postnatal development.

Pet1 was found in a screen in PC12 cells to identify novel ETS domain transcription factors (Fyodorov et al., 1998). Pet1 expression is restricted to the raphe nuclei and precedes the expression of 5-HT by approximately half a day (Hendricks et al., 1999). Furthermore, several genes specific for 5-HT neurons, such as the rate limiting enzyme tryptophan hydroxylase (Tph) and the 5-HT reuptake transporter (SERT) have a conserved Pet1 binding site in their promoter (Hendricks et al., 1999). This suggests that Pet1 is involved in the final

differentiation of 5-HT neurons. In a Pet1 KO mouse, there is an approximately 70% reduction in number of 5-HT neurons. The fact that there are still 30% of 5-HT neurons remaining suggests that these are either independent of Pet1 or there is another unknown transcription factor.

After this genetic cascade, at approximately E11.5, 5-HT neurons start expressing the enzymes necessary for 5-HT synthesis (see box II) and proteins necessary for reuptake (SERT) and degradation of 5-HT (Monoamine-A (MAO-A)). Shortly after initiation of expression of these genes, 5-HT neurons start to

Box II **Synthesis of 5-HT**

5-HT is a neurotransmitter which belongs to the group of the biogenic amines, to which also the catecholamines (dopamine, adrenaline and noradrenaline) belong. All biogenic amines are derived from amino acids: the catecholamines from tyrosine, and 5-HT from tryptophan. 5-HT and the precursor tryptophan belong to a group of chemical compounds called the indoles, with a benzene ring joined to a five-member ring containing nitrogen. Tryptophan is an essential amino acid, which means that this amino acid cannot be made in the body from other amino acids and must therefore be ingested through the diet.

For synthesis of 5-HT in the brain, tryptophan is actively transported through the blood brain barrier via the large neutral amino acid transporter (LAT1). Once in the brain, tryptophan is metabolized to 5-HT using two different enzymes. First, tryptophan is hydroxylated in the 5-position to generate 5-hydroxy-L-tryptophan. This reaction is catalyzed by Tph, which is the rate limiting enzyme in 5-HT synthesis. Subsequently 5-hydroxy-L-tryptophan is converted to 5-hydroxytryptamine (5-HT) by the enzyme 5-hydroxytryptophan decarboxylase. 5-HT is stored in synaptic vesicles and large dense cored vesicles and transported into these vesicles by the enzyme Vesicular Monoamine Transporter 2 (VMAT2). After being released, 5-HT is transported back into the terminal using SERT, and either recycled or converted to 5-hydroxyindolacetic acid (5-HIAA) by MAO-A.

The rate limiting enzyme Tph has a (6R)-L-erythro-5,6,7,8-Tetrahydrobiopterin (Tetrahydrobiopterin, BH4) binding site. BH4 is an essential cofactor for the proper functioning of Tph. Without BH4, Tph cannot hydroxylate its substrate and thus 5-HT cannot be synthesized. BH4 is also required for dopamine synthesis by acting as a cofactor for tyrosine hydroxylase. Mice deficient for the BH4 gene die within 48 hours after birth and have extremely low levels of 5-HT and dopamine.

It has long been thought that only one Tph gene was present in the mammalian brain, although in zebrafish, two Tph genes were identified. However, in a mouse deficient for the Tph gene, no difference in brain 5-HT levels was found compared to control mice. This led to the discovery of the second Tph gene, called neuronal Tph or Tph2 (Walther et al., 2003). By using immunohistochemical analysis and in situ hybridization, it has been shown that Tph1 is expressed in the periferic system in the enterochromaffin cells and in the pineal gland in the brain, whereas Tph2 is exclusively expressed in the raphe nuclei in brain and in the myenteric plexus in the gut (Patel et al., 2004; Malek et al., 2005; Gutknecht et al., 2009).

release 5-HT and start to send axon branches towards the spinal cord and via the medial forebrain bundle towards the cortex. Only after birth the 5-HT network is fully matured.

In the human brain, 5-HT neurons are for the first time detected when the embryo is five weeks old (Sundstrom et al., 1993). At ten weeks of age, 5-HT neurons are detected in all nuclei of the reticular formation and after 15 weeks, the 5-HT neurons are clustered in the raphe nuclei (Shen et al., 1989). After birth, 5-HT levels increase during the first two years and then decline to adult levels at the age of five years (Hedner et al., 1986).

5-HT release is both synaptic and extrasynaptic

Several classical neurotransmitters such as GABA, acetylcholine and glutamate are released from small synaptic vesicles (SSVs) at synaptic sites with a postsynapse in close proximity. However, neurotransmitters such as dopamine and 5-HT are released not only synaptically, but there is also so-called extrasynaptic release (also called paracrine transmission or volume transmission; here there will be referred to this type of release as volume transmission). A major difference between synaptic release and volume transmission is that there is no postsynapse present, so upon release the neurotransmitters diffuse away and can mediate effects distant from the release site. In this manner volume transmission can produce paracrine effects. In contrast to synaptic release, where SSVs are only released from axonal terminals, volume transmission of 5-HT occurs also in the soma and in dendrites, the so-called somatodendritic release. In 5-HT neurons, vesicles storing 5-HT are not only present in axonal terminals, but also in somata, dendrites and axonal varicosities (Hery and Ternaux, 1981). It was shown in Retzius neurons of the leech that 5-HT is released from both SSVs and large dense core vesicles (LDCVs), where there is an average discharge of 4700 and 80.000 molecules of 5-HT for SSVs and LDCVs respectively (Bruns and Jahn, 1995). In another study of the same authors, several differences between SSV and LDCV release of 5-HT were shown. First of all there was a difference in distribution: while SSVs were exclusively found in axons, LDCVs were also found at the soma. Furthermore LDCV release was slower compared to SSV release (Bruns et al., 2000).

There are several lines of evidence for volume transmission of 5-HT, some of which are indirect. First of all it has been shown that 5-HT varicosities contain 5-HT vesicles, but lack a postsynaptic specialization in close proximity (Moukhles et al., 1997). 5-HT receptors and the SERT are localized at sites away from release sites, indicating that 5-HT diffuses over larger distances to activate these receptors (Ridet et al., 1993). The presence of SERT distant from release sites

suggests that these are required to transport 5-HT back into the cells to terminate the action of 5-HT in the extracellular space. Secondly, it has been shown by carbon fiber amperometry that upon stimulation an increase of 5-HT levels in the extracellular medium occurs (Bunin and Wightman, 1998). This suggests that upon stimulation, not all 5-HT is buffered by synaptic receptors and transporters, but diffuses away into the extracellular medium (Bunin and Wightman, 1998).

In contrast to extrasynaptic release, synaptic release occurs predominantly from SSVs. Apart from vesicular release of 5-HT, there is also evidence for non-vesicular release of 5-HT using a carrier-mediated mechanism (Levi and Raiteri, 1993).

The ratio between volume transmission and synaptic transmission of 5-HT depends on the location in the brain. In the DRN itself, 5-HT release is predominantly paracrine, and here somatodendritic 5-HT release activates 5-HT_{1A} autoreceptors which are present at the somata and dendrites. These activated receptors activate a G protein-activated inwardly rectifying K^+ (GIRK) current. In target areas of the 5-HT system, the ratio between volume transmission and synaptic transmission is still under debate. However, there are electron microscopy studies that quantified the percentage of 5-HT varicosities which formed a complex with a postsynaptic specialization. This revealed that ~25%, 48% and 38% of 5-HT varicosities formed a synaptic complex in rat hippocampus, suprachiasmatic nuclei and supraoptic nuclei of the hypothalamus respectively (Oleskevich and Descarries, 1990; Boulaich et al., 1994). It is noteworthy that 5-HT is not released exclusively from 5-HT neurons. During development, thalamocortical fibers transiently express SERT, capture 5-HT and use it as a borrowed transmitter (Lebrand et al., 1996). In the striatum, dopamine transporters can take up 5-HT and subsequently co-release dopamine and 5-HT from dopaminergic terminals (Zhou et al., 2005a).

5-HT has several effects on synaptic transmission

5-HT not only functions to activate postsynaptic receptors, but also has diverse functions in the presynapse. Several studies were performed on invertebrates, such as crayfish, lamprey and aplysia. It was observed at the crayfish neuromuscular junction that a brief exposure to 5-HT resulted in increased release of glutamate (Dixon and Atwood, 1989a). Injection of a protein kinase A inhibitor or an adenylate cyclase inhibitor abolished this effect, suggesting that the facilitatory effect of 5-HT required the action of both the phosphatidylinositol and adenylate cyclase second messenger pathway (Dixon and Atwood, 1989b). In a study by Wang and colleagues, they made use of the styryl dye FM1-43, a fluorescent dye which is taken up by endocytosis following vesicle release, thus

specifically labeling recycled vesicles (Betz and Bewick, 1992). Using this approach, application of 5-HT at the crayfish neuromuscular junction did not affect the rate of recycling. Instead, 5-HT increased the number of vesicles which are available for release, the ready-releasable pool (RRP) of vesicles (Wang and Zucker, 1998).

In contrast to this stimulatory effect in the crayfish neuromuscular junction, 5-HT has an inhibitory action on synaptic transmission in the lamprey giant synapse (Buchanan and Grillner, 1991). This effect of 5-HT is likely mediated via G protein βγ subunits, since injection of these subunits mimic the effect of 5-HT (Blackmer et al., 2001). Furthermore the presynaptic protein SNAP25 is involved in this effect, since injection of Botulinum toxin A, which cleaves SNAP25, abolished the effect of 5-HT (Gerachshenko et al., 2005). Again using FM-dyes it was demonstrated that the inhibitory effect of 5-HT on the lamprey giant synapse is mediated via a change in vesicle fusion properties since 5-HT seemed to prevent full fusion of vesicles (Photowala et al., 2006).

In aplysia the effect of 5-HT on the gill-withdrawal reflex is well studied. In the 70's it was shown for the first time that local application of 5-HT sensitized the gill-withdrawal reflex. This effect was also shown after application of cyclic adenosine monophosphate (cAMP), suggesting that this effect of 5-HT is mediated via an increase in cAMP (Brunelli et al., 1976). Both 5-HT and cAMP can close single potassium channels in aplysia sensory neurons, resulting in an increased transmitter release (Siegelbaum et al., 1982). This K^+ channel, called the S (serotonin)-type K^+ channel is modulated by 5-HT, cAMP and protein kinase A (PKA) (Shuster et al., 1985). Using calcium imaging on isolated synapses in culture, it was shown that 5-HT application results in a calcium influx in the terminal (Eliot et al., 1993). A brief single pulse of 5-HT resulted in short-term facilitation lasting minutes, whereas five subsequent pulses of 5-HT over 1.5 hours resulted in long-term facilitation lasting more than one day (Montarolo et al., 1986). 5-HT does not only affect release characteristics at single synapses, but upon application also activates silent presynaptic terminals. This results in the generation of new functional release sites within hours after application (Kim et al., 2003). This 5-HT-related growth of new synapses requires Cdc42, N-WASP and PAK (Udo et al., 2005).

In summary, 5-HT affects synaptic transmission in several ways. In crayfish neuromuscular junctions, 5-HT increases synaptic transmission by increasing the size of the RRP of vesicles. In the lamprey giant synapse 5-HT inhibits transmission by affecting single vesicle release kinetics. Finally, in aplysia sensory neurons, application of 5-HT can induce both short term facilitation and long term facilitation via an increase in transmitter release and the formation of new functional release sites.

5-HT has a role during development in maturation of the brain.

An increasing amount of evidence points towards a role of 5-HT in brain development and maturation. Additionally, several studies show that 5-HT has an important role during early embryogenesis. 5-HT is present at early stages in sea urchins, chick embryos and rodents (Emanuelsson et al., 1988; Buznikov, 1991). Application of a 5-HT antagonist to sea urchin embryos significantly delayed cell division, implicating a role in the regulation of cell division (Renaud et al., 1983). In sea urchin blastulae, 5-HT, but also dopamine and noradrenaline stimulate ciliary activity. Application of these monoamines was associated with changes in intracellular calcium and adenylyl cyclase activity (Soliman, 1983). In the chick embryo, 5-HT is synthesized by the notochord (Wallace, 1982). Monoamine inhibitors cause malformation in the chick embryo such as neural tube defects. Exposure of mouse embryos to 5-HT resulted in craniofacial malformations and increased cell death in craniofacial and cardiac mesenchyme (Yavarone et al., 1993); (Shuey et al., 1993). Several studies in rodents used injections of either p-chlorophenylalanine (PCPA) to inhibit Tph or the 5-HT-specific neurotoxin 5,7-dihydroxytryptamine. Injection of PCPA in mouse embryos from E12-E17 resulted in subtle alterations in pyramidal and non-pyramidal neuronal populations (Vitalis et al., 2007). 5,7-dihydroxytryptamine was injected at the day of birth in mice embryos in the medial forebrain bundle. These injected mice had a decreased 5-HT innervation towards the cortex and hippocampus and male mice showed a decreased novelty-induced exploration, suggesting an anxiety-like phenotype (Hohmann et al., 2007).

KO mice have shown to be a valuable tool in studying the role of the 5-HT system in development. KO mice for tryptophan hydroxylase 1 (Tph1), the rate limiting enzyme involved in periferic 5-HT synthesis, lack periferic 5-HT (Walther et al., 2003). Although these KO mice do not have an overt phenotype, approximately 80% of pups born from homozygous mothers are smaller and show developmental abnormalities compared to embryos born from heterozygous or wildtype mothers (Cote et al., 2007).

This phenotype was only observed in Tph1 KO embryos born from Tph1 KO mothers, showing that maternal 5-HT is crucial for normal embryonic development. Tryptophan hydroxylase 2 (Tph2) is the Tph isoform that is exclusively expressed in the raphe nuclei (Gutknecht et al., 2009). Deletion of Tph2 does not result in an overt phenotype, nor does deletion of both Tph1 and Tph2, although in another study a reduced viability in these mice was observed (Savelieva et al., 2008; Alenina et al., 2009). Thus it appears that maternal 5-HT is indispensable for proper embryonic (brain) development, but 5-HT synthesis in the embryo is not.

5-HT as a neurotrophic factor

In addition to the role of 5-HT in (early) neurodevelopment, 5-HT also functions as a neurotrophic factor. Application of 5-HT to buccal ganglion neuron 19 in helisoma caused a cessation in filopodial motility and an inhibition of neurite outgrowth (Haydon et al., 1984). Application of 5-HT results in a rise in intracellular calcium levels in the growth cone which probably links directly to the inhibition in outgrowth (McCobb et al., 1988). Application of 5-HT to 5-HT neurons also resulted in decreased outgrowth (Whitaker-Azmitia and Azmitia, 1986). In contrast to this inhibitory effect of 5-HT on growth of 5-HT neurons, 5-HT acts as a stimulatory signal on thalamic neurons which display more processes per cell and increased length of processes upon 5-HT administration (Lieske et al., 1999).

Mouse models with an impaired 5-HT system

Since the introduction of the knockout technique in mice, several genes of the 5-HT system have been assessed for their effect on development and behaviour. The most important genes with striking effects on brain development or behaviour will be mentioned here. Mice lacking the MAO-A gene involved in the degradation of 5-HT to 5-hydroxyindolacetic acid (5-HIAA) display increased levels of 5-HT (Cases et al., 1996). These mice have a lack of barrels in the somatosensory cortex that is restored by application of PCPA, suggesting that this effect is due to excess levels of 5-HT (Cases et al., 1996). Additionally, mice lacking MAO-A display increased aggressive behaviour (Cases et al., 1995).

On the contrary, deleting the Vesicular Monoamine Transporter 2 (VMAT2) which is necessary for 5-HT packaging in vesicles, results in mice with brain 5-HT levels of ~1% compared to control (Wang et al., 1997; Fon et al., 1997). These KO mice are significantly smaller compared to wildtype littermates and the majority dies within a few days after birth. 5-HT fibers are almost completely absent in the cortex at that age. VMAT2 heterozygotes display prolonged immobility times in the forced swim test, suggesting depressive-like behaviour (Fukui et al., 2007). This behaviour was normalized by the antidepressant imipramine.

Deletion of the SERT which is essential for 5-HT reuptake results in mice with increased extracellular 5-HT levels (Mathews et al., 2004). These mice have a reduced number of 5-HT neurons in the DRN, a reduced firing rate of these neurons and these mice display behavioural alterations (Lira et al., 2003). Notably, these behavioural effects were mimicked by applying the SERT blocker fluoxetine to young control mice between postnatal day 4 and 21 (Ansorge et al., 2004).

In addition to these genes involved in 5-HT synthesis, packaging, breakdown or reuptake, several 5-HT receptor genes have also been ablated in mice (for an overview of 5-HT receptors see Box III). The 5-HT_{1A} receptor acts as a heteroreceptor on non-5-HT neurons, but also as a 5-HT autoreceptor in the DRN. Mice with a deletion of the 5-HT_{1A} receptor spent less time in the center of the open field in the open field test and avoid the open arms in the elevated plus maze, suggesting that these mice display an increased anxiety-related behaviour (Parks et al., 1998; Ramboz et al., 1998). A very elegant study demonstrated that this effect on behaviour in the 5-HT_{1A} receptor KO occurs during development. In this study a second mouse line was made in which the expression of the 5-HT_{1A} receptor could be restored specifically in the forebrain. Rescue of 5-HT_{1A} receptor expression in the forebrain was sufficient to restore normal anxiety-related behaviour (Gross et al., 2002). In contrast, deletion of the 5-HT_{1A} receptor during development and rescue of the receptor after postnatal day 21 did not restore normal anxiety-related behaviour (Gross et al., 2002). This clearly shows that the 5-HT_{1A} receptor is required during development to establish normal anxiety-related behaviour.

Box III **5-HT receptors**

At least 14 different 5-HT receptor subtypes are known which are divided into 6 different groups. The first and largest group of 5-HT receptors is the 5-HT_1 group consisting of the 1A, 1B, 1D, 1E, 1F and 1P receptors. All receptors in this group are coupled to G proteins, and activation results in a decrease in cAMP levels. The most important receptor of this group is the 5-HT_{1A} receptor, which is expressed in the raphe nuclei on 5-HT cell bodies and functions as a 5-HT autoreceptor. Binding of 5-HT to the 5-HT_{1A} receptor results in activation of a GIRK channel which results in hyperpolarization of the 5-HT neuron; in this way, the 5-HT_{1A} receptor is involved in fine-tuning 5-HT release.

To the 5-HT_2 receptor group belong the 2A, 2B and 2C receptors. These receptors are also coupled to G proteins, and activation of these receptors results in an increase of inositol 3-phosphate (IP3). The 5-HT_3 receptor is the only 5-HT receptor which is a (Na^+/K^+) ion channel. The 5-HT_4 and 5-$HT_{6,7}$ receptors are coupled to Gs proteins and activation results in an increase of cAMP. Finally, activation of the 5-$HT_{5A,B}$ receptor results in cAMP decrease. For the majority of these 5-HT receptors there are agonists and antagonists known. For example, the 5-HT_{1A} receptor is agonized by 8-OH-DPAT whereas it is antagonized by WAY-100635.

Mice lacking the 5-HT_{1B} receptor display an increased aggressive-related behaviour: they have a shorter latency to attack in the resident intruder paradigm (Saudou et al., 1994). The 5-HT innervation is increased in the hippocampus and the amygdala of these mice, possibly contributing to the aggressive phenotype of these mice (Ase et al., 2001). Another receptor known to

be localized in the DRN is the 5-HT_{2C} receptor (Clemett et al., 2000). This receptor is localized on GABA-ergic neurons and activation of this receptor results in a decreased firing of 5-HT neurons (Boothman et al., 2006). Mice that lack the 5-HT_{2C} receptor are overweight and suffer from seizures (Tecott et al., 1995). Moreover, studies have been performed to show that these mice display compulsive-like behaviour (Chou-Green et al., 2003).

An additional study showed that 5-HT_{2C} KO mice display decreased inhibition in the conflict anxiety paradigm (Weisstaub et al., 2006). This decreased inhibition was rescued by selective restoration of the 5-HT_{2C} receptor in the cortex, suggesting that 5-HT_{2C} receptors in the DRN are not involved in this effect (Weisstaub et al., 2006).

Thus, 5-HT receptor KO mice do not exhibit overt developmental defects, but anxiety- or aggressive-related behaviour of these animals is affected as assessed in various behavioural paradigms.

The 5-HT system in relation to psychopathological processes

Anxiety & depression

The notion that the 5-HT system could be involved in psychopathological conditions such as major depression and anxiety-related disorders came only after the discovery that drugs used to treat these conditions exerted their function (partially) via the 5-HT system. In the late 50's, compounds such as iproniazide and G22355 (later named imipramine) were prescribed to patients suffering from tuberculosis. However, serendipitously it was found that these compounds were also effective in treating depression (Bloch et al., 1954; Kuhn, 1958). Only several years after it was found that these drugs had an antidepressant response, the mode of action of these drugs was elucidated; iproniazide inhibited the degradation of mono-aminergic compounds (5-HT, noradrenaline and dopamine), whereas imipramine blocked the reuptake of 5-HT and noradrenaline into the terminal. These findings led to the postulation of the monoamine hypothesis of depression in the late 60's, which stated that a decreased concentration of 5-HT and noradrenaline in the extracellular space might be responsible for depression. It was poorly understood, however, why there was such a discrepancy between the immediate modes of action of these compounds in contrast to the delayed therapeutic effects. Also, it was not clear why only ~$^2/_3$ of all patients responded to these drugs.

These considerations abandoned the monoamine hypothesis and the so-called network hypothesis of depression was postulated. This hypothesis states that depression results from an alteration in certain neuronal networks, and antidepressants restore these networks, which explains the time lag between

starting antidepressant administration and the onset of therapeutic actions. Evidence for this hypothesis, however, is still limited and mostly indirect. Human studies using functional magnetic resonance imaging (fMRI) or positron emission tomography (PET) imaging on patients suffering from major depression have shown volume reductions in cortex and hippocampus. Although farfetched, one could argue that this reflects a decreased network density.

The last few years a new hypothesis was proposed, largely based on the work by Santarelli and colleagues: the neurogenesis hypothesis. It had already been shown that treatment with antidepressants increased adult neurogenesis in the dentate gyrus (Malberg et al., 2000; Perera et al., 2007). Santarelli and colleagues showed that blocking neurogenesis in the dentate gyrus by X-irradiation blocked the behavioural effects of the antidepressant fluoxetine (Prozac) (Santarelli et al., 2003). In other words neurogenesis is required for the behavioural effects of antidepressants. However, several studies have shown that neurogenesis is not involved in the etiology of depression.

Thus, although it is clear that there is a link between the 5-HT system and anxiety and depression, it is still far from clear what this exact link is.

Aggression

Aggressive behaviour is defined as violent and impulsive behaviour often resulting in breaking the law. 5-HT is one of the neurotransmitters which is implicated in the etiology of aggression. A mutation in the MAO-A gene caused aggression-related abnormal behaviour (impulsive aggression, arson, attempted rape, and exhibitionism) in five males of a large kindred (Brunner et al., 1993). Several studies have shown a reduced concentration of the 5-HT metabolite 5-HIAA in cerebrospinal fluid (CSF) of individuals suffering from aggressive behaviour (Asberg et al., 1976; Coccaro et al., 1997). In rhesus monkeys, there was a negative relation found between high aggression and CSF 5-HIAA levels (Higley et al., 1992). A number of studies implicate the 5-HT_2 receptor subtypes in aggressive behaviour. A study by Winstanley and colleagues showed that 5-HT depletion increased the number of premature responses in the 5-choice serial reaction time task in rats (Winstanley et al., 2004). Also, in this study it was found that application of a 5-HT_{2A} antagonist decreased impulsive behaviour, whereas application of a 5-HT_{2C} antagonist had the opposite effect (Winstanley et al., 2004). A clinical study showed that antipsychotic drugs which had anti-aggressive properties were 5-HT_{2A} antagonists (Krakowski et al., 2006). PET imaging studies show an increased 5-HT_{2A} receptor binding in aggressive patients or in borderline personality disorder patients (Siever et al., 2002; Soloff et al., 2007). These abovementioned studies indicate increased 5-HT_{2A} receptor sensitivity in aggressive behaviour patients.

Autism

Autism is a behavioural disorder with unknown etiology, although impaired brain development may underlie this disorder. The link between autism and 5-HT was made after the discovery that autistic patients had elevated platelet 5-HT levels (Boullin et al., 1970; Ritvo et al., 1970). Additionally, depletion of the 5-HT precursor tryptophan resulted in a deterioration in autistic patients (McDougle et al., 1993). Chugani and colleagues used alpha-[11C]methyl-L-tryptophan as a tracer for PET to measure 5-HT synthesis. They showed that in autistic boys there is a decreased 5-HT synthesis in frontal cortex, thalamus, and dentate nucleus of the cerebellum, although there is an increased 5-HT synthesis in the contralateral dentate nucleus (Chugani et al., 1997). Possibly increased levels of 5-HT during early brain development result in the loss of 5-HT terminals which could explain why several drugs which show great efficacy in treating autism enhance 5-HT neurotransmission (Brodkin et al., 1997; Fatemi et al., 1998). More evidence for this hypothesis is the fact that prenatal exposure to cocaine, which is a releaser of 5-HT, is a risk factor for developing autism (Davis et al., 1992). Furthermore, several studies using the 5-HT agonist 5-methoxytryptamine during development in rodents show that this results in autistic-like behaviour (Winslow and Insel, 1990; Kahne et al., 2002).

Polymorphisms in genes involved in the 5-HT system

SERT

In several genes involved in the 5-HT system, polymorphisms and short nucleotide polymorphisms (SNPs) have been found. An example of a gene in which polymorphisms have been found which correlates with psychopathological processes is SERT. Soon after the cloning of the human SERT, several polymorphisms have been found (Furlong et al., 1998; Battersby et al., 1999). These include two SNPs in the promoter region, a variable number of tandem repeats polymorphism in intron 2, and rare Ile-425-Val and Pro-339-Leu SNPs which affects function and surface expression (Kilic et al., 2003; Prasad et al., 2005). One polymorphism has been found in the promoter region. This polymorphism, a 44 bp insertion has been called the 5-HTT gene-linked polymorphic region, 5-HTTLPR. The two polymorphisms, called the short (s) and long (l) variant have been shown to modulate SERT transcriptional activity (Lesch et al., 1996). In a luciferase assay in human placental choriocarcinoma cells it was shown that the l variant has approximately a threefold higher transcriptional activity compared to the s variant (Heils et al., 1996). Additionally, cells expressing the l/l variant of the SERT displayed a twofold

higher uptake rate of 5-HT compared to cells expressing the s/s variant. In human postmortem brain material, reduced mRNA concentrations of SERT were found in the raphe nuclei from subjects having the s/s variant of the SERT promoter polymorphism. This shows that both in vitro and in vivo, the s/s variant is associated with a decreased SERT expression and reduced 5-HT reuptake (Little et al., 1998).

In 1996, the first study was published which demonstrated a link between the SERT promoter polymorphism and anxiety, aggression and depression traits (Lesch et al., 1996). Since then, several studies have demonstrated a link between the s/s promoter variant and aggression, anxiety and depression. Moreover, several studies show that individuals carrying the s/s variant have a poorer response to antidepressant treatment than individuals carrying the l/l or l/s variant (Smeraldi et al., 1998; Zanardi et al., 2000).

MAO-A

MAO is involved in the degradation of 5-HT to 5-HIAA, and there are 2 isoforms encoded by different genes, MAO-A and MAO-B. MAO-A has a higher affinity for 5-HT than MAO-B. These genes are both localized on the X chromosome (Lan et al., 1989). In humans a point mutation in the MAO-A gene has been found in the 8th exon, resulting in a truncated MAO-A protein. The family in which the point mutation was identified suffered from abnormal behaviour including disturbed regulation of impulsive aggressiveness (Brunner et al., 1993). In the promoter region of MAO-A a 30 bp repeat polymorphism was found. The low-activity 3-repeat allele was associated with antisocial behaviour in a German population (Samochowiec et al., 1999). In the rhesus macaque, a repeat polymorphism in the promoter affected transcription of the MAO-A gene and was associated with aggressive behaviour (Newman et al., 2005).

Tph2

Tph1 and Tph2 are the rate limiting enzymes for 5-HT synthesis. Tph1 is expressed in the enterochromaffin cells in the gut and in the pineal gland, whereas Tph2 is the neuronal form and exclusively expressed in the raphe nuclei (Patel et al., 2004; Zill et al., 2004a). In Tph2 a C1473G mutation, resulting in a Pro-447-Arg substitution results in a reduction in 5-HT synthesis. BALB/cJ mice, which are homozygous for the 1473G mutation, have a 50 to 70% reduction in 5-HT synthesis in the frontal cortex resulting in ~40% reduction in 5-HT content in the frontal cortex (Zhang et al., 2004). In the human Tph2 gene, a G1463A SNP resulting in a Arg441His replacement was identified which, when transfected in PC12 cells, resulted in a ~80% loss of function in 5-HT synthesis

(Zhang et al., 2005). In a cohort of 87 patients suffering from major depression, 9 were found to carry this SNP, while among 219 controls, only 3 subjects had this SNP (Zhang et al., 2005). Knockin mice carrying this human mutation displayed behavioural abnormalities (Beaulieu et al., 2008). In contrast to SNPs found in exons, also SNPs in introns of the Tph2 gene have been found. In intron 5, a SNP has been found which was associated with major depression and suicide (Zill et al., 2004b; Harvey et al., 2004).

As in the SERT promoter, also in the Tph2 promoter polymorphisms have been found. One of these polymorphisms, rs4570625, has been associated with increased amygdala reactivity to emotional stimuli (Canli et al., 2005). Two polymorphisms, rs4570625 and rs11178997, were linked to anxiety disorder and major depression (Zhou et al., 2005b). As shown by a luciferase assay in 5-HT neurons, the polymorphism rs11178997 significantly reduced Tph2 transcriptional activity by 22% (Scheuch et al., 2007).

Conclusions

5-HT is a transmitter that has several modes of action in the central nervous system. First of all, 5-HT has a role during brain development and maturation. Secondly, due to the volume transmission, 5-HT can influence several aspects of behaviour, depending on the receptor(s) to which 5-HT binds. 5-HT can also regulate synaptic transmission. KO mice for the 5-HT_{1A}, 5-HT_{1B} or SERT gene display altered anxiety-related, depression-related and aggression-related behaviour. Finally, polymorphisms in several genes functioning in the 5-HT system are implicated in depression, anxiety, aggression and autism.

Aim and outline of this thesis

In this thesis the outgrowth and connectivity of the brain 5-HT system is studied. To study these aspects we used a number of different approaches. *First* of all, we used organotypic slice cultures to study the outgrowth of the 5-HT system in vitro. *Secondly*, we used neuronal cultures to study trafficking of two components of the 5-HT system, SERT and Tph2. *Thirdly*, we investigated whether a SNP in a presynaptic gene which associates with major depressive disorder, affects trafficking of the SERT. *Fourthly*, we used a genetic approach in mice to study the effect of a silenced 5-HT system on mouse development. *Finally*, we describe our approach to generate a mouse model in which the 5-HT system is labeled with fluorescent reporter genes to study the outgrowth and connectivity in vivo.

In **chapter 2**, we used organotypic slice cocultures of the dorsal raphe nucleus and the hippocampus as a target area. We have used this model system to study the outgrowth of the 5-HT system in vitro. Moreover, we investigated whether this system could be used to assess the effect of pharmacological manipulations on the outgrowth of the 5-HT system.

In **chapter 3**, we tagged SERT with the red fluorescent protein mCherry, and expressed this protein in hippocampal neurons to investigate the trafficking dynamics of SERT.

In **chapter 4**, we tagged Tph2 with EGFP and took the same approach as in chapter 3 to investigate the subcellular distribution and trafficking of Tph2 in neurons.

In **chapter 5**, we studied whether knockdown of the presynaptic gene Piccolo affects SERT trafficking and incorporation into the membrane. A SNP in the C2A domain of this protein was found which associated with major depressive disorder. We expressed these two variants and investigated whether there is a difference in SERT localization. Also, we investigated whether knockdown of Piccolo affects SERT trafficking.

In **chapter 6**, we used a genetic approach to silence the 5-HT system. We crossed floxed Munc18-1 mice with mice expressing Cre recombinase in SERT expressing neurons, which include 5-HT neurons. We used these mice to study the effect of Munc18-1 removal in 5-HT neurons on the development of the 5-HT system and viability. We used heterozygous Munc18-1$^{wt/lox}$ mice to study whether deletion of one allele of Munc18-1 in the 5-HT system affects mouse behaviour.

In **chapter 7**, we describe an approach to specifically label 5-HT neurons and projections with fluorescent markers which could eventually be used to study 5-HT outgrowth and connectivity in vivo.

Finally, in **chapter 8**, the main findings of this thesis will be summarized, discussed and future directions are suggested.

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Chapter

2

Chronic activation of the 5-HT_2 receptor reduces 5-HT neurite density as studied in organotypic slice cultures

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Abstract

The serotonin system densely innervates the brain and is implicated in psychopathological processes. Here we studied the effect of serotonin and serotonin pharmacological compounds on the outgrowth of serotonergic projections using organotypic slice co-cultures of hippocampus and dorsal raphe nuclei. Immunohistochemical analysis showed that several serotonergic neurites had grown into the target slice within 7 days in culture, after which the neurite density stabilized. These projections expressed the serotonin-synthesizing enzyme tryptophan hydroxylase and the serotonin transporter and contained several serotonin positive varicosities that also accumulated presynaptic markers. Chronic application of a 5-HT_2 agonist reduced the serotonergic neurite density, without effects on survival of serotonergic neurons. In contrast, application of a 5-HT_{1A} agonist or the serotonin transporter inhibitor fluoxetine did not affect serotonergic neurite density. We conclude that serotonergic connectivity was reproduced in vitro and that the serotonin neurite density is inhibited by chronic activation of the 5-HT_2 receptor.

Introduction

The serotonin (5-hydroxytryptamine (5-HT)) system has its cell bodies clustered in the midbrain raphe nuclei and sends abundant projections to virtually every brain area (Hornung, 2003; Rubenstein, 1998). The 5-HT system influences a wide variety of physiological processes, such as sleep-wake rhythm, feeding, sexual behaviour and nociception (Jacobs and Azmitia, 1992). Moreover, the 5-HT system is implicated in several psychopathological processes, such as anxiety, aggression, obsessive compulsive disorder and depression (Castren, 2005).

During brain development, the 5-HT neurons are one of the first groups of cells expressing a specific neurotransmitter (Lauder, 1990; Gaspar et al., 2003). Accumulating evidence shows that alterations in brain 5-HT levels during development could result in alterations in brain development and behavioural alterations. 5-HT reuptake transporter (SERT) knockout (KO) mice have an increase in extra neuronal 5-HT but a reduction in overall brain 5-HT levels (Bengel et al., 1998; Mathews et al., 2004). They exhibit behavioural alterations, reduced number of 5-HT neurons in the dorsal raphe nuclei (DRN) and reduced firing of 5-HT neurons in the DRN (Lira et al., 2003). These behavioural alterations observed in SERT KO mice can be mimicked by application of the

SERT inhibitor fluoxetine during development between postnatal day 4 and 21 (Ansorge et al., 2004).

In contrast to reduction in brain 5-HT content after disruption of the SERT gene, there is a significant increase in brain 5-HT levels after inactivation of the Monoamine Oxidase-A (MAO-A) gene (Cases et al., 1996). The chronic elevation of 5-HT levels during brain development results in lack of barrels in the barrel cortex (Cases et al., 1996). These data suggest that either reductions in brain 5-HT levels or excess 5-HT levels during a critical period in the development of the brain can lead to abnormalities in brain development and behavioural alterations. In vitro experiments have shown that 5-HT can inhibit the outgrowth of neurites or even cause growth cone collapse on 5-HT neurons (Haydon et al., 1984; Koert et al., 2001). Whether alterations in brain 5-HT levels during development of the brain result in alterations in 5-HT outgrowth, branching or connectivity, or whether the behavioural alterations observed are a result of alterations in the 5-HT outgrowth and connectivity is currently unknown. Organotypic slice cultures of the DRN and a target slice could be a valuable reduced model system to study 5-HT outgrowth in vitro.

Therefore the aim of this study was to investigate whether organotypic slice co-cultures of DRN and hippocampus, a method that has previously been characterized, can be used to study 5-HT outgrowth and the effect of pharmacological manipulations in vitro (Papp et al., 1995; Guthrie et al., 2005). We show that within seven days of culturing several 5-HT neurites have grown into the hippocampal slice. Using these slice co-cultures, we studied whether chronic application of 5-HT pharmacological compounds affects the outgrowth of 5-HT neurites from the DRN. To this end we used a 5-HT_{1A} receptor agonist, a 5-HT_2 receptor agonist and the SERT inhibitor fluoxetine since it has been shown before, also on non-5-HT cells, that (chronic) activation of a 5-HT_{1A} or 5-HT_2 receptor or blockade of the SERT can affect (5-HT) outgrowth or synaptogenesis (Wilson et al., 1998; Kondoh et al., 2004; Fricker et al., 2005; Zhou et al., 2006). We show here that chronic application of a 5-HT_2 agonist results in a reduction in 5-HT neurite density. We conclude that these organotypic slice co-cultures can be used to study 5-HT outgrowth and the effect of 5-HT pharmacological compounds on this outgrowth in vitro.

Results

Development and outgrowth of 5-HT neurons in organotypic slice co-cultures

To study the outgrowth of 5-HT neurites ex vivo, we used organotypic slice co-cultures of the DRN and a hippocampal slice. We positioned the slices close to each other to allow 5-HT neurites to grow into the hippocampal slice. Within

seven days of culturing the slices flattened out and several non-neuronal cells migrated away from the slices (Fig. 1).

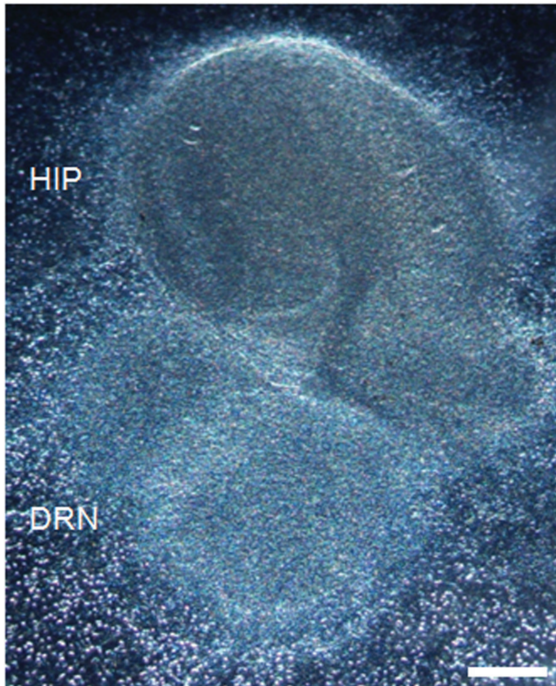


Figure 1. Overview of the culturing method
The DRN and hippocampal slice were positioned close to each other to allow 5-HT neurites to grow into the hippocampal slice. After culturing for 7 days, the slices flatten and several cells (e.g. macrophages) migrate away from the slices. Scale bar: 500 µm.

We performed immunohistochemistry for 5-HT to investigate whether 5-HT neurons survived in the slices. This showed that in several DRN slices 5-HT neurons were present, which resembled the topology of 5-HT neurons in the DRN in vivo (Fig. 2A). However, there were also DRN slices which contained only few 5-HT neurons, possibly resulting from a variation in the dissection of the DRN slice. A Z-stack of 5-HT cell somata showed that these cells have several primary neurites which contain numerous varicosities (Fig. 2B). In the growth cones of 5-HT neurites, 5-HT was also detected (Fig. 2C). Next, we focussed on the expression of tryptophan hydroxylase (Tph) and SERT, two markers for the 5-HT system. In 5-HT immunopositive cell bodies and in the 5-HT neurites and varicosities Tph was expressed (Fig. 2D,E). We found that all the 5-HT neurites were positive for SERT labelling, with the highest level of SERT staining on the soma and in the 5-HT varicosities (Fig. 2F,G). Moreover, SERT was also expressed in a punctate pattern in the 5-HT growth cones. (Fig. 2H). In vivo, 5-HT is released from varicosities in 5-HT axons and dendrites and 5-HT release is predominantly paracrine (Bunin and Wightman, 1999). In the slices, the 5-HT neurites contain several round and fusiform shaped varicosities (Fig. 3A,B).

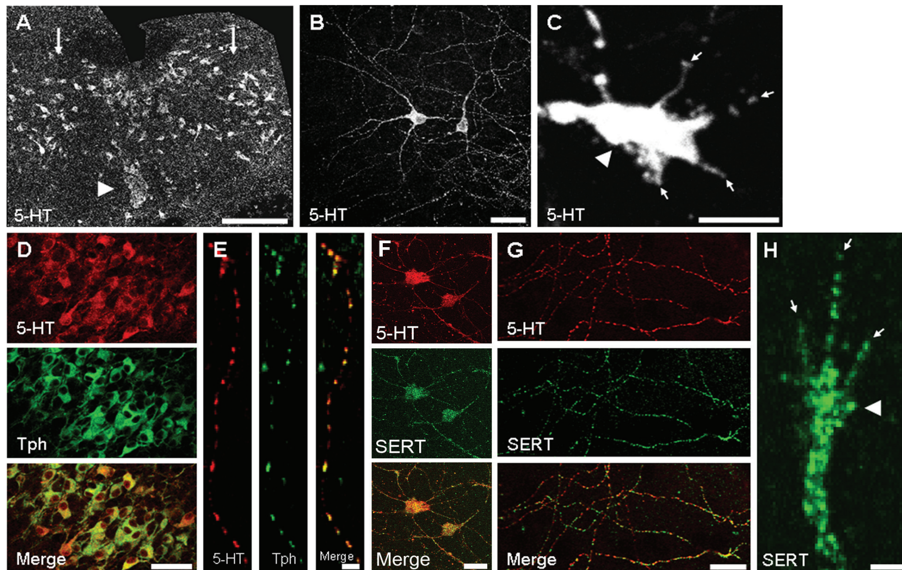


Figure 2. 5-HT neurons survive in the slice and grow out several 5-HT neurites
(A) A DRN slice cultured for 7 days which is stained for 5-HT shows that several 5-HT cells are present in the slice. Note the presence of the lateral wings of the DRN (arrows) and the group of 5-HT neurons in the ventral part of the DRN (arrowhead). (B) A Z stack of two cells shows that the cells project several neurites that are 5-HT positive. (C) The growth cones also contain 5-HT, both in the core (arrowhead) and in the filopodia (arrows). (D,E) Staining for the rate limiting enzyme Tph shows that Tph is present both in the cell bodies and in the varicosities in the neurites. (F,G) The other marker for the 5-HT system, SERT, is present in 5-HT cell bodies and 5-HT neurites. (H) Moreover, there is also a punctate SERT staining in 5-HT growth cones, both in the core (arrowhead) and in the filopodia (arrows). Scale bars: 200 µm in A, 50 µm in B and D, 20 µm in F and G, 5 µm in C and E, 2 µm in H.

Immunohistochemistry revealed that in these varicosities the calcium sensor Synaptotagmin and the active zone marker Bassoon are present, suggesting that the varicosities are presynaptic sites where calcium dependent 5-HT release occurs (Fig. 3C,D) (Perin et al., 1991; tom Dieck et al., 1998). This showed that in organotypic slice co-cultures of DRN and hippocampus 5-HT neurons survive and grow out neurites that contain 5-HT varicosities with immunoreactivity for all tested components of the 5-HT release machinery.

Dense ingrowth of 5-HT neurites into hippocampal slices
To study the outgrowth, we fixed slice cultures at different time points and quantified the 5-HT outgrowth. To this end the images were converted to binary images and the 5-HT neurite density in the slices was quantified (Fig. 4A-C).

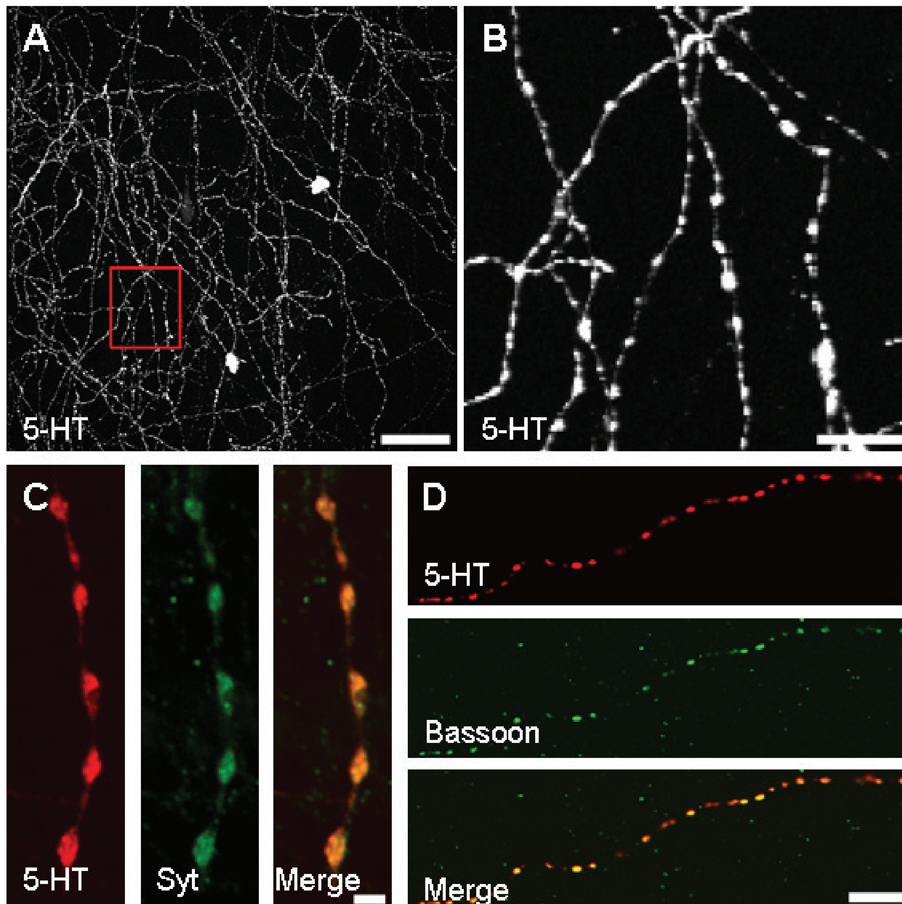


Figure 3. 5-HT neurites contain several varicosities which are positive for presynaptic markers

(A) A Z-stack in the hippocampal slice shows that several thin, highly branching 5-HT neurites have grown into the slice. (B) A blow-up of the red box in A shows that these neurites contain several varicosities. (C,D) These varicosities are positive for the protein involved in calcium dependent secretion, Synaptotagmin (Syt), and for the active zone marker Bassoon. Scale bars: 50 µm in A, 10 µm in B and D and 2 µm in C.

We quantified the 5-HT neurite density as the area in the slice occupied by 5-HT neurites compared to the total area of the slice. Immunohistochemistry for 5-HT on hippocampal slices cultured without the DRN for seven days revealed that there were no 5-HT neurites present (Fig. 4E). Thus, 5-HT neurites observed in the hippocampal slice in co-cultures are the result of re-growth of 5-HT neurites from the DRN slice. In co-cultures fixed at four days in vitro (DIV) we observed that the DRN slice already contained several 5-HT neurites, and the first 5-HT neurites started to grow into the hippocampal slice (Fig. 4F,I). At DIV7, also the

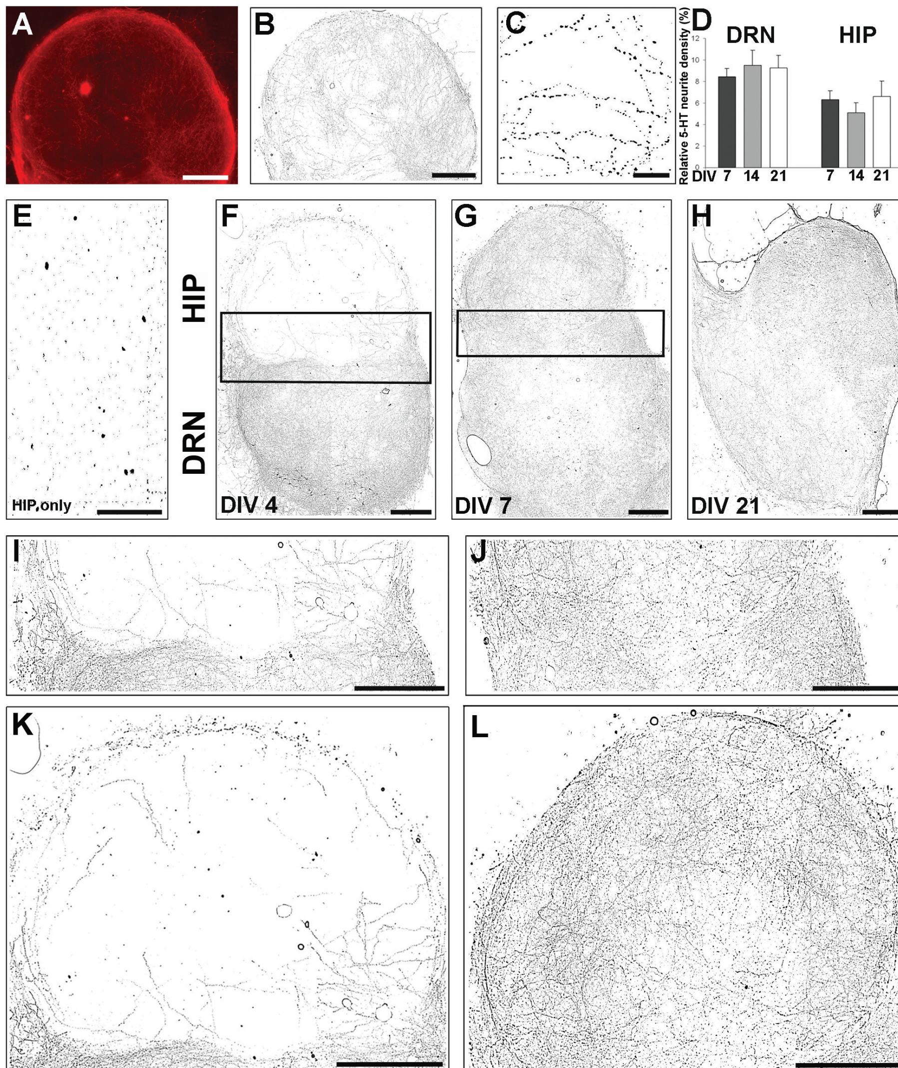
hippocampal slice contained several 5-HT neurites (Fig. 4G,J). Quantification of the 5-HT neurite density in the slices at DIV14 and DIV21 revealed that this was not significantly different from DIV7 (Fig. 4D,H; DIV7 DRN 8.42 ± 0.8%, HIP 6.31 ± 0.84% n=16; DIV14 DRN 9.49 ± 1.42%, HIP 5.12 ± 0.9% n=12; DIV21 DRN 9.26 ± 1.16%, HIP 6.61 ± 1.43% n=4). We investigated whether there is regional variation in the 5-HT neurite distribution in the hippocampus. At DIV4 the first 5-HT neurites which start growing into the hippocampal slice do not appear to display a preference for a certain region. (Fig. 4K). At DIV7, 5-HT neurites have grown in a uniform distribution into the hippocampus, and there are no regions which contain a higher density of 5-HT neurites than other regions (Fig. 4L). Thus, the initial outgrowth of the 5-HT neurites occurs in the first 7 days, after which the network stabilizes and the homogenous distribution suggests that 5-HT neurites do not have any preferential target region within the hippocampal slice in vitro.

The effect of 5-HT pharmacological compounds on the outgrowth of 5-HT projections

Finally, we studied whether this culture model system can be used to study the effect of chronic application of 5-HT pharmacological compounds on the outgrowth of 5-HT neurites. There are several 5-HT receptors which are expressed in both the DRN and the hippocampus.

Figure 4 next page. Dense ingrowth of 5-HT neurites into hippocampal slices within seven days of culturing

An original image of a hippocampal slice co-cultured with a DRN slice shows that there is abundant ingrowth of 5-HT neurites, but there is also a lot of red background staining. (B) When this image is converted to a binary image using the steps described in experimental procedures, the 5-HT neurites are positively labelled (black), whereas the background is removed (white). (C) A blow up of part of the binary image shows that all 5-HT neurites are detected, and the red background is removed. (D) Quantification of the 5-HT neurite density (Y-axis) in the DRN and hippocampal slices at DIV7, DIV14 and DIV21. (E) A hippocampal slice cultured without the DRN contains only background staining, and no 5-HT neurites. (F,I) Maximal 5-HT ingrowth of the slices is reached in the first seven days of culturing. At DIV4, the DRN slice already contains several 5-HT neurites, and the first 5-HT neurites start to grow into the hippocampal slice. (G,J) At DIV7, both the DRN and hippocampal slice contain abundant 5-HT neurites. (H) At DIV21 the 5-HT neurite density in the slices has not changed. (K,L) No regional variation in 5-HT neurite density in the hippocampal slice at DIV4 (K) and DIV7 (L). Figures I and J are a blow up of the black box in F and G respectively. Figures K and L are blow ups of the hippocampal slices in figure F and G respectively. Scale bars: 500 µm in A, B, E, F, G, H, I, J, K and L, 20 µm in C.



These include the 5-HT_{1A} receptor, the 5-HT_2 receptor and the 5-HT_7 receptor (Gustafson et al., 1996; Clemett et al., 2000; Xu and Pandey, 2000; Bickmeyer et al., 2002; Garcia-Alcocer et al., 2006). Since it has been shown before, also on non-5-HT cells, that (chronic) activation of a 5-HT_{1A} or 5-HT_2 receptor or blockade of the SERT can affect (5-HT-ergic) outgrowth or synaptogenesis, we decided to use a 5-HT_{1A} receptor agonist 8-hydroxy-2-(di-*n*-propylamino)tetraline (8-OH-DPAT), a 5-HT_2 receptor agonist (+-)-2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI hydrochloride) and the SERT inhibitor fluoxetine (Wilson et al., 1998; Kondoh et al., 2004; Fricker et al.,

2005; Zhou et al., 2006). We chronically applied the pharmacological compounds in 10 μM concentrations during the first seven days of outgrowth.
First of all, we studied the effect of chronic blockade of the SERT by fluoxetine. Fluoxetine did not affect 5-HT neurite density (Fluoxetine: DRN: 8.44 ± 0.72%, HIP: 6.12 ± 1.12% n=10) compared to control condition (Fig. 5A,B,E,F). Subsequently, we tested the effect of chronically activating the 5-HT_{1A} and 5-HT_2 receptor, two receptors which are present on 5-HT neurons. Moreover, it has been shown previously that activation of these receptors can affect outgrowth of Purkinje cells (Kondoh et al., 2004). First we investigated the effect of the 5-HT_{1A} receptor agonist 8-OH-DPAT. Activation of the autoreceptor by 5-HT or an agonist results in a reduction in 5-HT release (Quick, 2003). However, 8-OH-DPAT did not affect 5-HT neurite density (DRN 10.67 ± 0.79%, HIP 7.21 ± 0.9% n=9) (Fig. 5 C,E,F).

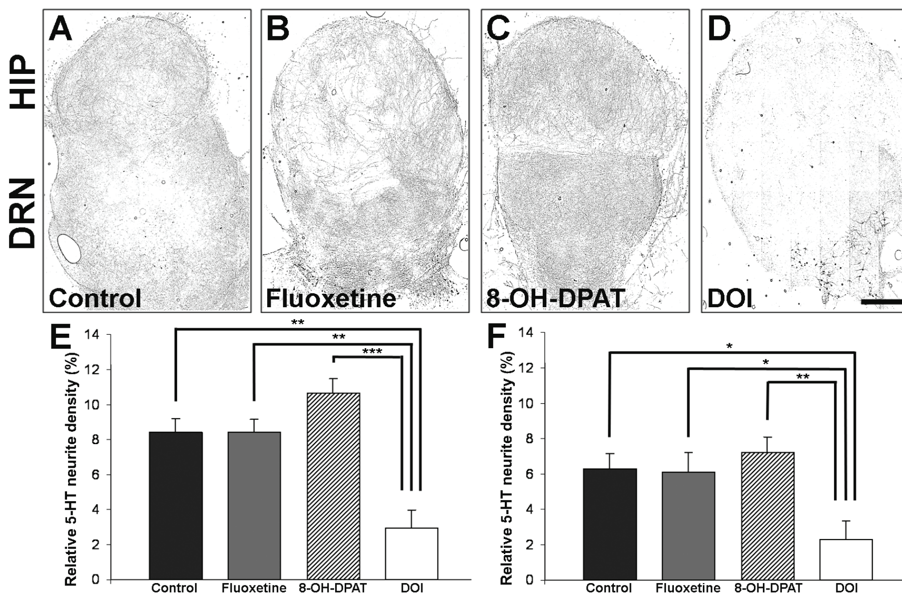


Figure 5. Chronic application of a 5-HT_2 agonist reduces 5-HT neurite density in the slices

(A) In control condition, after seven days of culturing several 5-HT neurites have grown into the slices. (B) Chronic blockade of the SERT with the SERT inhibitor fluoxetine did not affect 5-HT neurite density in the slices. (C) Chronic activation of the 5-HT_{1A} receptor with 8-OH-DPAT also did not affect 5-HT neurite density in the slices. (D) However, chronic activation of the 5-HT_2 receptor with DOI significantly reduced 5-HT neurite density both in the DRN and hippocampal slice. (E,F) Quantification of 5-HT neurite density in the DRN slice and hippocampal slice, respectively. Scale bar: 500 μm. * $p < 0.05$, ** $p<0.01$, *** $p<0.001$.

Next, we investigated the effect of chronic 5-HT_2 receptor activation. Activation of the 5-HT_2 receptor results in an increased protein kinase C activity and release of calcium from internal stores. We chronically activated the 5-HT_2 receptor by applying the 5-HT_2 receptor agonist DOI hydrochloride. Chronic application of the 5-HT_2 receptor agonist DOI hydrochloride resulted in a significantly decreased 5-HT neurite density (Fig. 5 D,E,F) (DRN 2.94 ± 1.01%, HIP 2.29 ± 1.05% n=5). To exclude the possibility that pharmacological treatment affected the survival of 5-HT neurons, we counted the number of 5-HT cells per group. This showed that treatment with fluoxetine, 8-OH-DPAT or DOI hydrochloride did not affect the number of 5-HT neurons in the slices (data not shown). Hence, changes in viability of 5-HT neurons cannot explain the effect of DOI hydrochloride. We conclude that these organotypic slice co-cultures can be used to study the 5-HT outgrowth and ingrowth into a target area in vitro and that chronic activation of the 5-HT_2 receptor results in a decreased 5-HT neurite density.

Discussion

An organotypic co-culture model system to study 5-HT outgrowth ex vivo

We used organotypic slice co-cultures to study the outgrowth of the 5-HT system ex vivo. Neurite projections of 5-HT neurons grew into the slices and at 7 days a dense network had formed with many serotonergic boutons that had accumulated presynaptic markers. The regional differences in 5-HT innervation observed in the hippocampus in vivo were not reproduced ex vivo and it has been observed that fewer synapses form in slices than in vivo (see discussion in (Papp et al., 1995). However, also in vivo the majority of the 5-HT release sites (70-80%, (Oleskevich and Descarries, 1990), are not classical synapses, i.e. with a juxtaposed postsynaptic specialization. Hence, the in vivo connectivity of the 5-HT system appears to depend largely on non-synaptic release. Therefore, despite some discrepancies compared to the in vivo situation, the slice co-culture system is an excellent, reduced model system to unravel the basic principles that orchestrate 5-HT neurite outgrowth and connectivity. Such a reduced system is a valuable supplementation of in vivo studies, which are often complicated by the exceptionally complex and dynamic connectivity of the 5-HT system, with for instance large fluctuations in innervation over the course of a few days (transient hyper-innervation, see (Fujimiya et al., 1986; D'Amato et al., 1987).

Chronic activation of the 5-HT_2 receptor decreased 5-HT neurite density

We found that chronic application of a 5-HT_2 agonist decreased the neurite density of 5-HT neurites. Since DOI hydrochloride treatment did not affect 5-HT neuron survival, 5-HT mediated cytotoxicity cannot explain the decrease in 5-HT

neurite density. Hence, the decreased neurite density appears to be the result of a specific effect of the activation of the 5-HT_2 receptor on the neurites (decreased outgrowth/increased pruning). In contrast, chronic activation of the 5-HT_{1A} receptor did not mimic the effects of 5-HT, although the 5-HT_{1A} receptor has often been implicated in shaping 5-HT connectivity (Gross et al., 2000; Gross et al., 2002). Activation of somatotodendritic 5-HT_{1A} receptors results in hyperpolarisation and a decrease in 5-HT release (Quick, 2003). However, chronic activation results in rapid desensitization (Assie et al., 2006) which may explain why we do not observe an effect of chronic 5-HT_{1A} receptor activation. On the other hand, 5-HT_2 receptor activation increases intracellular calcium and activates protein kinase C (Tamir et al., 1992). This suggests that (chronic) activation of the 5-HT_2 receptor affects outgrowth, since elevation of calcium levels in growth cones reduces outgrowth (Gomez and Spitzer, 1999). Since DOI hydrochloride has a comparable affinity for the 5-HT_{2A} and 5-HT_{2C} receptor subtype, based on these data we cannot conclude which of the two subtypes is involved in the effect of DOI hydrochloride.

However, although the 5-HT_{2A} receptor is widely expressed throughout the brain, the receptor seems not to be expressed in the DRN (Xu and Pandey, 2000). In contrast, the 5-HT_{2C} receptor is present in the DRN (Clemett et al., 2000; Serrats et al., 2005). Moreover, it has been shown previously, that activation of the 5-HT_{2C} receptor in the DRN results in a reduction in 5-HT neuron firing (Boothman et al., 2006). Therefore, based on the anatomical distribution we speculate that the effect of DOI hydrochloride is predominantly mediated via the 5-HT_{2C} receptor. Fluoxetine did not affect 5-HT neurite density. It could be expected that fluoxetine application mimics a 5-HT_{1A} receptor agonist, i.e. SERT blockade results in an increase in extracellular 5-HT which could activate 5-HT_{1A} autoreceptors. However, possibly upon application of fluoxetine extracellular 5-HT is rapidly diluted in the culture medium in vitro, and therefore extracellular 5-HT will not reach high enough concentration levels to mimic the effect of a 5-HT_{1A} receptor agonist. Also chronic fluoxetine treatment in vivo failed to produce effects similar to chronic 5-HT application (Zhou et al., 2006). In fact, chronic fluoxetine enhanced neurite density in this case (Zhou et al., 2006).

Conclusions

We have shown here that in DRN – hippocampus organotypic slice co-cultures 5-HT projections grow into the target slice. After four days of culturing in the DRN slice already several 5-HT projections were present, and the first 5-HT projections started to grow into the target slice. Within seven days of culturing also the target slice contained several 5-HT neurites. These 5-HT neurites,

contained several varicosities, which are presumably sites of 5-HT release, since presynaptic markers were present in these varicosities. Finally, we have shown that this culture model system can be used to study the effect of pharmacological manipulations on the outgrowth of 5-HT neurites, and that application of a 5-HT_2 agonist results in a reduction in 5-HT neurite density in the slices.

EXPERIMENTAL PROCEDURES

Laboratory animals

Wildtype C57BL/6 mouse fetuses were obtained from caesarean section at embryonic day 18 or postnatal day 1. Animals were housed and bred according to institutional, Dutch and U.S. governmental guidelines.

Pharmacological compounds and antibodies

Mouse monoclonal anti-Tph antibody, which detects both Tph1 and Tph2, was obtained from Sigma-Aldrich and used in a 1:1000 dilution. Rabbit anti 5-HT polyclonal and mouse anti-SERT monoclonal were obtained from Immunostar/Diasorin and used in a 1:1000 dilution. Bassoon and Synaptotagmin monoclonal antibodies were used in a 1:500 and 1:1000 dilutions respectively and obtained from Stressgen. As secondary antibodies Alexa593-conjugated goat anti rabbit and Alexa488-conjugated goat anti mouse were used in a 1:1000 dilution (Molecular probes). 8-OH-DPAT and DOI hydrochloride were obtained from Sigma-Aldrich. Fluoxetine was obtained from Tocris.

Organotypic slice co-cultures

Organotypic slice cultures were made as follows. After decapitation of the fetuses, the heads were immediately transferred to ice-cold dissection Gey's balanced salt solution (dGBSS, Invitrogen, supplemented with 0.65 g/l glucose and 200 μM kynurenic acid). For isolation of a DRN slice, the brains were dissected in dGBSS and the midbrain cut into 400 μm thick slices using a McIlwain tissue chopper (Mickle Engineering, Gomshall, UK). Individual slices were separated in dGBSS and the hindbrain slice containing the rostral DRN was identified by visual inspection. The DRN was dissected out using a dissection knife. The entire hippocampus was dissected out of the brain and individual slices (400 μm) were sagitally cut perpendicular to the hippocampus' longitudinal axis. The hippocampal and DRN slices were allowed to recuperate in dGBSS at 4˚C for 60 minutes. A hippocampal and DRN slice were cultured in close proximity on a poly-D-lysine coated 12x24 glass coverslip (O. Kindler GmbH & Co. Mikroskopische Gläser, Freiburg, Germany) in a plasma clot (chicken plasma, Cocalico Biologicals Inc. Reamstown, U.S.) which was coagulated with thrombin (Merck, Darmstadt, Germany). Culturing medium

consisted of 50% BME Hanks, 25% Hanks balanced salt solution and 25% horse serum supplemented with 1.3 g/l glucose and 200 μM glutamine. For culturing 700 μl of culturing medium was added to the slices in a Nunc flat-bottomed tube. The slices were incubated in a roller drum at 36˚C. After the first week, medium was changed twice a week.

Pharmacological treatments
All pharmacological compounds were dissolved in ddH_2O, filter sterilized and aliquots stored at -80˚C. After DIV1 10 μM of the pharmacological compound was added to the slices. Every day a new aliquot of pharmacological compound was added to the slices. In control slices only vehicle was added (sterile ddH_2O). At DIV7 the slices were fixed, processed and analyzed as described below.

Immunohistochemistry
Slices were fixed for 20 minutes in 4% paraformaldehyde dissolved in phosphate buffered saline. After fixation the slices were washed three times five minutes in phosphate buffered saline (PBS) and nonspecific binding was blocked by incubating the slices in PBS containing 0.1% Triton X-100 and 2% normal goat serum for two hours. The slices were incubated overnight with the primary antibodies diluted in PBS containing 0.1% Triton X-100 supplemented with 2% normal goat serum at 4˚C. After incubation in the primary antibodies, the slices were washed three times two hours in PBS on a shaking platform. The secondary antibodies were diluted in PBS supplemented with 2% normal goat serum and incubated for one hour. After incubation in the secondary antibodies, the slices were washed again three times two hours in PBS and mounted in Dabco-Mowiol (Sigma) on glass coverslips. All reactions were carried out at room temperature unless otherwise stated. No labeling was observed when omitting the primary antibodies.

Confocal analysis
Confocal analysis of the slices was performed on a LSM 510 microscope (Carl Zeiss b.v. Weesp, the Netherlands) and a 63x Plan-Neofluar lens (Numerical aperture 1.4, Carl Zeiss). To excite the Alexa 488 antibody a HeNe1 laser was used and for excitation of the Alexa 593 antibody a HeNe2 laser was used. For analysis of the 5-HT neuron morphology and the 5-HT neurites, Z-stacks of 1 μm were made. Images were analyzed and further processed in Zeiss CLSM software.

Histological quantification of 5-HT neurite density
Quantification of 5-HT immunopositive neurites and cells was performed using an MCID Elite imaging system (Imaging Research Inc., Ont., Canada). Images of the immunostained co-cultures were digitized using an objective magnification

of 20x on a Leica DM/RBE photo-microscope with a Sony (DXC-950P, 640 x 512 pixels) camera using epifluorescence microscopy. The 5-HT-immunopositive neurites were segregated from background using several point operators and spatial filters combined in an algorithm designed to detect local changes in relative optical density. Briefly, images underwent histogram equalization and smoothing (low-pass filter, kernel size 7x7). The unfiltered image was subtracted from the smoothed image, followed by a series of steps to optimize the processed image and make it a suitable measuring template for detecting objects the size and shape of 5-HT neurites or cell bodies. This algorithm was preferred over relative optical density thresholding since it does not involve an observer-dependent operation. Finally, a line was manually drawn around the DRN or hippocampal slice and the number of positive pixels (i.e. pixels representing 5-HT neurites) compared to the total number of pixels was calculated. The number of 5-HT neurons was counted manually in the slices.

Data analysis

In order to analyze differences in neurite density between different pharmacological treatments, ANOVA was used with the Bonferonni test for post hoc analysis. Data shown are mean values ± standard error of the mean (SEM). Significance levels were set at <0.05.

Acknowledgements

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Chapter

3

Dynamic vesicular trafficking of the serotonin reuptake transporter in hippocampal neurons

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In preparation

Abstract

The serotonin (5-HT) reuptake transporter (SERT) has an important role in 5-HT transmission by terminating the action of 5-HT in the synaptic cleft. Here we address the question how SERT is transported in neuronal cells. We studied the trafficking of SERT in hippocampal neurons by tagging SERT with a fluorescent protein. We show that SERT displays a punctate distribution in axons and a more uniform distribution in dendrites. The majority of SERT puncta do not co-localize with the synaptic marker Synapsin, showing that SERT is predominantly localized extra-synaptically. SERT is transported in secretory vesicles as SERT co-localizes with the secretory vesicle marker tissue plasminogen activator (tPA). Live cell imaging revealed a highly dynamic trafficking of SERT along the neurites. Approximately 35% of the vesicles moved, with an average velocity of ~ 1.2 μm/s. SERT vesicles do not display a preference for direction. We conclude that SERT, tagged with a fluorescent protein, displays a highly dynamic character in hippocampal neurons. These data are important to achieve a better understanding of the dynamics of treatment with 5-HT reuptake inhibitors.

Introduction

The projections of the 5-HT system abundantly innervate several brain areas. 5-HT is released both synaptically and extra-synaptically. After being released, 5-HT is cleared from the extracellular space by the SERT. Since this protein regulates the termination of the action of 5-HT, it is very important in regulating efficacy of 5-HT transmission. Moreover, SERT is the primary target for the selective serotonin reuptake inhibitors (SSRIs) class of antidepressants, several tricyclic antidepressants, and may mediate the effects of amphetamines such as 3,4-methylenedioxy-N-methylamphetamine (MDMA).

SERT is a sodium and chloride neurotransmitter symporter and belongs to the solute carrier family 6, to which also transporters for dopamine, noradrenaline/adrenaline, glycine and GABA belong (Chen et al., 2004). In the adult brain, SERT is expressed in 5-HT neurons, but during development also transiently in the cortex, hippocampus, thalamocortex and the lateral geniculate nucleus (Lebrand et al., 1996; Zhuang et al., 2005). In 5-HT neurons, SERT is predominantly localized in axons but also in the cytoplasm of the soma and dendrites (Zhou et al., 1998; Tao-Cheng and Zhou, 1999). In axons SERT is not only associated with varicosities, sites where 5-HT is released, but also with the plasma membrane of the axons (Zhou et al., 1998).

Several lines of evidence indicate that 5-HT has a role during brain development. In mice, deletion of SERT results in a 60-80% reduction in total

brain tissue 5-HT level, although there is a 7-8 fold increase in extracellular 5-HT levels in the striatum and substantia nigra (Fabre et al., 2000; Mathews et al., 2004). Analysis of emotional behaviour of these mice revealed that deletion of SERT results in abnormalities in anxiety-related behaviour (Holmes et al., 2003a; Holmes et al., 2003b). Additionally, blockade of SERT during development with the SSRIs fluoxetine, clomipramine or citalopram results in mice which display altered emotional behaviour in adulthood (Ansorge et al., 2004; Ansorge et al., 2008). These studies show that SERT is important in establishing normal emotional behaviour during development, presumably by regulating the extracellular concentration of 5-HT in the brain.

The level of SERT present at the cell membrane at synaptic boutons determines the rate of 5-HT reuptake and hence the efficacy of 5-HT transmission. Therefore, there is extensive regulation of the level of cell-surface expressed SERT. The trafficking and regulation of SERT cell-surface expression has been studied in many different cell types, including HEK293 cells, 1C11 cells, basophilic leukemia 2H3 cells and 5-HT neurons (Zhu et al., 2004; Lau et al., 2008a; Lau et al., 2008b). A study using biotinylated SERT in 5-HT neurons showed that citalopram reduced the amount of cell surface-expressed SERT (Lau et al., 2008b) However, live cell imaging to investigate the trafficking dynamics of SERT has not been studied in neurons before.

Here we investigated the trafficking of SERT in hippocampal neurons. To this end we expressed SERT tagged with the fluorescent protein mCherry in hippocampal neurons. SERT displays a polarized distribution, as in axons SERT is distributed in a punctate manner, whereas in dendrites SERT is present in a uniform distribution. SERT is transported in secretory vesicles through the neurites, as it co-localized with the secretory vesicle marker tPA. Live cell imaging revealed that SERT displays a highly dynamic vesicular trafficking. Analysis of direction of movement showed that SERT does not display a preference for a direction of movement. In future experiments, live cell imaging of mCherry-SERT can be used to address whether 5-HT reuptake inhibitors affect the highly dynamic SERT trafficking.

Results

Localization of mCherry-SERT in hippocampal neurons differs between axons and dendrites

To study the trafficking we tagged the N-terminus of SERT with the red fluorescent protein mCherry, which is an improved variant of monomeric red fluorescent protein in terms of maturation and photostability (Shaner et al., 2004). Tagging the N-terminus of SERT with a fluorescent protein does not interfere with the function of SERT (Schmid et al., 2001; Just et al., 2004;

Fjorback et al., 2009). We used hippocampal neurons that do not express SERT, although it is expressed transiently in a subpopulation of hippocampal neurons during development (Narboux-Neme et al., 2008).

The expression of mCherry-SERT in dissociated hippocampal neurons was analyzed between 9-12 days in vitro (DIV) using live cell imaging and immunocytochemistry on fixed neurons. This revealed that the whole neuron was extensively labelled with mCherry-SERT (Fig. 1A). Immunocytochemistry for the dendritic marker Microtubule Associated Protein 2 (MAP2) was used to discern between the dendrite and axon (Fig. 1B,C). SERT localization differed between axons and dendrites. In dendrites SERT is predominantly uniformly distributed where only occasionally a puncta of SERT is observed. However in axons, SERT displays a more punctate distribution suggesting that SERT is transported in vesicles through the axon (Fig. 1D,E,F). Alternatively, the punctate pattern may reflect local accumulation of SERT in varicosities. To further explore whether these puncta are varicosities or vesicles, we used immunocytochemistry and live cell imaging.

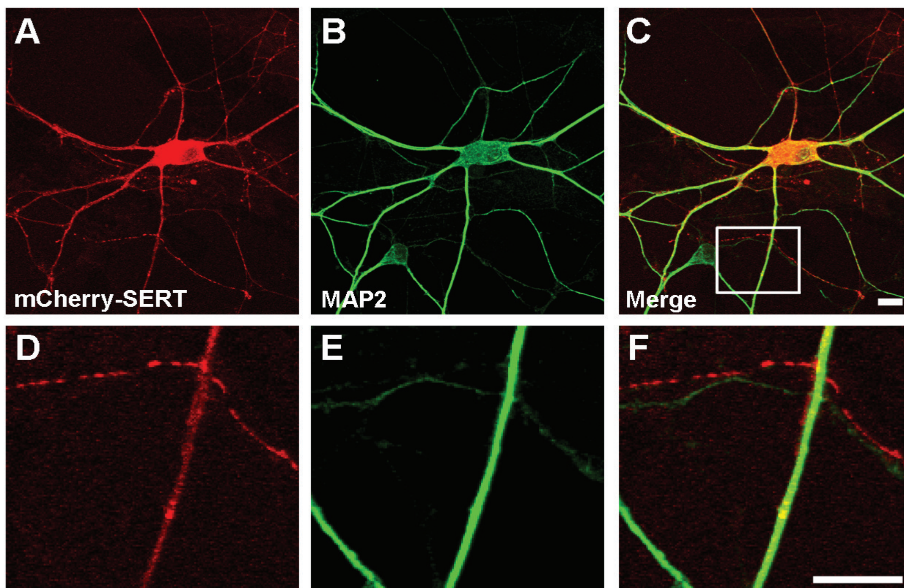


Figure 1. Distribution of mCherry-SERT differs between axons and dendrites
(A-C) Expression of mCherry-SERT in a hippocampal neuron (DIV11). (A) mCherry-SERT is present in all the neurites and the cell body. (B) MAP2 labelling to discern between axons and denrites. (C) Merged image. (D-F) Blow up of the white box in C. (D) mCherry-SERT distribution differs between axons and dendrites, as in the axon mCherry-SERT displays a punctate distribution, whereas in dendrites mCherry-SERT displays a more uniform distribution where only occasionally a mCherry-SERT puncta is observed. (E) MAP2 labelling. (F) Merged image. Scale bars in C and F are 10 μm.

SERT is transported in secretory vesicles

Since SERT displayed a punctate distribution in neurites, we investigated whether these puncta are secretory vesicles. To this end mCherry-SERT was co-expressed with tPA-GFP, which is transported in secretory vesicles in hippocampal neurons (Lochner et al., 1998; Silverman et al., 2005). In neurons expressing both mCherry-SERT and tPA-GFP, the majority of SERT puncta co-localized with tPA-GFP (Fig. 2A-F). Therefore, these data strongly suggest that SERT is transported in secretory vesicles along the axons in hippocampal neurons.

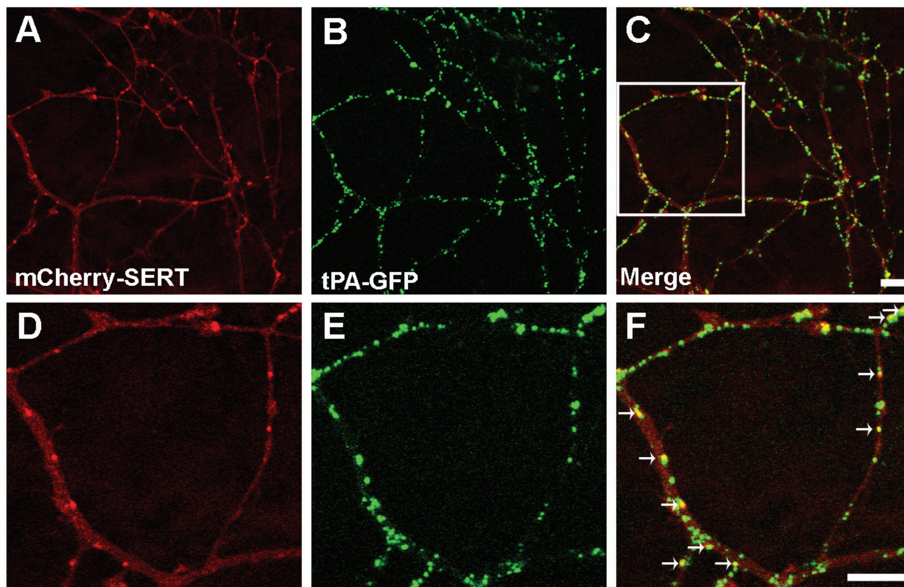


Figure 2. mCherry-SERT is transported in secretory vesicles
(A-C) Co-expression of mCherry-SERT and tPA-GFP in a hippocampal neuron (DIV11). (A) mCherry-SERT displays a punctate distribution. (B) tPA-GFP is transported in secretory vesicles. (C) The majority of mCherry-SERT puncta co-localize with tPA-GFP, suggesting that mCherry-SERT is transported in secretory vesicles. (D-F) Blow-up of the white box in C. (D) mCherry-SERT expression. (E) tPA-GFP expression. (F) Merged image. Arrows show the co-localization between mCherry-SERT and tPA-GFP. Scale bars in C and F are 10 μm.

To investigate whether vesicles of SERT co-localized with presynaptic terminals, we performed immunocytochemistry for the synaptic marker Synapsin on neurons transfected with mCherry-SERT. Although several mCherry-SERT puncta are in close vicinity of Synapsin puncta (Fig. 3 A-F), only few mCherry-SERT puncta co-localized with Synapsin. Thus, mCherry-SERT is transported in secretory vesicles and several of those vesicles localize to the vicinity of presynaptic terminals.

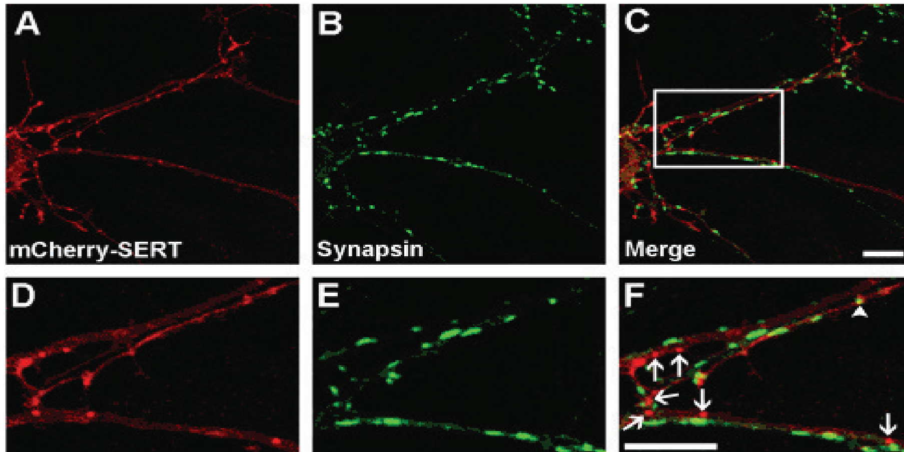
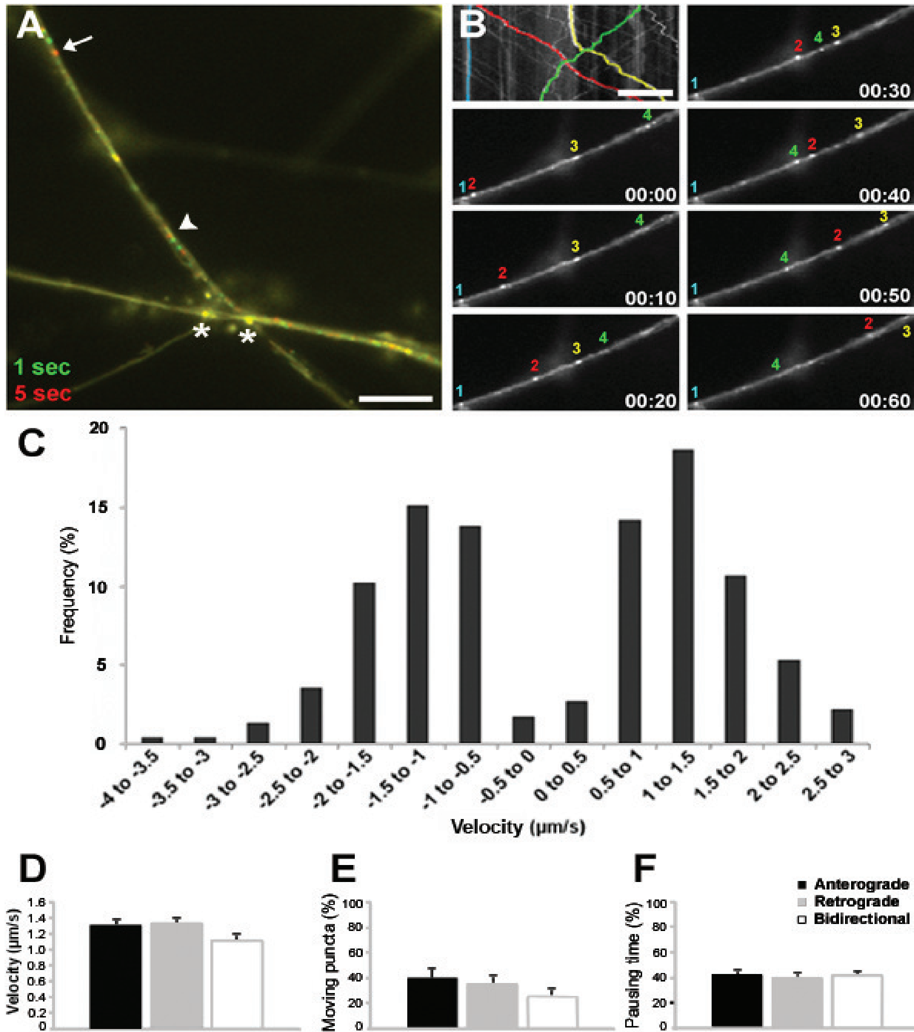


Figure 3. Several mCherry-SERT vesicles are localized in the vicinity of presynaptic terminals

(A-C) Expression of mCherry-SERT in a hippocampal neuron (DIV11). (A) mCherry-SERT expression. (B) Synapsin labelling. (C) Merged image. Several mCherry-SERT vesicles are localized in the vicinity of presynaptic terminals, and some co-localized with the presynaptic marker Synapsin. (D-F) Blow up of the white box in C. (D) mCherry-SERT expression. (E) Synapsin labelling. (F) Merged image. Arrows in F show mCherry-SERT vesicles which are localized in the vicinity of presynaptic terminals. Arrowhead shows a mCherry-SERT vesicle which co-localizes with a Synapsin puncta. Scale bars in C and F are 10 µm.

Figure 4 next page. Trafficking dynamics of mCherry-SERT secretory vesicles

Live cell imaging of mCherry-SERT trafficking dynamics in hippocampal neurons (DIV 9-11). (A) Two frames 5 seconds apart (green is 1 s, red is 5 s) from a 1 minute movie reveal the different modes of trafficking of mCherry-SERT. There are mCherry-SERT vesicles which move in the anterograde direction (arrow), in the retrograde direction (arrowhead) or are stationary (yellow puncta, asterisks). (B) Corresponding kymograph of part of the neurite displays the different trafficking modes of mCherry-SERT. Vesicle 1 is a stationary vesicle, vesicle 2 moves in the anterograde direction, whereas vesicle 4 moves in the retrograde direction. Vesicle 3 is a vesicle which pauses for a while and then continues moving. Vesicle numbers are shown in the individual images taken 10 seconds apart and vesicle trajectories are highlighted in the kymograph. (C) No difference between average anterograde and retrograde velocity as can be observed in the histogram of the average velocity. (D) No difference between average velocity of mCherry-SERT vesicles which move in the anterograde or retrograde direction or which display bidirectional movements. (E) Percentage moving vesicles does not differ between vesicles which move anterogradely, retrogradely or bidirectionally. (F) Also, percentage pausing time for moving vesicles is not different between vesicles which move anterogradely, retrogradely or bidirectionally. Scale bars in A and B are 10 µm.



SERT trafficking is highly dynamic

We analyzed the trafficking dynamics of SERT using live-cell imaging of hippocampal neurons expressing mCherry-SERT. We observed rapid movements of mCherry-SERT vesicles in both the anterograde and retrograde direction. Additionally, also stationary mCherry-SERT puncta were observed (Fig. 4A). To illustrate different types of movement we show part of a neurite with 10 second time intervals. In this neurite a stationary vesicle can be observed (vesicle 1), but also a vesicle which moves in an anterograde direction (vesicle 2) and a vesicle which moves in a retrograde direction (vesicle 4). Additionally, vesicles are

observed which are temporarily stationary but then continue moving (vesicle 3 in Fig. 4B). In the corresponding kymograph of this part of the neurite the trajectories of the numbered vesicles are shown (Fig. 4B). We quantified the direction of vesicle movement, percentage of moving vesicles, average velocity of moving vesicles and percentage pausing time of moving vesicles using the automated vesicle detection program Fluotrack (Broeke et al., 2008). First we measured the average velocity of anterogradely and retrogradely moving vesicles. The frequency histogram plotting the velocities of mCherry-SERT vesicles moving in the anterograde or retrograde direction showed that the velocity distributions for anterogradely and retrogradely moving vesicles did not differ (Fig. 4C). The average velocity of all moving vesicles was 1.22 ± 0.03 µm/s. The average velocity of anterograde vesicles was 1.31 ± 0.05 µm/s compared to 1.34 ± 0.06 µm/s in retrograde direction. Vesicles moving bidirectional (vesicles that changed direction during imaging) moved with an average velocity of 1.12 ± 0.055 µm/s (Fig. 4D). Next, we determined the percentage of puncta which moved in the anterograde or retrograde direction, moved bidirectional or were stationary. This revealed that on average 34.52 ± 5.31% of mCherry-SERT puncta were moving during one minute of imaging. Further analysis of fraction of moving mCherry-SERT vesicles which moved in the anterograde or retrograde direction or moved bidirectional, revealed that there was no difference (Fig. 4E, see also table 1). Finally, we calculated the pausing time for the moving vesicles, which is the fraction of time moving vesicles pause during the 1 minute movie. There was no difference in percentage pausing time between mCherry-SERT vesicles which moved anterogradely, retrogradely or bidirectionally (Fig. 4F, see also table 1).

We conclude that mCherry-SERT displays highly dynamic vesicular trafficking in hippocampal neurons and that these mCherry-SERT vesicles do not have a preference for a direction of movement.

Table 1: Overview of mCherry-SERT vesicle dynamics

	Velocity (µm/s)	Percentage of total	Percentage pausing time
Anterograde	1,31 +/- 0.05	13.83	42.13
Retrograde	1,34 +/- 0.06	12.06	39.21
Bidirectional	1,12 +/- 0.06	8.63	41.04
Stationary	-	65.48	-

Velocity data are shown as mean ± standard error of the mean (SEM). Data of 927 mCherry-SERT puncta in 6 neurons.

Discussion

Here we studied the trafficking of SERT in hippocampal neurons. We used human SERT cDNA tagged at its N-terminus with the red fluorescent protein mCherry. Overexpression of mCherry-SERT in hippocampal neurons resulted in a distinct pattern of expression between axons and dendrites; in dendrites SERT displayed a uniform distribution, whereas in axons SERT displayed a punctate distribution. Using electron microscopy, it has been shown that in 5-HT neurons endogenous SERT predominantly localized to varicosities and the membrane in axons, and in soma and dendrites SERT localized predominantly in the cytoplasm (Zhou et al., 1998; Tao-Cheng and Zhou, 1999). Thus, although we are using hippocampal neurons, mCherry-SERT distribution seems to mimic the distribution of endogenous SERT in 5-HT neurons.

Ultrastructural studies revealed that SERT labeling was associated with vesicular organelles, which possibly are large dense cored vesicles (LDCVs) (Pickel and Chan, 1999). Here, we co-transfected mCherry-SERT with tPA-GFP for which it has been shown that these are transported in LDCVs (also called secretory vesicles) (Silverman et al., 2005). The majority of mCherry-SERT puncta co-localized with tPA-GFP, strongly suggesting that SERT is transported in secretory vesicles. Several mCherry-SERT vesicles are localized in close proximity (~1 μm), and some co-localized, with presynaptic terminals. This suggests that the majority of SERT is not localized in the presynaptic terminal itself, but rather is localized close to a synapse. Indeed, previous research found that presynaptic membranes lacked SERT, but SERT was present on extrasynaptic domains, either in close proximity or at some distance away from synapses (Zhou et al., 1998).

Live cell imaging revealed that mCherry-SERT vesicles display highly dynamic transport, i.e. there are vesicles which move anterogradely or retrogradely without interruptions, stationary vesicles or vesicles which move bidirectional. Analysis of average velocity revealed that both anterograde and retrograde vesicles moved with an average velocity of ~1.3 μm/s. The average velocity is in analogy with other transport or transport associated proteins which are transported in secretory vesicles, like the p75 neurotrophin receptor and Gephyrin (Maas et al., 2006; Formaggio et al., 2008). Surprisingly, we found that mCherry-SERT vesicles did not display a preference for direction of movement. For other releasable fluorescent cargos that are transported in secretory vesicles, such as Sema3A, BDNF and CCL21 it has been shown that transport is predominantly anterogradely (Adachi et al., 2005; de Wit et al., 2006; de Jong et al., 2008). In contrast, the p75 neurotrophin receptor, which is transported in vesicular structures, does not display a preference for direction of movement (Formaggio et al., 2008). Moreover, the scaffold protein Gephyrin,

which is associated and co-transported with the glycine receptor also does not display a preference for anterograde or retrograde movement (Maas et al., 2006). Therefore, it appears that proteins which can be secreted and are transported in secretory vesicles predominantly move in the anterograde direction, whereas receptor/transporter proteins move both in the anterograde and retrograde direction.

An explanation for this could be that cargos which can be secreted are transported towards synaptic release sites distant from the cell soma. After a vesicle has been released, the secreted compounds either diffuse in the extracellular space (as is the case for Sema3A) or are taken up again and degraded in the presynaptic terminal. Hence, releasable cargos are hardly transported back towards the cell soma. On the other hand, the level of membrane expression for receptor and transporter protein is tightly regulated, and these proteins can be rapidly internalized (Loder and Melikian, 2003; Melikian, 2004; Zhu et al., 2004). Upon internalization, transporters are packaged in vesicles again and are recycled. Finally, receptors and transporters can maintain functioning without a need for intracellular transport, whereas secreted components are dependent on a new supply of vesicles. Therefore, vesicles containing transporters or receptors can be transported both in anterograde and retrograde directions.

In conclusion, expression of mCherry-SERT in hippocampal neurons differs between axons and dendrites. In axons mCherry-SERT is distributed in puncta, which co-localize with tPA-GFP and therefore presumably are secretory vesicles. Several of these mCherry-SERT vesicles are localized in close proximity of presynaptic terminals, but only few mCherry-SERT vesicles co-localized with presynaptic terminals. Live cell imaging revealed that mCherry-SERT displays highly dynamic trafficking behaviour with no preference for direction of movement.

EXPERIMENTAL PROCEDURES

Plasmids

Construction of the human SERT cDNA with ECFP at the 5' end (ECFP-SERT in the pECFP-C1 backbone (Clontech)) has been described before ((Schmid et al., 2001); a kind gift of Dr. H. Sitte). To construct mCherry-SERT, ECFP was excised with AseI and BsrGI and replaced for mCherry.

Neuronal culturing and transfection

Hippocampi from embryonic day 18 C57/Bl6 mouse embryos were dissected free of meninges and collected in Hanks Balanced Salt Solution (HBSS; Invitrogen) supplemented with 7 mM HEPES (Invitrogen). Hippocampi were digested with

0.25% trypsin (Invitrogen) in HBSS supplemented with 7 mM HEPES for 15 minutes at 37ºC. After digestion tissue was washed three times with HBSS supplemented with 7 mM Hepes and subsequently triturated with a fire-polished glass pipette. Dissociated neurons were plated at a density of 25.000 cell/well on rat glial cells on 18 mm glass coverslips (Menzel Glaser, Braunschweig, Germany) in 12 well plates. Cells were cultured in neurobasal medium supplemented with B27 supplement, 18 mM HEPES, 0.5 mM Glutamax and penicillin/streptomycin (Invitrogen). Once a week half of the medium was replaced. The hippocampal cultures were transfected using Lipofectamine 2000 (Invitrogen). Before transfection, half of the neurobasal medium was collected from each well and replaced with new neurobasal medium. For one well, 1 µl of Lipofectamine 2000 was added to 25 µl of serum free glutamax medium (Invitrogen) and incubated for 5 minutes. Subsequently, from each construct 1 µg of DNA was added to 25 µl of serum free glutamax and immediately mixed with the 25 µl of serum free glutamax containing Lipofectamine 2000. This mixture was incubated for 30 minutes at room temperature before adding to the cells. After 4-6 hours of incubation on the cells at 37°C, half of the neurobasal medium was replaced again with the earlier collected neurobasal medium. Cells were routinely transfected at DIV2-7 and imaged at DIV9-12.

Live cell imaging
Cells were imaged in a chamber perfused with Tyrode's solution (119 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 30 mM glucose, 25 mM HEPES, pH 7.4) on an Axiovert II Microscope (Zeiss, Oberkochen, Germany) equipped with a Coolsnap HQ camera (Photometrics, Tucson, Arizona, USA) and a Polychrome IV illumination unit (TILL photonics, Grafelfing, Germany). Images were acquired in MetaMorph 6.2 software (Universal Imaging, Downingtown, Philadelphia, USA) using a 100x oil objective.

Immunocytochemistry and confocal microscopy
For immunocytochemistry, neurons were fixed by incubation in 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 minutes. Subsequently, to block aspecific binding and for permeabilization, the neurons were incubated in PBS containing 0.2% Triton X-100 and 4% fetal calf serum for 20 minutes. Neurons were incubated in primary antibody diluted in PBS containing 0.2% Triton X-100 for one hour. After washing three times in PBS, neurons were incubated in secondary antibodies in PBS for one hour. Neurons were washed again three times in PBS and mounted in Dabco-Mowiol. All reactions were carried out at room temperature. As primary antibodies, monoclonal MAP2 (Chemicon) and polyclonal Synapsin (E028) were used. As secondary antibodies Alexa fluor-conjugated antibodies were used (Invitrogen).

For confocal analysis, neurons were examined on a confocal LSM510 microscope (Zeiss, B.V. The Netherlands)

Trafficking analysis
In order to analyze the trafficking dynamics of SERT, one minute movie image stacks with 1 s interval between images, were analyzed in the custom written software program Fluotrack (Broeke et al., 2008). To this end, metamorph files (.stk) were converted to .avi files using ImageJ, and analyzed using Fluotrack. Data analysis was performed using the SPSS statistical package. Data shown are mean values ± SEM.

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Chapter

4

Presynaptic localization of tryptophan hydroxylase 2 in mature hippocampal neurons

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In preparation

Abstract

Tryptophan hydroxylase (Tph) is the rate limiting enzyme in serotonin (5-HT) synthesis. Two genes named Tph1 and Tph2 synthesize 5-HT in peripheral tissues and the central nervous system, respectively. When expressed in neurons, Tph1 displays a cytoplasmic distribution in the cell body and is associated with microtubules in the axon and dendrites. Axonal Tph1 is associated with 5-HT varicosities. Here, we studied the subcellular distribution of Tph2. To this end we expressed Tph2-EGFP in hippocampal neurons. In young neurons, before synaptogenesis has occurred, Tph2-EGFP displayed a cytoplasmic distribution throughout the neurites. Live cell imaging revealed that Tph2-EGFP is not transported in vesicles. In mature neurons, Tph2-EGFP accumulated in presynaptic terminals of axons, whereas in dendrites it was distributed throughout the cytoplasm, also in spines. In live cell imaging, Tph2-EGFP hardly displayed any movement. These results suggest that Tph2 is freely diffusible in the dendrites and soma, but accumulates at the synaptic terminal by a mechanism that deserves further study.

Introduction

The neurons of the 5-HT system are among the first neurons in the brain synthesizing a specific neurotransmitter. Two enzymes are necessary to synthesize 5-HT from its precursor L-tryptophan. Of these, the rate limiting enzyme is Tph which converts L-tryptophan to 5-hydroxy-L-tryptophan. For decades only one Tph isoform was known in the mammalian brain. However, deletion of this gene did not result in a significant decrease in brain 5-HT levels, which led to the discovery of a second Tph gene called Tph2 (Walther et al., 2003). Tph2 is exclusively expressed in the raphe nuclei, whereas Tph1 is expressed in the pineal gland and in the peripheral 5-HT system (Patel et al., 2004; Gutknecht et al., 2009).

Since 5-HT projections are distributed throughout the brain, it is unlikely that Tph2 is only present in the cell body to synthesize 5-HT. Indeed, previous research showed that Tph was present in the cytoplasm of the cell body, and in axons and dendrites where it was associated with microtubules (Joh et al., 1975). Additionally, Tph was associated with 5-HT varicosities, suggesting local synthesis of 5-HT (Pickel et al., 1976; Pickel et al., 1977).

Although the subcellular distribution of Tph1 is known, it is currently unknown how Tph is transported to synaptic terminals and whether Tph2 also

localizes to presynaptic terminals. Therefore we tagged Tph2 with EGFP, and expressed this chimaeric protein in hippocampal neurons. We show here that in young neurons (before synaptogenesis), Tph2-EGFP is homogenously distributed throughout the neurites. However, in mature neurons, Tph2-EGFP accumulated at presynaptic terminals in the axon. In the dendrites of mature neurons, Tph2-EGFP displayed a homogenous distribution, and also localized to spines. Live cell imaging suggested that Tph2 is a soluble protein and is not transported in vesicles to presynaptic terminals. We conclude that Tph2-EGFP accumulates at presynaptic terminals in mature hippocampal neurons.

Results

Cytoplasmic distribution of Tph2-EGFP in young neurons

To study the distribution of Tph2 in neurons, we inserted EGFP at the N-terminus of Tph2 and expressed Tph2-EGFP in hippocampal neurons. First, we started with imaging Tph2-EGFP in neurons at 9 days in vitro (DIV), which are still immature neurons in terms of synapse development (Basarsky et al., 1994) Transfected neurons showed a uniform distribution of Tph2-EGFP through the whole neuron, indicating that Tph2-EGFP is cytoplasmic (Fig. 1A). We did not observe Tph2-EGFP puncta, suggesting that Tph2-EGFP is not transported in

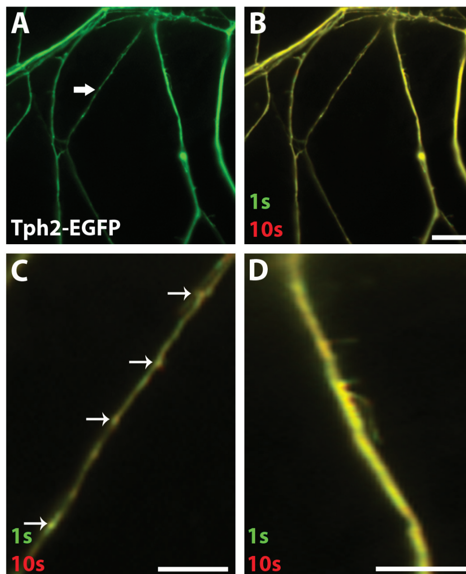
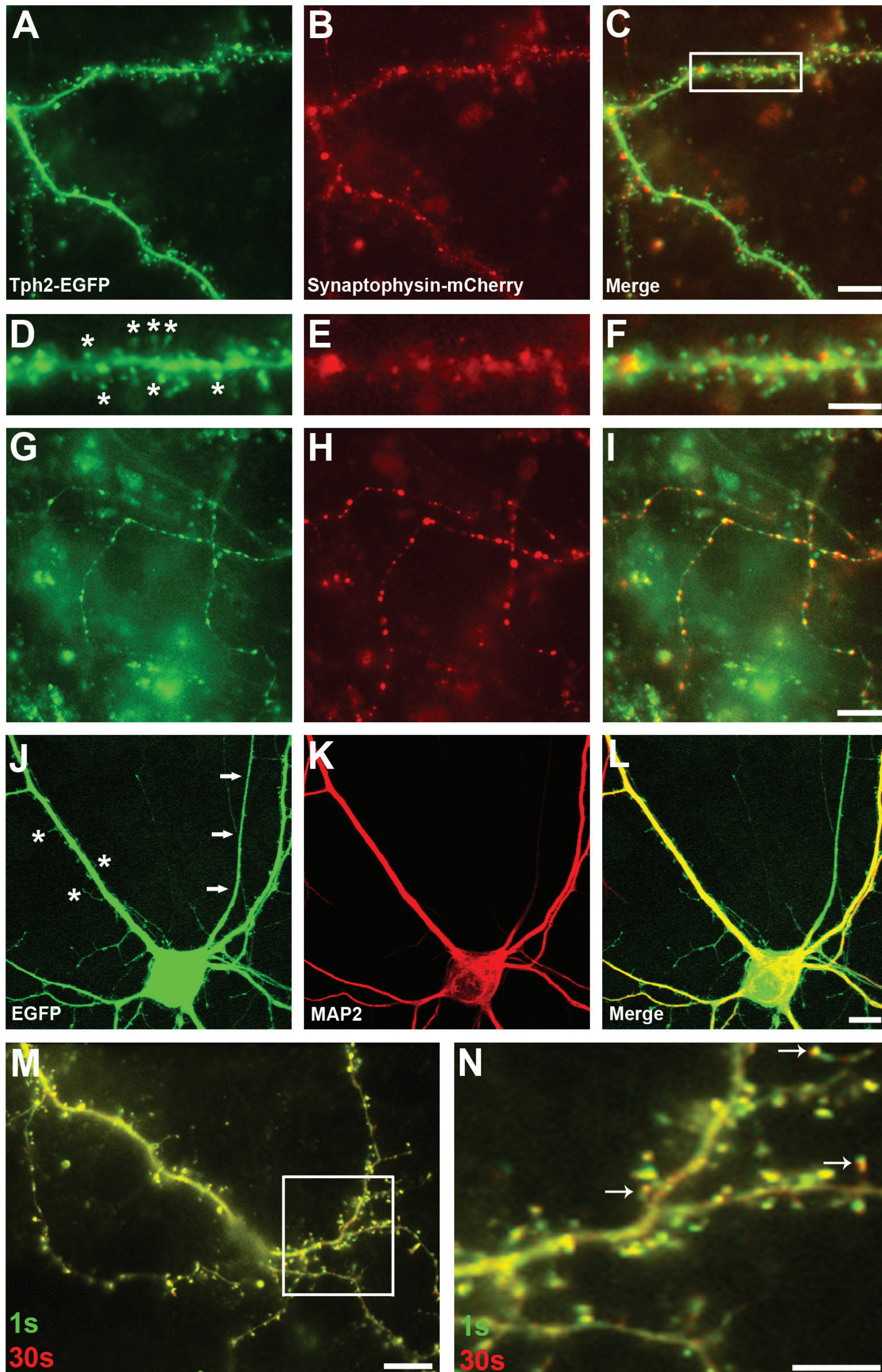


Figure 1. Tph2-EGFP expression in young neurons

Tph2-EGFP was expressed in young hippocampal neurons before synaptogenesis and imaged at DIV9. (A) Tph2-EGFP displays a cytoplasmic distribution throughout all the neurites. (B) Live cell imaging revealed that Tph2-EGFP is not transported in vesicles, and only little movement is detected. Images are taken 10 seconds apart, first image is green and image 10 seconds later is in red. (C) Zooming in on the axon (arrow in A), as identified by its smaller diameter compared to dendrites shows that already puncta are observed which might be immature synapses or varicosities (arrows). (D) In the dendrites, Tph2-EGFP distribution is cytoplasmic, and Tph2-EGFP also localized to dendritic filopodia. Scale bars: 10 µm in B, 5 µm in C and D.


A
B
C
Tph2-EGFP
Synaptophysin-mCherry
Merge
D
E
F
G
H
I
J
K
L
EGFP
MAP2
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M
N
1s
30s
1s
30s

Figure 2 previous page. Tph2-EGFP localizes to presynaptic terminals in mature neurons

Neurons expressing Tph2-EGFP and the presynaptic marker Synaptophysin-mCherry were imaged at DIV23. (A) Tph2-EGFP was expressed in the cytoplasm of dendrites. (B) Expression of Synaptophysin-mCherry in the same neuron. (C) Merged image. (D) Blow up of the white box in C shows that some Tph2-EGFP appears to accumulate in the tip of dendritic spines (asterisks). (E) Synaptophysin-mCherry expression. (F) Merged image. (G) Axon of a neuron expressing Tph2-EGFP. Tph2-EGFP displays a punctate expression, indicative of presynaptic localization. (H) Indeed, Tph2-EGFP co-localizes with Synaptophysin-mCherry. (I) Merged image. (J) A EGFP expressing neuron as a control shows that EGFP is homogenously distributed in the dendrites (asterisks) and in the axon as identified by the absence of MAP2 staining (arrows). (K) MAP2 staining. (L) Merged image. (M) Live cell imaging revealed that Tph2-EGFP is static. Images are taken 30 seconds apart, with the green image after 1 second, and the red image after 30 seconds. (N) Blow up of the white box in J shows that some dendrites which contain Tph2-EGFP displayed some movement (arrows). Scale bars: 10 μm in C, 5 μm in F, 10 μm in I, 10 μm in L, 10 μm in M and 5 μm in N.

vesicles, but instead is a soluble protein. Live cell imaging revealed that Tph2-EGFP is rather static, and no Tph2-EGFP puncta or other movements were observed (Fig. 1B). The axon, as identified by its smaller diameter and larger length as compared to dendrites, also had a uniform distribution of Tph2-EGFP, although some Tph2-EGFP appeared to accumulate in puncta along the axon (arrows in Fig. 1C). As in axons, in dendrites Tph2-EGFP displayed a uniform distribution and also filopodia were labelled (Fig. 1D).

Tph2-EGFP localizes to presynaptic terminals in mature neurons

Next, we investigated the distribution of Tph2-EGFP in mature neurons after synaptogenesis had occurred. To this end we transfected hippocampal neurons with Tph2-EGFP at DIV18 and imaged Tph2-EGFP at DIV23. Tph2-EGFP was uniformly distributed in the cell body, dendrites and dendritic spines (Fig. 2A-C). We expressed EGFP in neurons as a control to compare the distribution of EGFP with Tph2-EGFP (Fig. 2J-L). In contrast to EGFP, which labels the whole dendritic spine, it appeared that in neurons transfected with Tph2-EGFP in some spines Tph2-EGFP accumulated in the tip of the spine, suggesting that Tph2-EGFP was enriched in postsynaptic structures (Fig. 2D-F). We co-transfected Tph2-EGFP with Synaptophysin-mCherry, which is a marker for synaptic vesicles and presynaptic terminals (Jahn et al., 1985; Granseth et al., 2006). In the axon, identified by the punctate labelling of Synaptophysin-mCherry, Tph2-EGFP displayed a punctate distribution (Fig. 2G). The Tph2-EGFP puncta co-localized with Synaptophysin-mCherry puncta, strongly suggesting that Tph2-EGFP accumulated at presynaptic terminals (Fig. 2H,I). In neurons transfected

with EGFP as a control, EGFP displayed a uniform distribution throughout the axon (Fig. 2J-L). Next, we imaged the neurons expressing Tph2-EGFP during 1 minute. Hardly any Tph2-EGFP movement could be observed (Fig. 2M). The only movement which could be observed was some motility in postsynaptic spines (Fig. 2N).

To further test if Tph2-EGFP is localized to presynaptic terminals in mature neurons, we fixed neurons expressing Tph2-EGFP at DIV23 and performed immunocytochemistry for the synaptic marker Synapsin and the dendritic marker MAP2 to discriminate dendrites and axons (Fig. 3A-C). In the axon, as identified by the absence of MAP2 labelling, Tph2-EGFP displayed a punctate distribution (Fig. 3D-F). Immunocytochemistry for Synapsin showed that in dendrites of neurons expressing Tph2-EGFP, spines were labelled, and some spine heads were localized in close proximity with Synapsin puncta, suggesting that this is a synapse (Fig. 3G-I). Finally, we focussed on the axon of a neuron expressing Tph2-EGFP which we stained for Synapsin. The boutons with Tph2-EGFP co-localized with Synapsin, strongly suggesting that Tph2-EGFP localized to presynaptic terminals (Fig. 3J-L).

Discussion

We have shown here that Tph2, the rate limiting enzyme in 5-HT synthesis localized to presynaptic terminals in mature hippocampal neurons. Moreover, in dendrites and in the cell body Tph2-EGFP had a cytoplasmic distribution and appeared to accumulate in the tip of some dendritic spines. Thus, it appears that Tph2-EGFP displayed a polarized distribution in mature hippocampal neurons. Live cell imaging revealed that hardly any Tph2-EGFP movement could be observed, and no Tph2-EGFP vesicles were observed, indicating that Tph2 is a soluble protein. Accordingly, Tyrosine hydroxylase, an enzyme related to Tph2, also was shown to be a soluble protein (Mockus et al., 1997). Electron microscope studies on Tph and TH localization revealed that these proteins were enriched on presynaptic terminals, and were associated with microtubules in somatodendritic compartments (Pickel et al., 1976). Therefore, Tph2-EGFP seems to mimic the endogenous distribution of Tph in 5-HT neurons.

Tph2-EGFP displayed a polarized distribution in mature hippocampal neurons, i.e. in dendrites the protein is distributed throughout the cytoplasm, whereas in the axon the protein accumulated in presynaptic terminals. This suggests that in presynaptic terminals and in 5-HT varicosities, there is local synthesis of 5-HT. In dendrites, Tph2-EGFP localized to spines, which prompts the question whether 5-HT could be synthesized in dendrites.

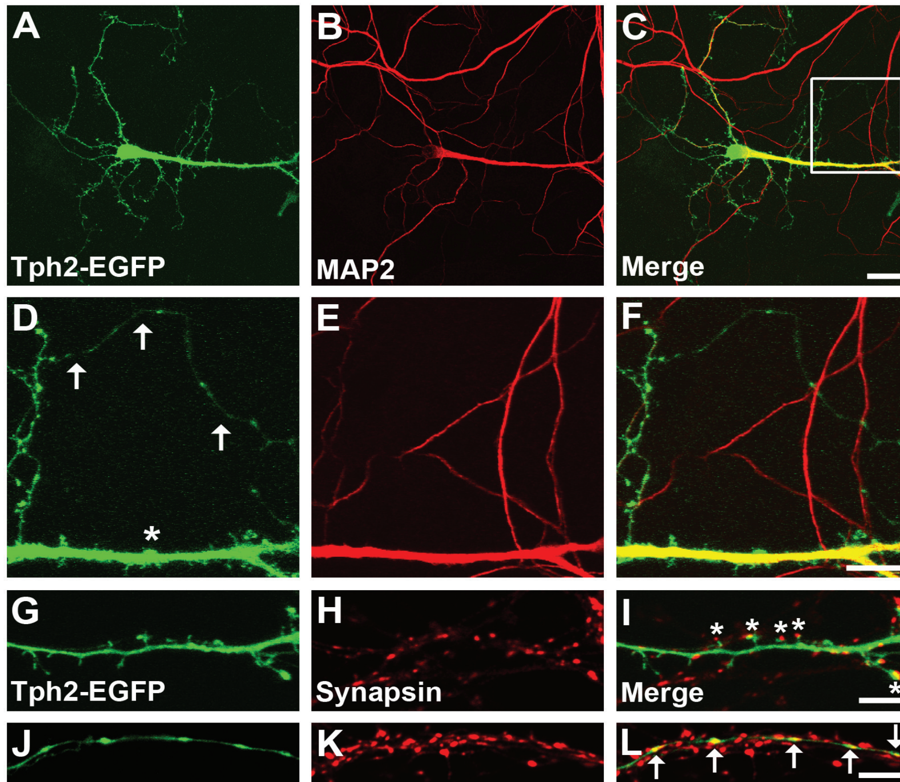

Figure 3. Immunocytochemistry on a mature neuron expressing Tph2-EGFP
Neurons (DIV23) expressing Tph2-EGFP were fixed and immunocytochemistry was performed for MAP2 and Synapsin. (A) Tph2-EGFP expression. (B) MAP2 immunostaining to distinguish between dendrites and the axon. (C) Merged image. (D) Blow up of the white box in C shows a dendrite (asterisk) which has Tph2-EGFP through the whole cytoplasm, and the axon (arrows) in which Tph2-EGFP displays a punctate distribution. (E) MAP2 staining. (F) Merged image. (G) Tph2-EGFP expressing neurons were stained for the presynaptic marker Synapsin. (H) Synapsin staining. (I) Merged image, which shows that in the close proximity of some dendritic spines there is a Synapsin puncta, suggesting that these are true synapses (asterisks). (J) Distribution of Tph2-EGFP in an axon. (K) Synapsin staining. (L) Tph2-EGFP puncta were positive for Synapsin (arrows), showing that these are presynaptic terminals. Scale bars: 20 μm in C, 10 μm in F, 5 μm in I and L.

Tph is localized in dendrites, where it associated with microtubules (Joh et al., 1975). Correspondingly, 5-HT vesicles are detected in dendrites, either associated with a synaptic specialization or with the membrane (Chazal and Ralston, 1987). After stimulation of Retzius neurons in the leech, which contain large dense core vesicles (LDCVs) filled with 5-HT, several vesicles are localized close to the cell membrane as studied by electron microscopy (Trueta et al.,

2004). Therefore, this strongly suggests that 5-HT release can occur from the somatodendritic compartment. Our results indicate that 5-HT can also be synthesized in the somatodendritic compartment.

Tph2-EGFP was not associated with vesicles and the protein interactions for its presynaptic accumulation remain unclear. Tph is associated with microtubules, suggesting it could interact with molecular motors of the dynein and kinesin family which move along microtubules (Hirokawa and Takemura, 2005). For Tph2, several polymorphisms are known, some of which associate with neuropsychiatric conditions (Zhang et al., 2006). Both in humans and in mice a polymorphism in the Tph2 gene is found which results in a marked reduction in Tph2 activity (Zhang et al., 2004; Zhang et al., 2005). Finally, it would be interesting to investigate whether these polymorphisms not only affect Tph2 activity, but could also affect Tph2 transport and localization.

EXPERIMENTAL PROCEDURES

Plasmids

Tph2 cDNA in pCR4-TOPO vector was obtained from Geneservice. To clone Tph2 in an expression vector, EGFP was digested using PstI and cloned in the PstI site of pBiotag-EGFP-C1. Synaptophysin-mCherry was a kind gift from Dr. A. Jeromin (Allen Brain Institute).

Neuronal culturing and transfection

Hippocampi from embryonic day 18 C57/Bl6 mouse embryos were dissected free of meninges and collected in Hanks' Balanced Salt Solution (HBSS; Invitrogen) supplemented with 7 mM HEPES (Invitrogen). Hippocampi were digested with 0.25% trypsin (Invitrogen) in HBSS supplemented with 7 mM HEPES for 15 minutes at 37°C. After digestion tissue was washed three times with HBSS supplemented with 7 mM Hepes and subsequently triturated with a fire-polished glass pipette. Dissociated neurons were plated at a density of 25.000 cell/well on rat glial cells on 18 mm glass coverslips (Menzel Glaser, Braunschweig, Germany) in 12 well plates. Cells were cultured in neurobasal medium supplemented with B27 supplement, 18 mM HEPES, 0.5 mM Glutamax and penicillin/streptomycin (Invitrogen). Once a week half of the medium was replaced. The hippocampal cultures were transfected using Lipofectamine 2000 (Invitrogen). Before transfection, half of the neurobasal medium was collected from each well and replaced with new neurobasal medium. For one well, 1 µl of Lipofectamine was added to 25 µl of serum free glutamax medium and incubated for five minutes. Subsequently, from each construct 1 µg of DNA was added to 25 µl of serum free glutamax and immediately mixed with the 25 µl of serum free

glutamax containing Lipofectamine. This mixture was incubated for 30 minutes before adding to the cells. After 4-6 hours of incubation on the cells, half of the neurobasal medium was replaced again with the earlier collected neurobasal medium. Cells were routinely transfected at DIV2-7 and DIV18 and imaged at DIV10-14 and DIV23.

Live cell imaging
Cells were imaged in a chamber perfused with Tyrode's solution (119 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 30 mM glucose, 25 mM HEPES, pH 7.4) on an Axiovert II Microscope (Zeiss, Oberkochen, Germany) equipped with a Coolsnap HQ camera (Photometrics, Tucson, Arizona, USA) and a Polychrome IV illumination unit (TILL photonics, Grafelfing, Germany). Images were acquired in MetaMorph 6.2 software (Universal Imaging, Downingtown, Philadelphia, USA) using a 100x oil objective.

Immunocytochemistry
For immunocytochemistry, primary antibodies against MAP2 (monoclonal, Chemicon) and synapsin (polyclonal E028) were used in a 1:1000 dilution. As secondary antibodies, goat-anti-mouse Alexa488 and goat-anti-rabbit Alexa 543 were used (Invitrogen). To perform immunocytochemistry, cells were incubated in 4% paraformaldehyde dissolved in phosphate buffered saline (PBS, pH 7.4), for 20 minutes. Subsequently, cells were washed two times with PBS and incubated in PBS containing 0.2% Triton X-100 and 4% fetal calf serum for 20 minutes. Cells were incubated in primary antibody in PBS containing 0.2% Triton X-100 for one hour, washed three times with PBS and incubated in the secondary antibody in PBS for one hour. After washing the cells three times with PBS, coverslips were mounted using Dabco-Mowiol. All reactions were performed at room temperature. Cells were analyzed on a confocal LSM510 microscope (Zeiss, B.V. The Netherlands) using a 63x oil immersion lens and appropriate lasers and filters to visualize EGFP fluorescence and secondary antibodies.

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Chapter

5

The effect of a polymorphism in Piccolo on localization and trafficking dynamics of the serotonin reuptake transporter

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Abstract

The active zone protein Piccolo is involved in formation of synapses and stabilization of existing synapses. Recently, a short nucleotide polymorphism (SNP) in the C2A domain of Piccolo was found in association with depression. In this study we addressed whether knockdown of Piccolo affected the localization and trafficking of the serotonin reuptake transporter (SERT) in hippocampal neurons. We co-expressed a short nucleotide RNA which resulted in an efficient knockdown of Piccolo with mCherry-SERT to evaluate the effect of knockdown of Piccolo on SERT localization and trafficking in hippocampal neurons. We found that knockdown of Piccolo did not alter SERT trafficking dynamics. Subsequently, we expressed the two variants of the Piccolo C2A domain together with SERT in heterologous cells. SERT displayed a uniform distribution at the membrane upon co-expression of the wildtype C2A variant. However, upon co-expression of the mutant C2A variant, SERT displayed a more patchy distribution at the membrane, indicating that membrane levels of SERT might be decreased. These data suggest that knockdown of Piccolo does not alter SERT trafficking dynamics, but the mutant variant of the Piccolo C2A domain might alter SERT membrane localization.

Introduction

Major depression is a neuropsychiatric disorder affecting millions of people world wide each year. The serotonin (5-HT) system is implicated in depression. An important protein in the 5-HT system is the SERT which regulates the transport of 5-HT back into the synapse after its release, thus regulating the extracellular 5-HT concentration. Therefore, SERT is an important factor in determining efficacy of 5-HT neurotransmission. Several polymorphisms have been described in SERT which associate with psychopathological processes such as depression, anxiety, aggression and autism (Murphy et al., 2004; Serretti et al., 2006).

Several decades ago it was hypothesized that psychopathological processes such as depression and anxiety were the result of an alteration in extracellular 5-HT content. This hypothesis, the so-called monoamine hypothesis, was based on the fact that blockade of the SERT by selective 5-HT reuptake inhibitors (SSRIs) resulted in an alleviation in depressive symptoms, possibly due to the increase in extracellular 5-HT levels. However, between starting SSRI treatment and alleviation of depressive symptoms was a time period of 3-4 weeks, whereas 5-

HT levels increased immediately after SSRI intake. Therefore the monoamine hypothesis was abandoned and shifted towards the so-called network hypothesis, which stated that psychopathological processes such as anxiety and depression could be the result of an altered 5-HT network connectivity (Castren, 2005). The evidence for this hypothesis was based on human imaging studies which showed that humans with depression had a reduced grey matter volume in cortex and hippocampus. However, evidence for this hypothesis on a cellular level is still lacking. Recently, a SNP in the presynaptic gene Piccolo was found which might be associated with major depressive disorder (Sullivan et al., 2008). Therefore, this revitalizes the network hypothesis for depression and opens up new opportunities to study the relation between the presynaptic terminal, proper formation of networks, and the relation between 5-HT network (formation) and psychopathological processes such as depression.

Piccolo is a 420 kDa protein which is enriched in presynaptic terminals where it is a structural component of the presynaptic cytoskeletal cytomatrix (PCM) (Cases-Langhoff et al., 1996). Piccolo is a multidomain zinc finger protein which is structurally related to Bassoon, another component of the PCM (tom Dieck et al., 1998; Fenster et al., 2000). Piccolo contains several coiled coil domains, a PDZ domain and two C2 domains (Fenster and Garner, 2002). The C2A domain functions as a low-affinity calcium sensor in presynaptic terminals (Gerber et al., 2001). Piccolo and Bassoon, along with other presynaptic constituents, are transported in 80 nm large dense core vesicles (LDCVs) to nascent synapses, and 2 or 3 of these vesicles are sufficient to form an active synapse (Zhai et al., 2001; Shapira et al., 2003; Tao-Cheng, 2007). The recent described nonsynonymous SNP rs2522833 in Piccolo encodes a common variation in the C2A domain, where a serine is substituted for an alanine (Sullivan et al., 2008).

Piccolo is involved in the regulation of the internalization of the dopamine transporter (DAT) in heterologous cells. Specifically, expression of the C2A domain of Piccolo reduces methamphetamine-induced DAT internalization. (Cen et al., 2008). DAT and SERT are structurally related proteins, which belong to the solute carrier superfamily. It could be likely that membrane incorporation and internalization of these proteins is regulated in the same manner. Additionally, Piccolo influences synaptic function by negatively regulating synaptic vesicle exocytosis (Leal-Ortiz et al., 2008). These data led us to hypothesize that possibly, Piccolo might contribute to the overall risk of depression by affecting the membrane incorporation of SERT.

To test this hypothesis we expressed mCherry-SERT in hippocampal neurons and investigated whether knockdown of Piccolo or the variation rs2522833 affected the localization and trafficking of SERT. We show here that

knockdown of Piccolo in neurons did not affect SERT trafficking. However, expression of the Piccolo C2A domain variation rs2522833 in heterologous cells might affect membrane localization of SERT.

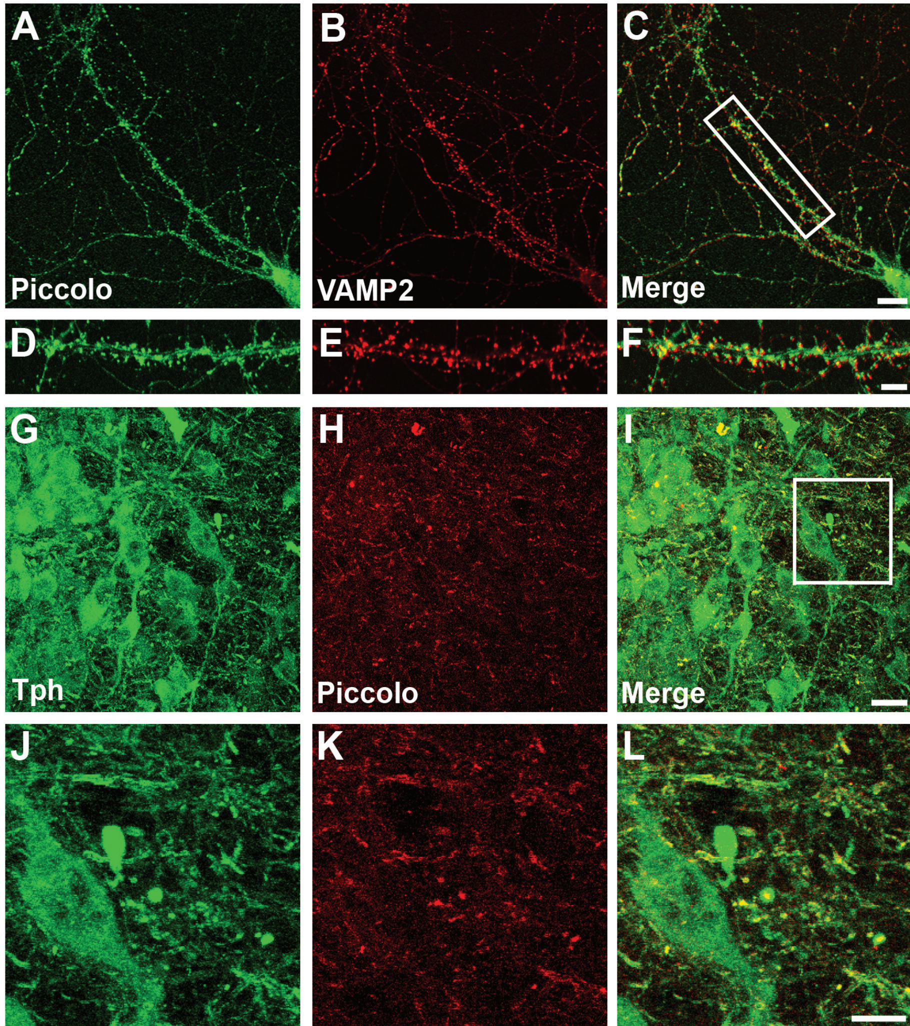
Results

Piccolo localized to neurotransmitter release sites in hippocampal and 5-HT neurons

First, we analyzed the cellular localization of Piccolo in hippocampal neurons and 5-HT neurons. Hippocampal neurons (14 days in vitro (DIV)) were fixed and immunocytochemistry was performed for Piccolo and for the presynaptic terminal marker Synaptobrevin-2 (VAMP2). Piccolo immunoreactivity was distributed in a punctate pattern, indicative for labeling of presynaptic terminals (Fig. 1A). Indeed, the majority of Piccolo puncta co-localized with VAMP2 puncta, showing that these represented presynaptic terminals (Fig. 1B,C). Zooming in on part of a neurite showed that >90% of Piccolo puncta co-localized with VAMP2 (Fig. 1D-F). Thus, Piccolo was present in presynaptic terminals.

Next, we investigated the localization of Piccolo in 5-HT neurons. We used a coronal brain slice from an adult mouse through the dorsal raphe nucleus (DRN), the region in the brain where the majority of 5-HT neurons is localized. We performed immunocytochemistry for tryptophan hydroxylase (Tph), the rate limiting enzyme for 5-HT synthesis and a marker for the 5-HT system. In a DRN slice, several 5-HT neurons were present (Fig. 1G). Immunocytochemistry for Piccolo resulted in a punctate labeling in the DRN slice (Fig. 1H). Piccolo staining was barely detectable in the 5-HT cell bodies (Fig. 1I). However, Piccolo puncta co-localized with Tph puncta. Tph is present in varicosities and presynaptic terminals (chapter 4 of this thesis) (Pickel et al., 1976).

Figure 1 next page. Piccolo localizes to presynaptic terminals in neurons
(A-C) Hippocampal neurons were fixed at DIV14, and immunocytochemistry was performed for Piccolo (A) and the presynaptic marker VAMP2 (B). (C) Merged image shows that several Piccolo puncta co-localized with VAMP2 puncta. (D-F) Blow up of the white box in C shows that almost every Piccolo puncta co-localizes with a VAMP2 puncta. (D) Piccolo staining. (E) VAMP2 staining. (F) Merged image. (G) A coronal brain slice through the DRN from an adult mouse was stained against the 5-HT marker Tph. This shows that 5-HT cell bodies and fibers are present. (H) Piccolo staining on this slice shows that Piccolo expression is low in 5-HT cell bodies. (I) Merged image. (J) Blow up of the white box in I shows a 5-HT cell body and several Tph puncta, which might be 5-HT varicosities. (K) Piccolo staining shows that in several of the Tph puncta Piccolo is present. (L) Merged image. Scale bars: 20 µm in C and I, 10 µm in F and L.



These Tph puncta might therefore represent 5-HT varicosities, axonal sites where 5-HT release occurs, often without a post-synaptic specialization. Therefore, it seems that also in 5-HT neurons Piccolo is localized in release sites (Fig 1J-L).

Efficient knockdown of Piccolo in hippocampal neurons

Next, we investigated whether knockdown of Piccolo affected trafficking of mCherry-SERT. In hippocampal neurons, mCherry-SERT is transported in secretory vesicles which move through the axon with no preference for direction of movement (Chapter 3, this thesis). To achieve efficient knockdown of Piccolo,

we designed six short hairpin RNA (shRNA) nucleotides, targeted towards the N-terminus of Piccolo. These short hairpins were inserted in a vector containing also an internal ribosomal entry site (IRES) and EGFP, as a control for transfected neurons. We tested the shRNAs for their Piccolo knockdown efficiency. To this end we transfected the shRNA constructs in hippocampal neurons, and four days later fixed and processed the neurons for immunocytochemistry. Several constructs tested did not show any knockdown of Piccolo. We decided to use shRNA#5 as a negative control. Analysis of Piccolo expression in MAP2 negative axons of neurons transfected with shRNA#5 was not reduced compared to untransfected cells (Fig. 2A-D). The axon had a punctate labeling of Piccolo, which was indistinguishable from untransfected neurons. On the other hand, transfection of shRNA#6 caused a reduction in levels of Piccolo in axons (Fig. 2E-H, 2I-L).

Quantification of the number of Piccolo puncta per µm neurite revealed that in control condition there was on average one Piccolo puncta per two µm neurite, whereas in shRNA#6 mediated knockdown there was on average one Piccolo puncta per ten µm neurite (control 0.49 ± 0.11 Piccolo puncta per µm axon; knockdown 0.11 ± 0.02 Piccolo puncta per µm axon, $p=0.002$) (Fig. 2M). Thus, shRNA#6 resulted in an efficient, approximately 80%, reduction in number of Piccolo puncta. Therefore, we used this construct to test the effect of Piccolo knockdown on mCherry-SERT localization and trafficking.

Knockdown of Piccolo does not affect SERT trafficking

We used shRNA#6 to investigate the effect of Piccolo knockdown on mCherry-SERT localization and trafficking. SERT tagged with mCherry is transported in secretory vesicles, which move in anterograde and retrograde direction, with no preference for direction of movement (chapter 3 of this thesis). We transfected mCherry-SERT and shRNA#6 in hippocampal neurons, and used EGFP as a control. One minute movies of mCherry-SERT trafficking were made, and the movies were analyzed using the custom written Fluotrack software program (Broeke et al., 2008). The velocity of moving puncta was quantified, as well as the direction of movement. For direction we discerned anterograde direction, retrograde direction and bidirectional, vesicles which changed direction during imaging. Both in neurons expressing shRNA#6 and EGFP, we observed several puncta of mCherry-SERT, which displayed rapid movements in both the anterograde and retrograde direction. Additionally, also stationary vesicles were observed (Fig. 3A-D). We quantified the average velocity of mCherry-SERT moving puncta in control neurons expressing EGFP, and neurons expressing shRNA#6. A velocity histogram shows the distribution of the average velocity and direction of movement between control and knockdown (Fig. 3E).

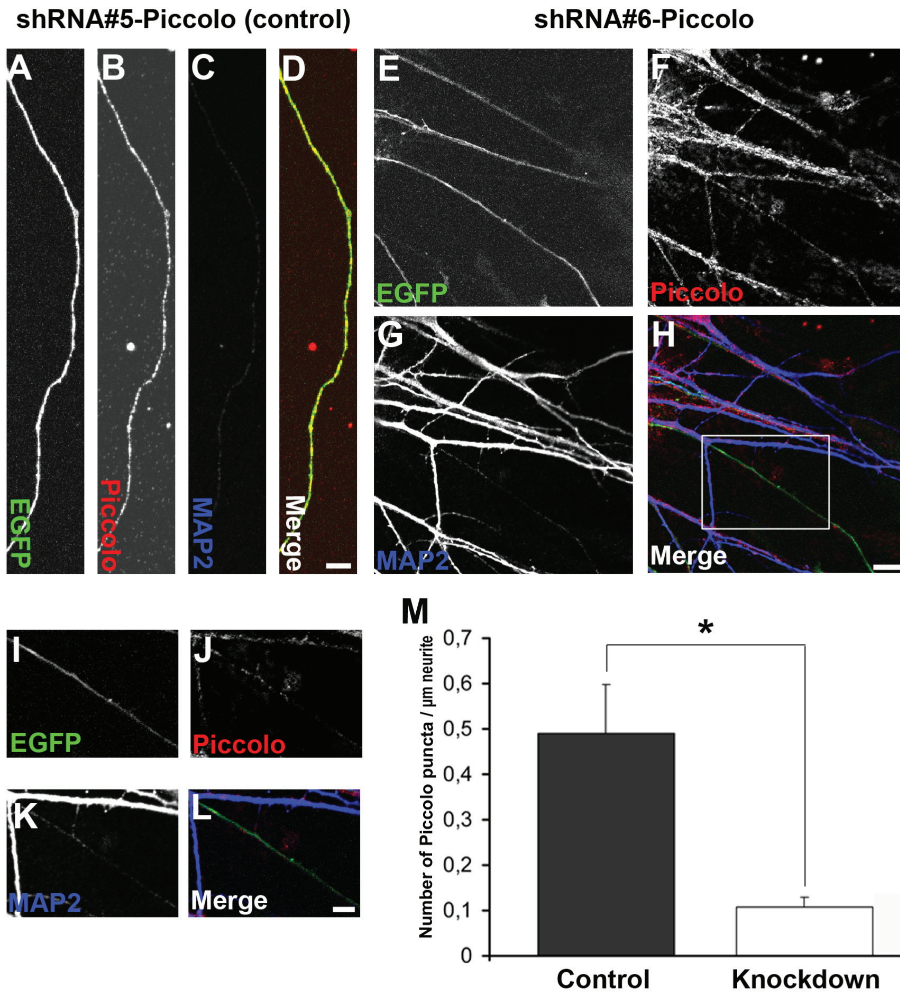


Figure 2. Efficient shRNA-mediated knockdown of Piccolo
Several shRNA constructs against Piccolo were tested for their efficacy to reduce Piccolo expression in cultured hippocampal neurons. ShRNA#5 did not result in Piccolo knockdown. (A) EGFP expression, showing that the shRNA construct is expressed. (B) Piccolo staining. (C) Neurite is MAP2 negative, showing that this is an axon. (D) Merged image. (E) EGFP expression in a neuron transfected with shRNA#6-EGFP. (F) Piccolo staining. (G) MAP2 staining. (H) Merged image, showing that shRNA#6 results in efficient knockdown of Piccolo in the axon. (I-L) Blow up of the white box in H, with EGFP, Piccolo staining, MAP2 staining and merged image respectively. (M) Quantification of the level of Piccolo knockdown shows that shRNA#6 resulted in a ~80% reduction in Piccolo puncta (control 0.49 ± 0.11 Piccolo puncta per µm axon (n=7); knockdown 0.11 ± 0.02 Piccolo puncta per µm axon (n=8)). Scale bars: 5 µm in D and L, 10 µm in H.

Trafficking of mCherry-SERT did not differ between control and knockdown (average velocity control 1.30 ± 0.042 µm/s (n=8 neurons); knockdown 1.36 ± 0.039 µm/s, (n=18 neurons) (Fig. 3F). Also, percentage of moving vesicles was not different between control and knockdown (control 46.69 ± 5.03%; knockdown 39.88 ± 3.39%) (Fig. 3G). Additionally, percentage of vesicles moving anterogradely, retrogradely or bidirectionally did not differ between control and knockdown (data not shown).

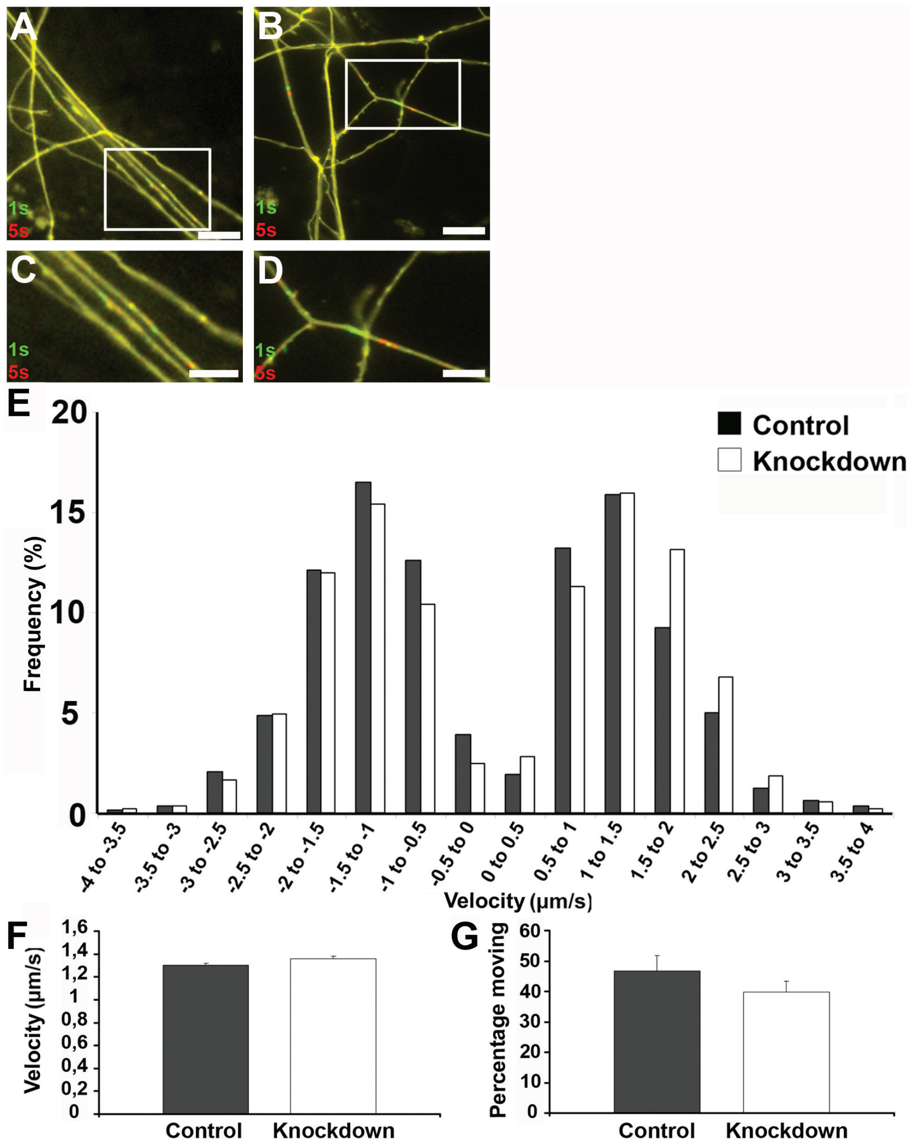


Figure 3 previous page. Piccolo knockdown does not affect SERT trafficking dynamics

(A) Neurons transfected with EGFP and mCherry-SERT. Images are taken 5 seconds apart. Several mCherry-SERT puncta are observed which are either stationary (yellow), or are moving anterogradely or retrogradely. (B) A neuron transfected with shRNA#6EGFP and mCherry-SERT. Images taken 5 seconds apart show that after Piccolo knockdown also several mCherry-SERT puncta are observed, some of which are stationary, and other which display anterograde or retrograde movements. (C) and (D) are blow ups of the white boxes in A and B, respectively. (E) Velocity histogram of mCherry-SERT puncta in control neurons and Piccolo knockdown neurons. Negative values are retrograde movements, positive values are anterograde movements. (F) Average velocity of mCherry-SERT puncta in control neurons and Piccolo knockdown neurons. (G) Percentage moving mCherry-SERT puncta in control neurons and Piccolo knockdown neurons. Scale bars: 10 µm in A and B, 5 µm in C and D.

Expression of C2A variant rs2522833 alters SERT distribution at the membrane in heterologous cells

Finally, we were interested whether the SNP in the C2A domain of Piccolo which was found to associate with major depression, affected the membrane localization of SERT. Therefore, we constructed the $C2A_{wt}$ domain of Piccolo in an IRES2-EGFP vector and a $C2A_{mut}$ domain with the rs2522833 SNP which results in a serine to alanine substitution at amino acid 4684. We transfected these constructs, together with mCherry-SERT, in heterologous Neuro2A cells, which do not express endogenous Piccolo. In Neuro2A cells transfected with mCherry-SERT together with Piccolo $C2A_{wt}$, SERT was localized predominantly at the cell membrane. The majority of cells displayed this SERT distribution, although also in some cells mCherry-SERT was homogenously distributed. This may be due to the level of expression, since in several cells which showed this mCherry-SERT distribution, also EGFP fluorescence intensity was increased compared to other cells (Fig. 4A-C). Zooming in on a few cells expressing EGFP and mCherry-SERT showed that SERT is localized at the membrane, as mCherry fluorescence appeared like a ring at the membrane. Moreover, mCherry-SERT also seemed to associate with intracellular organelles, as some mCherry-SERT appeared as puncta in the cytoplasm. (Fig. 4D-F). Expressing mCherry-SERT together with Piccolo $C2A_{mut}$ resulted in cells in which mCherry-SERT was localized at the membrane, but also some cells in which mCherry-SERT displayed a more uniform distribution in the cytoplasm which might again be due to the level of expression (Fig. 4G-I). A typical example of three cells expressing both constructs showed that in one cell mCherry-SERT was localized at the membrane, and in the other cells mCherry-SERT displayed a more uniform distribution in the cytoplasm (Fig. 4J-L). Strikingly, membrane bound mCherry-SERT in Piccolo $C2A_{mut}$ expressing cells displayed a more patchy

distribution than in Piccolo C2A$_{wt}$ expressing cells (Fig. 4M-O). A line scan profile of a typical cell co-expressing mCherry-SERT with Piccolo C2A$_{wt}$ showed that the highest level of fluorescence was at the membrane (Fig. 4P). In contrast, a line scan profile of a cell co-expressing mCherry-SERT with Piccolo C2A$_{mut}$ showed that fluoresence levels in the cytoplasm were comparable with fluorescence levels at the membrane (Fig. 4Q).

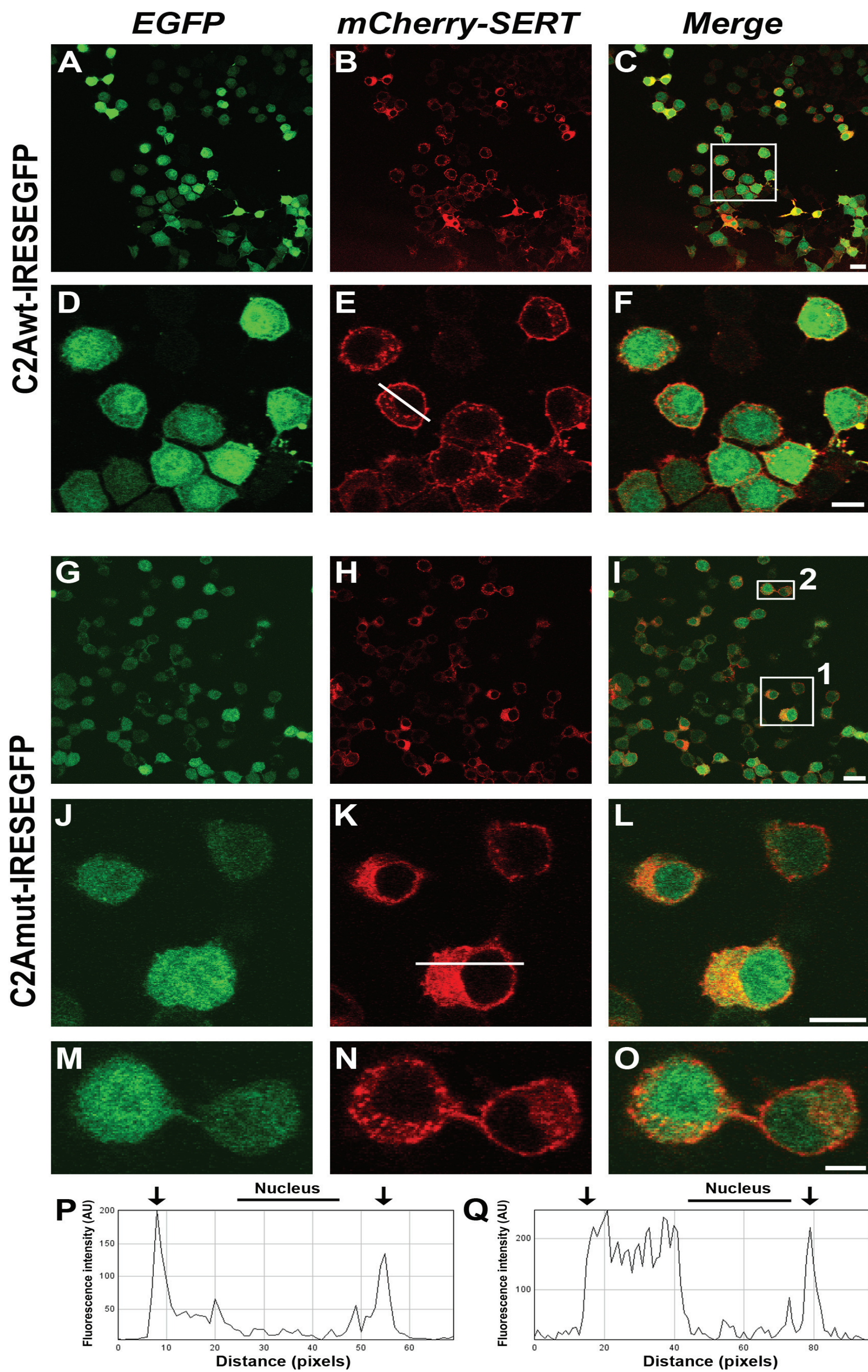
Thus, it appeared that co-expression of the Piccolo C2A$_{mut}$ with the rs2522833 SNP resulted in an altered distribution of mCherry-SERT at the membrane. These data suggest that Piccolo is not involved in the trafficking of SERT, but might be involved in regulating SERT membrane levels.

Discussion

Piccolo is a large protein, which is part of the active zone in the presynaptic terminal. There, Piccolo probably has a scaffolding role, but Piccolo also contains a PDZ domain and two C2 domains. Piccolo can be alternatively spliced, resulting in absence or presence of the C2B domain (Wang et al., 1999). Additionally, in the C2A domain a nine-residue sequence has been found which is subject of alternative splicing, resulting in variants of the C2A domain with different calcium affinities (Garcia et al., 2004). In this study we focused exclusively on the long C2A variant. The gene region in which Piccolo is localized has been linked to autism (Nabi et al., 2003). The SNP rs2522833 is localized in the C2A domain (Sullivan et al., 2008). The C2A domain has a low calcium affinity, suggesting an involvement in presynaptic plasticity processes (Gerber et al., 2001). It is currently unknown whether the SNP affects PIP_2 or Ca^{2+} binding to the C2A domain.

Figure 4 next page. Expression of Piccolo C2A$_{wt}$ and C2A$_{mut}$ with mCherry-SERT in heterologous cells

(A-C) Expression of C2A$_{wt}$–IRES-EGFP and mCherry-SERT in Neuro2A cells. (D-F) Blow up of white box in C shows that in the majority of cells mCherry-SERT is localized at the membrane. In the cytoplasm, mCherry-SERT displays a punctate distribution, suggesting association with intracellular organelles. (G-I) Expression of C2A$_{mut}$-IRES-EGFP and mCherry-SERT in heterologous cells. (J-L) Blow up of white box #1 in I shows that in several Neuro2A cells expressing C2A$_{mut}$-IRES-EGFP, mCherry-SERT seems to have a more uniform cytoplasmic distribution. (M-O) Blow up of white box #2 in I shows that in cells in which mCherry-SERT is localized at the membrane, mCherry-SERT is not uniformly distributed at the membrane, but instead is distributed in patches along the membrane. (P,Q) Line scan profiles of a typical cell expressing mCherry-SERT with C2A$_{wt}$–IRES-EGFP or C2A$_{mut}$-IRES-EGFP (lines in figures E and K respectively). Arrows indicate cell membrane. Scale bars: 20 µm in C and I, 10 µm in Fand L and 5 µm in O.


EGFP
mCherry-SERT
Merge
C2Awt-IRESEGFP
C2Amut-IRESEGFP
A
B
C
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O
1
2
P
Q
Nucleus
Nucleus
Fluorescence intensity (AU)
Distance (pixels)
Distance (pixels)

The regulation of SERT membrane localization and internalization is important in determining efficacy of 5-HT transmission. Increases in membrane localization of SERT increase 5-HT reuptake and thereby reduce extracellular levels of 5-HT and binding of 5-HT to postsynaptic 5-HT receptors. In contrast, internalization of SERT results in increased extracellular levels of 5-HT and increased postsynaptic activation of 5-HT receptors.

Protein kinase C (PKC) reduces the expression level of SERT, presumably by altering the membrane localization of SERT (Qian et al., 1997). Indeed, application of phorbol esters results in increased internalization of SERT. In contrast, substrates of SERT, such as 5-HT, reduce PKC dependent SERT internalization, whereas SERT antagonists, such as antidepressants, block this 5-HT mediated reduction in SERT internalization (Ramamoorthy and Blakely, 1999). On the other hand, protein kinase G (PKG) activity increases the membrane localization of SERT (Zhu et al., 2004). Therefore, there is an extensive level of regulation of membrane expression of SERT. There is an indirect link between Piccolo and SERT; Syntaxin1A is known to interact with SERT, and to regulate SERT activity (Quick, 2003). The C2A domain of Synaptotagmin, which shares similarity with the C2A domain of Piccolo, interacts with Syntaxin1A (Shao et al., 1997). Therefore this makes it likely that also the C2A domain of Piccolo is able to interact with Syntaxin1A, and thus possibly with SERT.

We used heterologous Neuro2A cells, which do not express Piccolo endogenously, to study the effect of expression of both C2A variants on SERT localization. We showed that distribution of SERT at the membrane might be altered after co-expression with the mutant C2A variant. Previously it has been shown that DAT trafficking and internalization is not altered in heterologous cells upon Piccolo knockdown. However, upon treatment of cells with 3,4-methylenedioxy-N-methylamphetamine (MDMA), there is a significant increase in DAT internalization. Thus, Piccolo seems to regulate the MDMA dependent internalization of DAT. For SERT, it has been shown that application of antidepressants and the PKC activator β-phorbol 12-myristate 13-acetate (β-PMA) results in an increased internalization of SERT (Lau et al., 2008a; Lau et al., 2008b). In view of this, it would be interesting to challenge SERT with an antidepressant after expression of both C2A variants, to investigate whether this results in an altered distribution of SERT between the two C2A variants.

Several studies show that the expression level, membrane level, or transport activity of SERT might be affected in psychopathological processes. Levels of SERT expression in DRN are reduced in brains of depressed suicide victims (Arango et al., 2001). In contrast, long-term administration of antidepressants seemed to also reduce SERT surface expression (Hirano et al., 2005). Recent

studies showed that a SNP in SERT which associated with obsessive compulsive disorder resulted in increased transport activity of SERT (Kilic et al., 2003). Thus, further studies are required to investigate the relation between the SNP in Piccolo, SERT expression and activity, and major depression disorder.

EXPERIMENTAL PROCEDURES

Plasmids

Human SERT (hSERT) cDNA tagged with ECFP at the 5' end in the Clontech ECFP-C1 vector was a kind gift from Dr. H. Sitte. To clone a red fluorescent version of hSERT we replaced ECFP for the red fluorescent protein mCherry. To this end mCherry was digested out of the vector using AseI and BsrGI and cloned sticky in the ECFP-hSERT vector from which ECFP had been excised. For construction of pSuper-CMV-EGFP-shRNA-Piccolo, pSuper was digested using EcoRI and PstI and CMV-EGFP was inserted after isolation of this fragment from pEGFP-C3 (Clontech) using BsrGI and NsiI. The shRNA against Piccolo was constructed using the following primers: forward 5'-GATCCCCACCCCACAAATATAAAGCTTTCAAGAGAAGCTTTATATTTGTGG GGTTTTTTGGAAA-3' and reverse 5'-AGCTTTTCCAAAAAACCCCACAAA TATAAAGCTTCTCTTGAAAGCTTTATATTTGTGGGGTGGG-3'. These primers were annealed by heating to 100°C, cooling down to room temperature and inserted in pSuper-CMV-EGFP by digesting this vector with BglII and HindIII. For construction of $C2A_{wt}$, this fragment was isolated from a Yeast-2-Hybrid mouse cDNA bank using the following primers: forward 5'-GGCGTGGATCCCAAGCAGCTG-3' and reverse 5'-GTTTTGACTGGAGTGAGACTTG-3' and subsequently a PCR was performed using the following primers: forward 5'-AAGGATCCGCCTCTCACCCAATTACAGGA-3' and reverse 5'-TTTGCGGCCGCATGCTTTCAGTCTGTTCTTTCAG-3' to add BamHI and NotI sites. For $C2A_{mut}$ (S4684A), the C2A domain was isolated using two PCR reactions using the following primers: forward 5'-AAGGATCCGCCTCTCACCCAATTACAGGA-3' and reverse 5'-CGAGGAGTGTTGTCCAGATGAGCAGTACTAGATAAATCAATCAATAC-3' and forward 5'-GTATTGATTGATTTATCTAGTACTGCTCATCTGGACAACACTCCTCG -3' and reverse 5'- TTTGCGGCCGCATGCTTTCAGTCTGTTCTTTCAG-3'. These two PCR products were annealed and a PCR was performed on this product using primers: forward 5'-AAGGATCCGCCTCTCACCCAATTACAGGA-3' and reverse 5'-TTTGCGGCCGCATGCTTTCAGTCTGTTCTTTCAG-3' to add BamHI and NotI sites and $C2A_{wt}$ and $C2A_{mut}$ fragments were ligated in pGEX. To construct pCMV-C2A-IRES2EGFP, both fragments were isolated from pGEX by

PCR using the following primers: forward 5'-AAAGGATCCACCATGGCCTCTCACCCAATTACAGG-3'and reverse 5'-TTTGAATTCTCAGCTTTCAGTCTGTTCTTTCAG-3'to add BamHI and EcoRI sites and a Kozak and stop sequence. Fragments were inserted in pIRES2EGFP by ligating this vector with BglII and EcoRI.

Cell culture and transfections

Hippocampi from embryonic day 18 C57/Bl6 mouse embryos were dissected free of meninges and collected in Hanks' Balanced Salt Solution (HBSS; Invitrogen) supplemented with 7 mM HEPES (Invitrogen). Hippocampi were digested with 0.25% trypsin (Invitrogen) in HBSS supplemented with 7 mM HEPES for 15 minutes at 37°C. After digestion tissue was washed three times with HBSS supplemented with 7 mM Hepes and subsequently triturated with a fire-polished glass pipette. Dissociated neurons were plated at a density of 25.000 cell/well on rat glial cells on 18 mm glass coverslips (Menzel Glaser, Braunschweig, Germany) in 12 well plates. Cells were cultured in neurobasal medium (Invitrogen) supplemented with B27 supplement (Invitrogen), 18 mM HEPES, 0.5 mM Glutamax (Invitrogen) and penicillin/streptomycin (Invitrogen). Once a week half of the medium was replaced. The hippocampal cultures were transfected using Lipofectamine 2000 (Invitrogen). Before transfection, half of the neurobasal medium was collected from each well and replaced with new neurobasal medium. For one well, 1 µl of Lipofectamine was added to 25 µl of serum free glutamax (Invitrogen) medium and incubated for 5 minutes. Subsequently, from each construct 1 µg of DNA was added to 25 µl of serum free glutamax and immediately mixed with the 25 µl of serum free glutamax containing Lipofectamine. This mixture was incubated for 30 minutes before adding to the cells. After 4-6 hours of incubation on the cells, half of the neurobasal medium was replaced again with the earlier collected neurobasal medium. Cells were routinely transfected at DIV2-7 and imaged at DIV10-14.

Immunocytochemistry

For immunocytochemistry, neuronal cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) (pH 7.4) for 20 minutes at room temperature. Cells were washed in PBS and blocked in PBS containing 4% fetal calf serum and 0.2% Triton X-100 for 20 minutes at room temperature. Cells were incubated overnight with primary antibodies at 4°C in PBS containing 0.2% Triton X-100. As primary antibodies monoclonal MAP2 (Chemicon), and polyclonal Piccolo (Synaptic Systems) and Synapsin (E028) were used. After incubation in primary antibodies, cells were washed three times using PBS. Subsequently, cells were incubated in Alexa Fluor-conjugated secondary antibodies (Invitrogen) in PBS

for one hour at room temperature. All antibodies were used in a 1:1000 dilution. Finally, cells were washed three times in PBS and mounted using Dabco-Mowiol.

For immunohistochemistry on mouse brain slices, mice were anaesthetized and transcardially perfused with 4% paraformaldehyde (PFA) in PBS (pH 7.4). Brains were post fixed in PFA for one day, and subsequently cryoprotected in increasing concentrations of sucrose. Thirty µm coronal adult brain slices were made using a Cryostat (Leica). Midbrain slices containing the dorsal and medial raphe nucleus were blocked for two hours in PBS containing 10% normal goat serum and 0.5% Triton X-100. Subsequently, the slices were incubated overnight in primary antibodies in PBS containing 0.5% Triton X-100 at 4ºC. Brain slices were washed three times two hours in PBS and incubated in secondary antibodies in PBS overnight at 4ºC. After washing three times two hours, slices were mounted using Dabco-Mowiol. As primary antibodies monoclonal Tph (Sigma, recognizing both Tph2 and Tph2) and polyclonal Piccolo (Synaptic systems) were used in a 1:1000 dilution. Alexa Fluor-conjugated secondary antibodies (Invitrogen) were used in a 1:1000 dilution. Cells and slices were examined on a Zeiss LSM 510 confocal laser scanning microscope (Zeiss, BV The Netherlands).

Live cell imaging

Cells were imaged in a chamber perfused with Tyrode's solution (119 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 30 mM glucose, 25 mM HEPES, pH 7.4) on an Axiovert II Microscope (Zeiss, Oberkochen, Germany) equipped with a Coolsnap HQ camera (Photometrics, Tucson, Arizona, USA) and a Polychrome IV illumination unit (TILL photonics, Grafelfing, Germany). Images were acquired in MetaMorph 6.2 software (Universal Imaging, Downingtown, Philadelphia, USA) using a 100x oil objective.

Image analysis

To analyze the trafficking of mCherry-hSERT, image stacks were converted to .avi movie files using ImageJ software. Movies were analyzed using the Fluotrack software program (Broeke et al., 2008) to quantify the average number of moving puncta, the average velocity of moving puncta, the average pausing time of moving puncta and the direction of moving puncta.

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Chapter

6

Deletion of Munc18-1 in 5-HT neurons results in rapid degeneration of the 5-HT system and early postnatal lethality

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Submitted

Abstract

The serotonin (5-HT) system densely innervates many brain areas and is important for proper brain development. To specifically ablate the 5-HT system we generated mutant mice carrying a floxed Munc18-1 gene and Cre recombinase driven by the 5-HT-specific serotonin reuptake transporter (SERT) promoter. Mutant mice were smaller than their control littermates and the majority died within a few days after birth. Immunohistochemical analysis of brains of these mice showed that initially 5-HT neurons are formed and the cortex is innervated with 5-HT projections. From embryonic day (E) 16 onwards, however, 5-HT neurons started to degenerate and at postnatal day (P) 2 hardly any 5-HT projections were present in the cortex. The 5-HT system of mice heterozygous for the floxed Munc18-1 allele was indistinguishable from control mice. Behavioural analysis of these heterozygous mice showed that there is no difference in basal behaviour, conditioning and anxiety-related behaviour compared to control mice. These data show that deletion of Munc18-1 in 5-HT neurons results in rapid degeneration of the 5-HT system and suggests that the 5-HT system is important for postnatal survival.

Introduction

The 5-HT system consists of clusters of cell bodies in the midbrain raphe nuclei, with the largest clusters in the median raphe nucleus (MRN) and the dorsal raphe nucleus (DRN). Several brain areas receive dense 5-HT innervation and 5-HT is released both synaptically and as volume transmission (Ridet et al., 1993; Bunin and Wightman, 1998). Due to this and to the several 5-HT receptor subtypes which are present in the brain, 5-HT has many roles and influences many processes in the brain (Jacobs and Azmitia, 1992).

Neurogenesis of 5-HT neurons in the mouse brain occurs in the ventral rhombencephalon around E10 (Wallace and Lauder, 1983). One day later, 5-HT neurons begin to synthesize and secrete 5-HT and start growing out axons. Around birth, target areas such as the forebrain and the hippocampus are densely innervated with 5-HT projections. Only after birth, the maturation of the 5-HT network is completed.

Several studies have addressed the role of 5-HT on the development of the 5-HT system and brain development. In a conditional Lmx1b knockout (KO) mouse, almost all 5-HT neurons fail to survive, resulting in a significant decrease in brain tissue 5-HT levels (Zhao et al., 2006). However, these mice do not show

an overt phenotype and survive to adulthood (Zhao et al., 2006). In contrast, it was shown that maternal 5-HT is required for embryonic development (Cote et al., 2007). Furthermore, in tryptophan hydroxylase 2 (Tph2) KO mice 5-HT neurons are completely devoid of 5-HT, but the morphology and neurite distribution of the 5-HT system is not affected, and these mice do not show any behavioural phenotype (Gutknecht et al., 2008). Neonatal depletion of 5-HT by the neurotoxin 5,7-Dihydroxytryptamine results in rather subtle changes in behavioural response and brain development (Hohmann et al., 2007; Bennett-Clarke et al., 1995).

In this study we silenced the 5-HT system by conditional deletion of Munc18-1 in 5-HT neurons. Neurons that lack the presynaptic protein Munc18-1 have a complete absence of neurotransmitter secretion and these mice are born paralyzed and die immediately after birth (Verhage et al., 2000). In these mice, initially synapses are formed and the assembly of the brain is normal. However, in later stages of brain development there is massive neuronal cell death and brain degeneration (Verhage et al., 2000).

In SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ mice 5-HT neurons were initially generated and 5-HT projections innervated the midbrain and cortex, later followed by degeneration and loss of 5-HT projections in the cortex. The majority of these mice died within a few days after birth. These data suggest that the 5-HT system contributes importantly to postnatal brain development.

Results

Deletion of Munc18-1 in SERT expressing neurons results in postnatal lethality

We have shown previously that Munc18-1 mutant mice lack regulated secretion and have a lethal phenotype (Verhage et al., 2000). Therefore, we have generated Munc18-1$^{lox/lox}$ mutant mice in order to conditionally delete Munc18-1. Crossing these mice with a L7-Cre line, with Cre expressed in Purkinje neurons in the cerebellum, resulted in mice which developed severe ataxia, suggesting a cerebellar phenotype (Heeroma et al., 2004). Here we crossed Munc18-1$^{lox/lox}$ mice with SERT-Cre mice, which express Cre in SERT expressing neurons. These are the 5-HT neurons in the raphe nuclei, but also some hippocampal neurons and thalamocortical neurons which express SERT transiently during development (Zhuang et al., 2005; Narboux-Neme et al., 2008). Crossing SERT-Cre mice with Munc18-1$^{lox/lox}$ mice results in mice in which Munc18-1 is specifically removed in SERT expressing neurons (Fig. 1A). We crossed SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/wt}$ mice with SERT-Cre$^{wt/wt}$ Munc18-1$^{lox/wt}$ mice which should result in 12.5% of offspring which have SERT-Cre$^{cre/wt}$ and Munc18-1$^{lox/lox}$

genotypes. Genotyping mice sacrificed at E16 or E18 revealed that there were more SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ and SERT-Cre$^{wt/wt}$ Munc18-1$^{lox/lox}$ mice and less SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/wt}$ mice then expected ($p= 0.007$) (Fig. 1B). Genotyping 101 mice three weeks after birth revealed that genotype frequencies were not distributed at Mendelian ratio ($p= 0.027$). Only three SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ mice were found, whereas based on Mendelian ratio the expected number of SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ mice should be 13, giving a 77% mortality rate in SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ mice (Fig. 1C). Also, SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ mice were smaller compared to littermates (data not shown). These three remaining SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ mice all died in the fourth postnatal week. This shows that Munc18-1 deletion in SERT expressing neurons results in postnatal lethality.

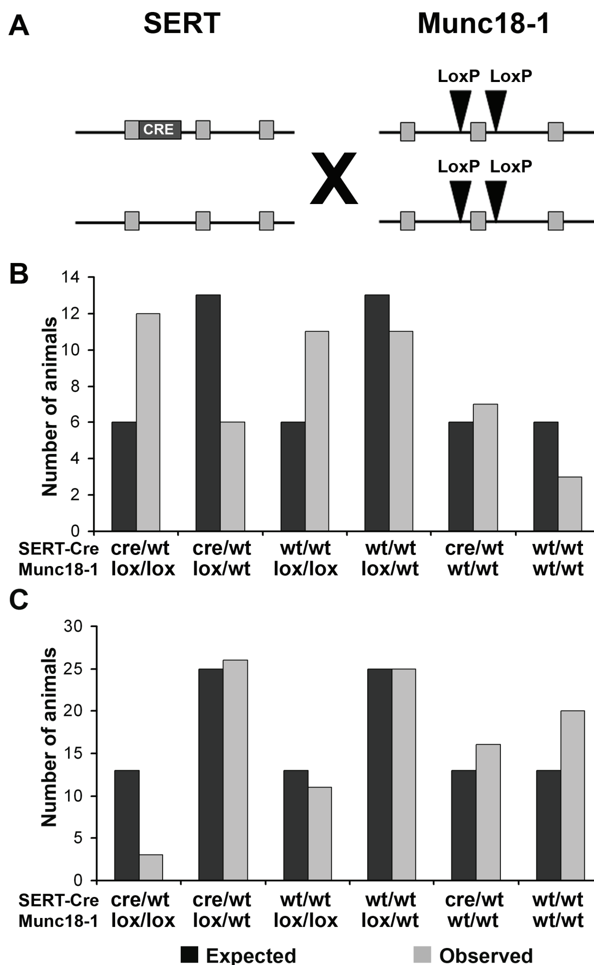

Figure 1. Postnatal lethality in SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ mice
(A) Overview of breeding approach. Crossing SERT-Cre with Munc18-1$^{lox/lox}$ mice results in deletion of Munc18-1 only and specifically in SERT expressing neurons, whereas all other neurons still express Munc18-1.
(B) Genotypes of mice sacrificed at E16 or E18 differed from the expected Mendelian ratio. (C) Genotyping mice after weaning at 3 weeks of age revealed that only few SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ mice survived up to three weeks.

5-HT neurons are initially generated but then quickly degenerate

To assess the effect of Munc18-1 deletion in 5-HT neurons on the development of the 5-HT system, we made coronal sections of paraformaldehyde (PFA) fixed brains at different developmental stadia and performed immunohistochemistry for 5-HT. As developmental time points we chose E16, E18 and P2. Expression of SERT, and thus of Cre recombinase, starts at E11 and the earliest recombination observed in the DRN in SERT-Cre mice was at E12.5 (Narboux-Neme et al., 2008). Since it will take some days before all remaining Munc18-1 mRNA and protein is degraded, we chose E16 as the first time point. We first focused on the 5-HT cell bodies in the DRN. Immunohistochemistry for 5-HT on brain slices from E16 SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ and control mice showed that in the DRN 5-HT neurons are present and these showed the characteristic DRN topology (Fig. 2A,B). The number of 5-HT neurons did not differ from control (control 312 ± 17.67, SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ 305 ± 25.43, Fig. 2G,H,O). However, morphological analysis of the neurons showed that these were already degenerating, as assessed by the round morphology and reduced number of primary neurites. At E18, there was a ~80% decrease in the number of 5-HT neurons in SERT-Cre$^{cre/wt}$ M18$^{lox/lox}$ mice compared to control (control 358 ± 10.82, SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ 59 ± 4.84, Fig. 2C,D,O). At P2 there were only few 5-HT neurons left in the DRN (control 364 ± 19.68, SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ 25 ± 2.89, Fig. 2E,F,O). At E18 and P2 the remaining 5-HT neurons in SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ brain sections displayed an altered morphology compared to control 5-HT neurons (Fig. 2I,J,K,L). 5-HT neurons in control brains had a characteristic fusiform or ovoid shape and grew out several primary neurites (Fig. 2M). In contrast, the mutant 5-HT neurons had a rounded morphology and the majority did not contain primary neurites, indicative of degenerating neurons (Fig. 2N). This showed that between E16 and P2 there is a massive degeneration of 5-HT neurons in the DRN.

5-HT projections innervate the DRN and cortex but are degenerated at P2

Next, we focused on the innervation of the midbrain and cortex with 5-HT projections. Midbrain and cortex are the first regions which are densely innervated with 5-HT projections during development. We quantified 5-HT neurite density first in DRN midbrain sections at E16, E18 and P2. At E16 the sections from both control and SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ mice contained several 5-HT neurites and there was no significant difference in 5-HT innervation density (1.31 ± 0.51% and 0.63 ± 0.24% respectively, Fig. 3A,B). At E18, in control sections the 5-HT innervation density was slightly increased compared to E16 (Fig. 3C). However, in SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ sections the 5-HT innervation density was decreased compared to control (control 2.32 ± 0.86%, SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ 0.31 ± 0.27%, Fig. 3D).

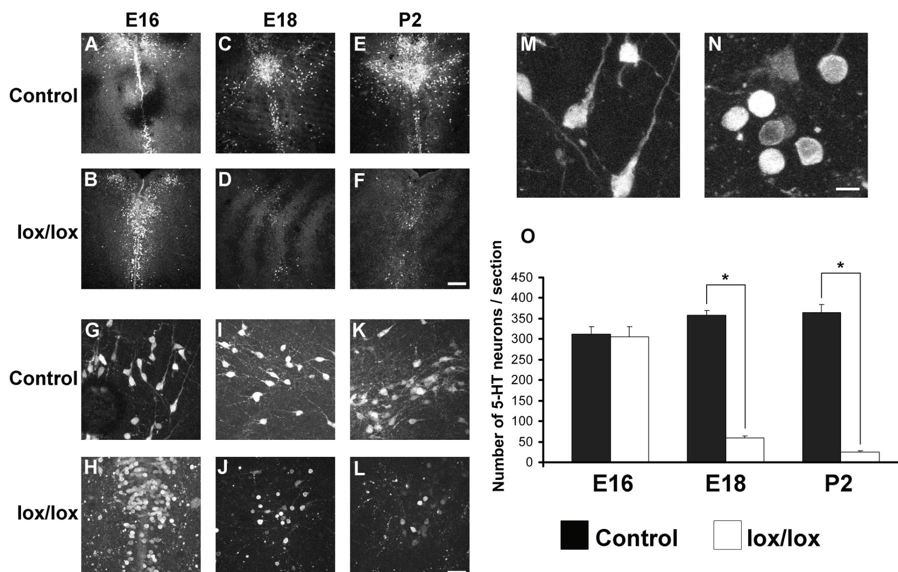

Figure 2. Rapid degeneration of 5-HT neurons in SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ mice

We compared the number of 5-HT cell bodies in the DRN in control and SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ mice at E16, E18 and P2. (A-F) At E16, in both control mice (A) and SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ (lox/lox) mice (B) the 5-HT cell bodies are distributed in the DRN topology. At E18 and P2, however, in midbrain sections from SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ brains (D, F respectively) only few 5-HT neurons are present compared to control sections (C, E respectively). (G-L) Zooming in on 5-HT cell bodies shows that in control sections at E16 (G), E18 (I) and P2 (K) the cell bodies grow out several neurites and have a fusiform or ovoid morphology. However, remaining 5-HT cell bodies in SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ sections at E16 (H), E18 (J) and P2 (L) have a round morphology with hardly any neurites. (M,N) Blow up of some 5-HT cell bodies in control and SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ mice sections shows the differences in 5-HT cell body morphology. (O) Analysis of the number of 5-HT cell bodies in the sections showed that there is no difference at E16, but at E18 and P2 there is a decrease of 70% to 80% in SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ sections. Scale bars: 200 μm in F, 50 μm in L and 20 μm in N. Data shown are mean ± standard error of the mean (SEM). * $p < 0.05$.

At P2 only few 5-HT neurites are left in SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ sections (control 1.6 ± 0.41%, SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ 0.33 ± 0.14%, Fig. 3E,F). Next, we focused on the 5-HT innervation density in the cortex. This revealed that already at E16 there is a reduction in 5-HT innervation density in SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ sections (control 3.27 ± 1.14%, SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ 0.65 ± 0.19%, Fig. 3G,H). At E18 and P2 the reduction in 5-HT innervation density is augmented, with only very few 5-HT neurites left at P2 in the cortex (E18 control 1.73 ± 0.45%, SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ 0.36 ± 0.18%, P2 control

1.78 ± 0.52%, SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ 0.19 ± 0.04%, Fig. 3I,J,K,L). Quantification showed that in the midbrain sections the 5-HT innervation density was reduced to ~20% and ~10% at E18 and P2 respectively (Fig. 3M). In the cortex, the 5-HT innervation density was reduced to ~20%, ~10% and ~5% at E16, E18 and P2 respectively (Fig. 3N).

In summary, deletion of Munc18-1 in 5-HT neurons resulted in rapid degeneration of 5-HT cell bodies and the absence of 5-HT projections in the postnatal brain.

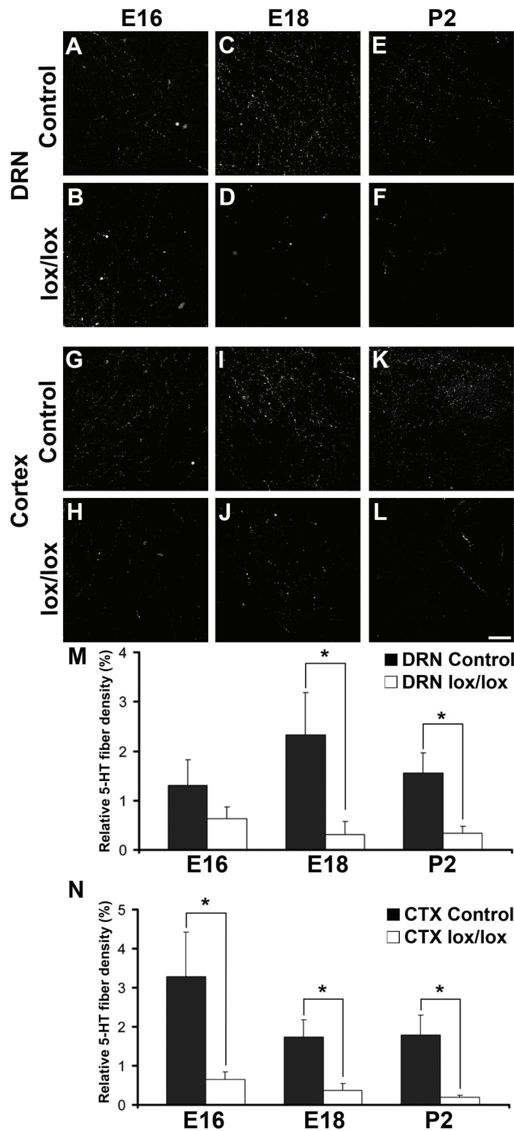

Figure 3. Degeneration of 5-HT neurites in DRN and cortex
(A-F) At E16 in the midbrain containing the DRN, there was no difference in 5-HT neurite density between control (A) and SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ (B) mice. In control sections at E18 (C) and P2 (E) several 5-HT neurites were present. However, in SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ sections at E18 (D) and P2 (F) only very few 5-HT neurites are left. (G-L) In the cortex already at E16 there is a reduction in 5-HT neurite density in sections from SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ (H) mice compared to control (G). At E18 (J) and P2 (L), in cortical sections from SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ mice only very few remaining 5-HT neurites are present, in contrast to E18 (I) and P2 (K) control cortical sections. (M) Quantification of the 5-HT neurite density in the DRN revealed that at E16 there is no difference, but at E18 and P2 5-HT neurite density is reduced to ~20% and ~10% respectively. (N) In cortical sections, at E16 the 5-HT neurite density is reduced to ~20% and at E18 and P2 the 5-HT neurite density is reduced to ~10% and ~5% respectively. Scale bar 50 µm in L. Data shown are mean ± SEM * $p < 0.05$.

Basal behaviour is not affected in SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/wt}$ mice

Finally, we were interested whether deletion of one allele of Munc18-1 in SERT expressing cells affected mouse behaviour. Previously, we have shown that Munc18-1 expression levels regulate the readily releasable pool (RRP) size. In Munc18-1 heterozygous mice there is a faster synaptic rundown during episodes of high activity, a decreased RRP size and a decrease in docked vesicles at the synapse (Toonen et al., 2006). Therefore it is likely that deletion of one functional Munc18-1 allele in SERT expressing neurons affects the efficacy of 5-HT neurotransmission.

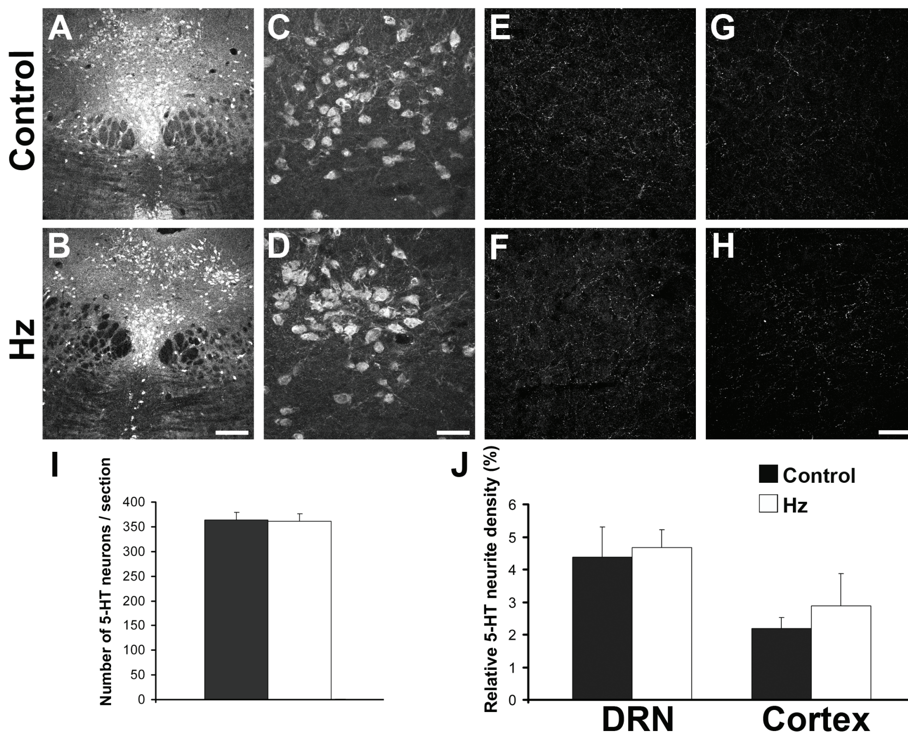

Figure 4. Deletion of one functional allele of Munc18-1 in SERT expressing neurons does not affect number of 5-HT cell bodies and 5-HT innervation density

(A-D) Midbrain sections containing the DRN from control (A) and hz (B) mice shows that 5-HT neurons are distributed in the characteristic DRN topology. Zooming in on 5-HT cell bodies show that both in control (C) and hz sections (D) these neurons have a fusiform or ovoid morphology and grow out several neurites. (E-H) 5-HT neurite density is not different between control and hz mice in the midbrain containing the DRN (E and F respectively) or in cortical sections from control and hz mice (G and H respectively). (I) Number of 5-HT cell bodies per section does not differ between control and hz. (J) 5-HT neurite density in midbrain containing DRN and cortical sections is not different between control and hz. Scale bars: 200 µm in B, 50 µm in D and H.

First, we investigated whether deletion of one allele of Munc18-1 in SERT expressing neurons affected the number of 5-HT cell bodies in the DRN, or the 5-HT fiber density in the DRN and cortex. Analysis of midbrain sections from control and SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/wt}$ (hz) mice showed that 5-HT neurons are distributed in the characteristic DRN topology, and control and hz are indistinguishable (Fig. 4A,B). 5-HT cell bodies in both control and hz had a fusiform or ovoid shape (Fig. 4C,D). Quantification of the number of 5-HT cell bodies in the DRN revealed no difference between control and hz (control 364 ± 14.78, hz 361 ± 15.01, Fig. 4I). Likewise, the 5-HT neurite density in the midbrain and cortex were similar (midbrain control 4.38 ± 0.93%, hz 4.67 ± 0.55%; cortex control 2.18 ± 0.35%, hz 2.87 ± 0.99%, Fig. 4E-H,J). Thus, deletion of one allele of Munc18-1 does not affect 5-HT neuronal survival, 5-HT neurite outgrowth or 5-HT projection density.

We tested several behavioural characteristics of hz mice by monitoring them for six days in home cages equipped with a video camera on top to visualize and analyze mouse behaviour. We analyzed the behaviour of eleven hz mice and compared several behavioural parameters with eleven control mice. First, we analyzed whether there was a difference in total distance moved in the open space and time spent under the shelter. Control and hz mice moved a similar total distance in the open space (control dark phase 2231.78 ± 139.39 cm/hour, light phase 483.91 ± 32.30 cm/hour; hz dark phase 2150.61 ± 112.70 cm/hour, light phase 433.69 ± 26.15 cm/hour; Fig. 5A,B), spent a similar proportion of time in a sheltered compartment (control dark phase 58.99 ± 2.03%, light phase 86.37 ± 2.36%; hz dark phase 60.04 ± 1.11%, light phase 90.38 ± 0.45%; Fig. 5C) and spent a similar time drinking (control dark phase 33.92 ± 1.99 s/hour, light phase 7.50 ± 0.52 s/hour; hz dark phase 41.06 ± 4.72 s/hour, light phase 8.06 ± 0.79 s/hour; Fig. 5D). Also, time spent in the feeder was indistinguishable between control and hz (control dark phase 476.77 ± 15.17 s/hour, light phase 160.55 ± 6.58 s/hour; hz dark phase 497.73 ± 18.41 s/ hour, light phase 165.33 ± 6.99 s/hour; Fig. 5E). Thus, basal behavioural parameters were indistinguishable between control and hz.

Conditioning and anxiety related behaviour is not affected in SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/wt}$ mice

To investigate whether hz mice display altered conditioning behaviour, a free operant conditioning task was performed, where a jump on the shelter resulted in the drop of a sucrose pellet. The number of directed short movement bouts (SMBs) increased during day four and five compared to day 1-3, but this conditioning behaviour did not differ between control and hz.

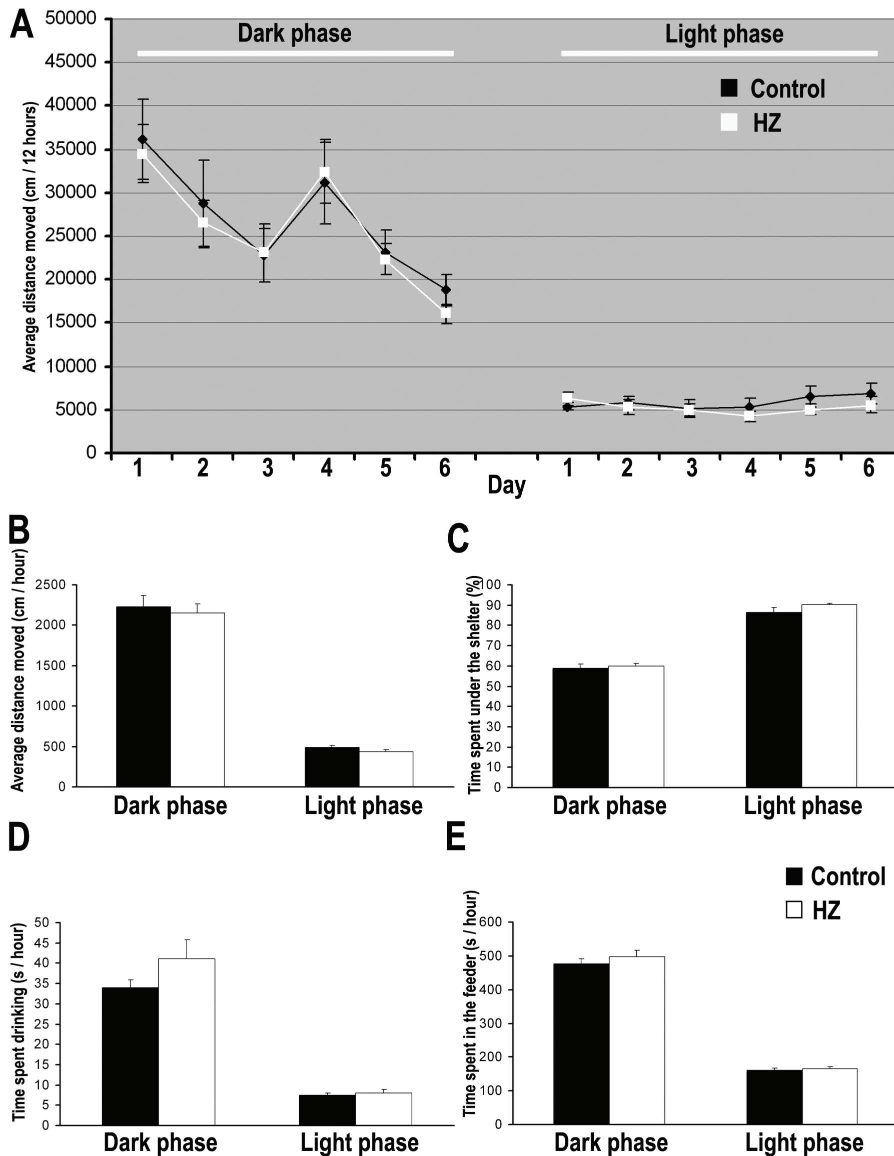

Figure 5. Hz mice do not display altered basal behaviour
(A) Six day plot of average distance moved (cm / 12 hours) during dark and light phase. Note the increase in distance moved during the dark phase at day 4 due to the free operant conditioning test. (B) No difference in average distance moved (cm/hour) between control and hz mice during the dark and light phase. (C) Proportion of time spent under the shelter does not differ between control and hz mice. (D) There is no difference in time spent drinking (s/hour) between control and hz mice. (E) Also, there is no difference in time spent in the feeder (s/hour). Data shown are mean ± SEM.

(day 1-3 control 17.07 ± 2.12, hz 16.33 ± 2.17; day 4-5 control 45.3 ± 5.27, hz 46.32 ± 5.78; Fig. 6A). Number of pellets dropped during pre-training phase (session 1-10, day four) did not differ between control and hz mice (control 4.88 ± 0.43, hz 5.99 ± 0.45; Fig. 6B). Also during the sessions at day five (session 11-12), there was no difference in the number of pellets dropped (control 20.36 ± 2.81, hz 19.95 ± 2.34; Fig. 6B).

To assess whether hz mice displayed altered anxiety related behaviour, a one trial anxiety task was performed at day six. To this end a lightspot was created at the feeder place, and the duration of time the animal spent in the lightspot and the feeder was recorded and compared with the time the animal spent at the feeder without a lightspot on. At day five (baseline) and day six (test) there was no difference between control and hz, but both groups spent less time in the lightspot at day six (day five control 1798.72 ± 208.1 s, hz 2108.01 ± 284.63 s; day six control 668.76 ± 61.28 s, hz 682.19 ± 83.98 s; Fig. 6C). Next, we investigated whether the frequency of entering the feeder zone at day five (baseline) and day six (test) was different. Frequency of entering the feeder zone did not differ between control and hz mice, but both groups displayed a decreased frequency during the lightspot test during day six (day five control 16.64 ± 2.51, hz 23.09 ± 3.51; day six control 7.15 ± 1.40, hz 8.12 ± 1.56; Fig. 6D). Also, time spent in the feeder zone during day five and day six did not differ between control and hz (day five control 528.15 ± 50.21 s, hz 621.91 ± 65.99 s; day six control 191.01 ± 30.36 s, hz 198.46 ± 33.53 s; Fig. 6E). Finally, analysis of latency to enter the lightspot zone after lightspot off revealed that this did not differ between control and hz (control 1567.71 ± 377.86 s; hz 1293.87 ± 329.17 s; Fig. 6F). Thus, mice in which one allele of Munc18-1 is deleted in SERT expressing neurons do not display altered basal, conditioning or anxiety-related behaviour.

Discussion

In this study, we showed that SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ mice display a postnatal lethality phenotype, accompanied by a rapid degeneration of the 5-HT system. Inactivation of one allele of Munc18-1 in SERT expressing neurons does not affect 5-HT cell number or 5-HT innervation density, and does not affect anxiety related behaviour or conditioning related behaviour.

Crossing Munc18-1$^{lox/lox}$ mice with SERT-Cre mice results in deletion of Munc18-1 from SERT expressing cells. It was shown previously that crossing SERT-Cre mice with loxSTOPlox-EYFP mice results in >99% of 5-HT neurons in the DRN which are EYFP positively labeled (Zhuang et al., 2005).

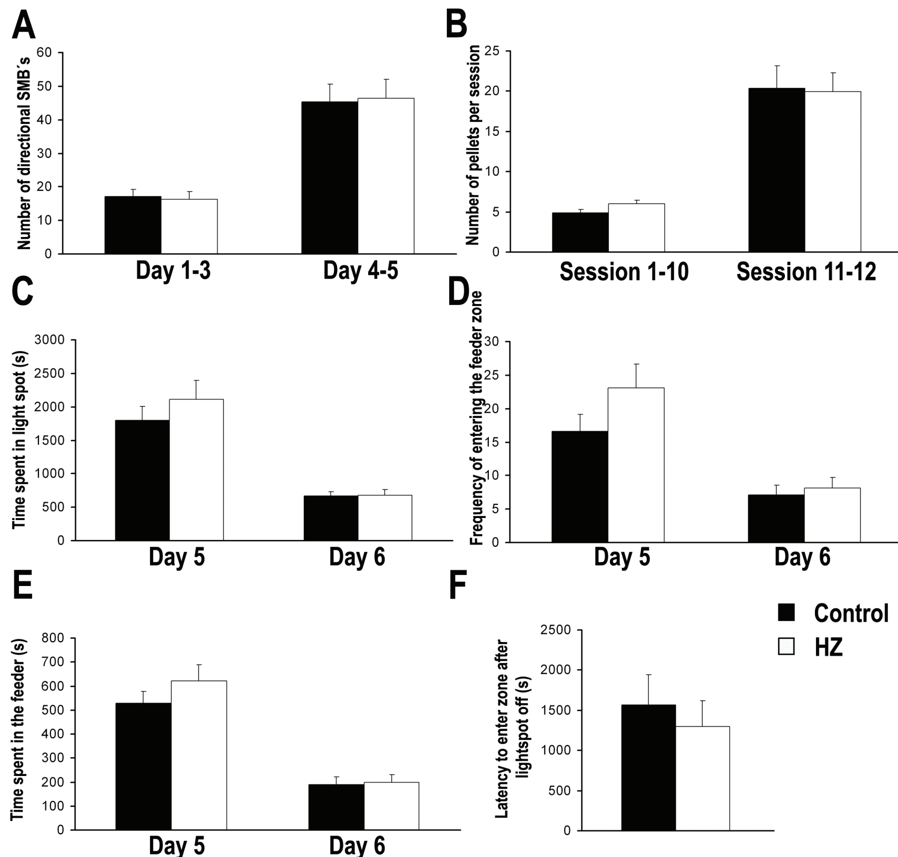

Figure 6. Hz behaviour on free operant conditioning test and one trial anxiety test does not differ from control

(A) The number of directional SMB's is increased at day 4 and 5 compared to day 1-3, but does not differ between control and hz. (B) Number of sucrose pellets acquired is increased at session 11 and 12 (day 5), compared to session 1-10 (day 4, pre-training day), but does not differ between control and hz. (C) Time spent in the lightspot (s) is decreased at day 6 (lightspot on) compared to day 5 (baseline, without lightspot on), but does not differ between control and hz. (D) Frequency of entering the feeder zone is decreased at day 6 compared to day 5, but there is no difference between control and hz mice. (E) During lightspot on on day 6 mice spent less time in the feeder (s) which does not differ between control and hz. (F) No difference in latency (s) to enter the lightspot zone after lightspot off. Data shown are mean ± SEM.

However, SERT is also transiently expressed in some other brain regions during development, such as the thalamocortical neurons (Lebrand et al., 1996). In SERT-Cre ROSA-loxstoplox-LacZ double positive mice, strong LacZ expression was not only observed in the DRN, but also in the thalamus, cingulate cortex and

in the CA3 region of the hippocampus (Zhuang et al., 2005; Narboux-Neme et al., 2008). Therefore, in SERT-Cre Munc18-1$^{lox/lox}$ mice also in some non-5-HT neurons Munc18-1 will be deleted. Genotyping sacrificed mice at E16 or E18 revealed that there were more SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ mice then expected, showing that these mice do not die during embryonic development. However, genotyping mice three weeks after birth showed that the majority of SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ mice had died, and the remaining SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ mice died within the fourth postnatal week. SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ mice were smaller compared to control mice which is in analogy with the VMAT2 KO mice which are hypoactive, smaller compared to control littermates, do not feed and die within a few days after birth (Fon et al., 1997; Wang et al., 1997).

Previously we showed that in Munc18-1 KO mice, the neuromuscular junction initially develops, but at later stages most of the motor neuronal cell bodies in the spinal cord degenerate (Heeroma et al., 2003). In analogy with this, in the absence of regulated secretion the hypothalamo-neurohypophysial system is initially normally formed, but in later stages of development degenerates (Korteweg et al., 2004). This is consistent with our observation that Munc18-1 is dispensable for initial generation and outgrowth of 5-HT neurons, but required for their survival.

Since also in some other neurons Munc18-1 is deleted, it is unclear whether the observed phenotypes can be attributed to the degeneration of the 5-HT system. In Tph2 KO mice, 5-HT neurons are completely devoid of 5-HT, yet there are no alterations in 5-HT neuron morphology and 5-HT neurite distribution (Gutknecht et al., 2008). Although these mice can survive into adulthood, approximately 50% does not survive the first 4 weeks and surviving mice have growth retardation (Alenina et al., 2009). Additionally, in a conditional Lmx1b KO mouse, 5-HT neurons are initially formed but almost all central 5-HT neurons fail to survive and brain 5-HT levels are reduced to ~10% (Zhao et al., 2006). Remarkably, these mice display normal locomotor behaviour and show no abnormalities in gross brain morphology. Recently, it was shown that these mice have severe apnea and have a ~20% mortality rate during the neonatal period, although the majority of these mice survive beyond P28 (Hodges et al., 2008; Hodges et al., 2009).

Apparently, even the absence of central 5-HT synthesis or an almost complete removal of 5-HT neurons does not result in a severe (postnatal) lethality phenotype. Thus, although several of these mice display high (postnatal) mortality, the phenotypes observed are not as severe as in the SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ mice.

On the other hand, mice which lack GAP43, display disrupted barrel cortex formation and thalamocortical connections fail to form. These mice display a

postnatal lethality phenotype, with 50% of homozygotes which die between P0 and P2, and >95% of homozygotes which die before P21 (Maier et al., 1999). Although these mutant mice display a reduced 5-HT innervation in the cortex and hippocampus, they have no significant difference in the number of 5-HT neurons, and other areas such as the piriform cortex and amygdale receive normal 5-HT innervation (Donovan et al., 2002). Thus, based on these data it is unclear whether the postnatal lethality phenotype observed in this study is attributable to a degeneration of thalamocortical or 5-HT neurons. It would be very interesting to delete the Munc18-1 gene specifically in 5-HT neurons using the Pet-Cre transgenic line, in which expression of Cre is restricted to 5-HT neurons (Scott et al., 2005). This approach could be used to investigate whether the phenotype we observed could indeed be attributed to degeneration of the 5-HT system rather than degeneration of thalamocortical neurons.

We have shown that mice in which one allele of Munc18-1 is deleted in SERT expressing neurons did not display altered basal, conditioning or anxiety-related behaviour. In these mice number of 5-HT neurons and 5-HT neurite density was indistinguishable from control mice. However, we have shown previously in neurons cultured from Munc18-1 heterozygous mice that there is a subtle secretion phenotype (Toonen et al., 2006). Thus, one could argue that possibly deletion of one functional allele of Munc18-1 affects 5-HT release and thus efficacy of 5-HT neurotransmission.

There could be a few explanations why reduced 5-HT neurotransmission in the heterozygous mice used in this study does not affect behaviour. First of all, the reduction in 5-HT neurotransmission might just be too subtle to affect mouse behaviour. In Munc18-1$^{+/-}$ neuronal cultures, there is a ~25% reduction in synaptic vesicle release (Toonen et al., 2006). In contrast, expression of tetanus toxin light chain in 5-HT neurons in vivo results in a robust (>90%) inhibition in 5-HT neurotransmission and these mice display decreased anxiety-related behaviour and enhanced associative learning (Kim et al., 2009). This suggests that a substantial reduction in 5-HT release is required to affect behaviour and that deletion of only one allele of Munc18-1 is not sufficient to achieve this reduction in 5-HT release.

Secondly, in Munc18-1 null mice synaptic vesicle release is completely abolished. However, at least in chromaffin cells lacking Munc18-1, there is still some large dense core vesicle (LDCV) release, although this is reduced to ~10% (Voets et al., 2001). In Munc18-1 heterozygous mice LDCV release is presumably largely unaffected. In the 5-HT system, the bulk amount of 5-HT is released from LDCVs, which contain up to ~17 times as much 5-HT molecules as a single synaptic vesicle (Bruns and Jahn, 1995). Thus, if 5-HT released from synaptic vesicles is more severely affected than 5-HT released from LDCVs, there is only a

minor reduction in release of 5-HT and thus in efficacy of 5-HT neurotransmission. Finally, (minor) reductions in extracellular 5-HT levels might be compensated for, such as by reduced SERT levels at the synaptic membrane and/or increased expression of postsynaptic 5-HT receptors, to guarantee proper levels of 5-HT neurotransmission.

In conclusion, deletion of one allele of Munc18-1 in 5-HT neurons does not affect mouse behaviour presumably due to the fact that there is a too subtle reduction in 5-HT neurotransmission in these mice. However, deletion of both alleles of Munc18-1 in SERT expressing neurons results in a rapid degeneration of the 5-HT system and postnatal lethality.

EXPERIMENTAL PROCEDURES

Laboratory animals

Generation and characterization of SERT-Cre mice has been described (Zhuang et al., 2005). Briefly, immediately upstream of the SERT translational start codon a cassette containing Cre and a FRT-flanked neomycin cassette was inserted. To generate conditional Munc18-1 mice (Heeroma et al., 2004), floxed Munc18-1 mice were generated by insertion of LoxP sites flanking the second exon using homologous recombination. To obtain SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ mice, SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/wt}$ mice were crossed with SERT-Cre$^{wt/wt}$ Munc18-1$^{lox/wt}$ mice. Pregnant females were sacrificed by cervical dislocation. Mouse embryos were obtained by caesarean section of pregnant females from timed matings. To investigate the effect of deletion of Munc18-1 in the 5-HT system on the development of the 5-HT system, SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/lox}$ mice were used. SERT-Cre$^{wt/wt}$ Munc18-1$^{lox/lox}$, SERT-Cre$^{wt/wt}$ Munc18-1$^{lox/wt}$, or SERT-Cre$^{cre/wt}$ Munc18-1$^{wt/wt}$ littermates were not different from SERT-Cre$^{wt/wt}$ Munc18-1$^{wt/wt}$ mice and were used as controls. SERT-Cre mice were genotyped as has been previously described (Zhuang et al., 2005). For genotyping of Munc18$^{lox/lox}$ mice the following primers were used: 5'-ttggtggtcgaatgggcaggtag-3', 5'-cctgtatgggtactgttcgttcactaaaata-3' and 5'-ttctgaacttgaggccagtctgagacacag-3'. Animals were housed and bred according to institutional and Dutch guidelines.

Immunohistochemistry

For immunohistochemical analysis of 5-HT, embryonic mouse brains were dissected and immediately fixed by immersion in 4% PFA in phosphate buffered saline (PBS, pH 7.4). For analysis of 5-HT neurons and projections in adult brains, mice were anaesthetized and transcardially perfused with 4% PFA in PBS. For post-fixation, brains were incubated in 4% PFA in PBS overnight. For cryoprotection, brains were incubated in increasing concentrations of sucrose.

Subsequently, coronal slices of 40 µm were made. For immunohistochemistry, brain slices were first incubated in blocking buffer containing PBS supplemented with 0.5% Triton X-100 and 10% normal goat serum for two hours. Subsequently, slices were incubated overnight with primary antibodies in PBS containing 0.5% Triton X-100 at 4°C in under gentle agitation. The next day, the slices were washed three times two hours in PBS and incubated with secondary antibodies in PBS for one hour under gentle agitation. Finally, slices were washed again three times two hours in PBS and mounted in Dabco-Mowiol for analysis. All procedures were performed at room temperature unless otherwise stated. Polyclonal anti-5-HT (1:1000) (Immunostar/Diasorin) was used as the primary antibody, and as a secondary antibody GAR-546 (1:1000) was used (Invitrogen). To analyze number of 5-HT cell bodies in DRN sections, at least three sections per experimental group were imaged using a CLSM 510 microscope. Number of 5-HT cell bodies was manually counted in the slices. For analysis of 5-HT fiber density in DRN and cortex a Z-stack of at least three positions per group was made. To analyze the 5-HT fiber density, the Z-stacks were projected in a single image and binarized. The 5-HT fiber density was quantified as the area in the image occupied by 5-HT neurites compared to the total area.

Mouse behaviour

Behavioural analysis of mice was tested in the Phenotyper ® (Noldus Information Technology, Wageningen, The Netherlands). For the home cage test, one animal per cage was tested for six days. Eleven SERT-Cre$^{cre/wt}$ Munc18-1$^{lox/wt}$ mice were used in the experimental group and eleven SERT-Cre$^{wt/wt}$ Munc18-1$^{lox/wt}$ mice were used as a control group. Each cage contains a top unit equipped with built-in hardware for videotracking, containing a digital infrared sensitive video camera and infrared lights. Using this method the animal behaviour can be continuously assessed, both in the light and in the dark phase. Ethovision 3.0 software was used as videotracking software. The phenotyper cages (30x30x35 cm) were made of transparent Perspex walls with an aluminium floor, and a feeding station and a water bottle attached to the outside. To the waterspout a lickometer was attached to determine the total duration the animal is touching the waterspout. A shelter unit (diameter: 9 cm, height: 10 cm, non-transparant material) was placed in the cage, which was defined as a hidden zone in Ethovision, and the software program could distinguish between in the shelter and on the shelter. Videotracking was performed at a rate of 12.5 samples per second and the following parameters were determined: average velocity of movement, total distance moved in the open space, proportion of time spent in the feeder, proportion of time spent in the shelter, and time spent drinking.

These parameters were calculated in two hour bins for both the dark and light phase. For the analysis of movement and velocity, only movement was scored when the mouse moved at a velocity of 3.5 cm/s and higher, averaged over twelve samples. Furthermore, total proportion of time spent in a zone of the home cage (on shelter, in shelter, feeder, pellet or waterspout), frequency of zone visits and frequency on the shelter during the dark period was quantified.

On day four and five a free operant conditioning task was performed as follows. The number of SMBs was quantified, which is a directed movement of the animal from one point in the cage to another. A distinction is made between raw and directional SMBs; When the animal moves with a higher velocity than the threshold value, which is determined by dividing the total distance moved by the total duration of movement, the SMB is considered to be a directional SMB. When the animal is moving at a lower velocity, it is considered as a raw SMB. Thus, in a directional SMB the animal is either moving with a greater velocity, or is taking a shorter route. At day four and five, when the mice gets on its shelter, this will result in the drop of a sucrose pellet. Thus, the mouse ‘learns’ that it has to jump onto its shelter in order to receive a new sucrose pellet.

One day six a one trial anxiety test was performed as follows. Fifteen minutes after the change from light to dark phase, a lightspot is on the feeder, thus creating a lightspot zone. The lightspot lasts for three hours, and the total amount of time the animal is in the lightspot zone in this period is recorded and compared with a baseline reference. The baseline reference is acquired by recording the amount of time the animal is in the feeder zone at day five at the corresponding hours, without a lightspot on.

Statistical analysis

Data were analyzed using SPSS 17.0. For analysis of number of 5-HT cell bodies in DRN and 5-HT neurite density in DRN and cortex, data was analyzed using the independent samples t-test. To analyze whether observed genotype frequencies differed significantly from a Mendelian ratio, a one-way chi-square test was performed. Mouse behaviour parameters were analyzed using the non-parametric Wilcoxon test after checking for normal distribution. Data shown are mean ± SEM. Significance level was set at $p < 0.05$.

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Chapter

7

Towards a genetic approach to study serotonergic outgrowth and connectivity in vivo

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Abstract

Altered connectivity of the serotonin (5-HT) system might underlie psychopathological processes such as anxiety and depression. Therefore, it would be very interesting to be able to visualize all 5-HT fibers, axons or presynaptic terminals/varicosities in living mouse brain. To specifically label 5-HT neurons and fibers, our aim is to express Cre recombinase in the 5-HT system. We describe here the generation of a tryptophan hydroxylase 2 (Tph2)-iCre targeting construct, the targeting of mouse embryonic stem (ES) cells and the blastocyst injections of positively targeted clones. Moreover, to study 5-HT connectivity in vivo, we generated several transgenic lines using the loxSTOPlox (LSL) methodology.

Eventually, we hope to use these techniques to create mice in which specifically in the 5-HT system reporter genes are expressed, so that these can be used to study the 5-HT outgrowth and connectivity in vivo.

Introduction

The 5-HT system has its cell bodies in the midbrain raphe nuclei. Although only few 5-HT neurons are present, virtually every brain area receives dense 5-HT innervation which triggers signaling pathways through several different 5-HT receptors. For these reasons the 5-HT system influences a wide variety of processes. Moreover, the 5-HT system is implicated in major depressive disorder and anxiety. One of the current hypotheses for psychopathological processes such as anxiety and depression is that 5-HT connectivity might be affected. Therefore, it would be very interesting to study the connectivity of the 5-HT system in vivo.

To study 5-HT outgrowth and connectivity in vivo, our aim is to generate a mouse line with expression of improved Cre recombinase (iCre) specifically in 5-HT neurons. As locus for the iCre knockin, we selected the Tph2 gene, which is selectively expressed in the 5-HT neurons in the raphe nuclei (Patel et al., 2004; Zhang et al., 2005; Clark et al., 2006). To be able to specifically express transgenes in the 5-HT system, we relied on the Cre-loxP technique (Tsien et al., 1996; Nagy, 2000). We selected to use iCre instead of Cre, since iCre gives improved Cre expression due to the application of mammalian codon usage (Shimshek et al., 2002).

In addition we generated several transgenic lines that carry a ubiquitous promoter, a LSL sequence and synaptically or axonally targeted fluorescent fusion proteins. Our ultimate goal is to combine the Tph2-iCre knockin allele with these transgenes, which would constitute an ideal model to study 5-HT

connectivity and dynamics in the living mouse brain. Here we describe the generation of the Tph2-iCre targeting construct, and the generation of Tph2-iCre chimaeric mice. Additionally, we describe the generation of several LSL transgenic lines.

Results

Construction and design of the Tph2-iCre targeting construct

In order to specifically express iCre in 5-HT neurons, we selected the locus for Tph2 which is localized at chromosome 10 D2. Tph2 is the rate limiting enzyme in 5-HT synthesis and is specifically expressed in 5-HT neurons in the brain. To generate mice with expression of iCre specifically in 5-HT neurons, our approach was to generate a targeting construct in which iCre cDNA is inserted at the endogenous Tph2 start codon We designed the targeting construct in a way that the 1.2 kb short arm at the 5' side of the construct stops just upstream of the endogenous Tph2 start codon. The 5.5 kb long arm was designed to start 53 bp downstream of the short arm, so that only 53 bp of endogenous DNA, including the endogenous Tph2 start codon, was deleted in case of homologous recombination (Fig. 1A). In between the short and long arm the iCreVenus cDNA was inserted and a neomycin phosphotransferase cassette (neo) for positive selection, flanked by FRT sites. These can be used to later on remove the neo cassette using the flippase/FRT system (O'Gorman et al., 1991).

In case of homologous recombination of the targeting construct in ES cells and subsequent generation of Tph2-iCre mice our approach was to use heterozygous Tph2-iCre mice. These contain one functional allele with Tph2 expression, whereas in the other allele, part of the first exon of Tph2 including the start codon is replaced for iCreVenus. In this case these mice will still have a functional Tph2 allele and expression of iCreVenus from the Tph2 promoter (Fig. 1B).

Generation of Tph2-iCre chimaeric mice

After designing and constructing the Tph2-iCre targeting construct, the construct was sequence verified. This showed that the construct had been correctly made and that the iCreVenus cDNA contained no mutations. The linearized targeting construct was used for ES cell transfection. The antibiotic G418 was used for positive selection of the neomycin resistance marker (Gossler et al., 1986). Eventually five clones were found in which homologous recombination had occurred. Blastocyst injection of clone 1C11 resulted in several chimaeric mice. Although we performed several rounds of chimaeric breeding, we have unfortunately not achieved germline transmission of the Tph2-iCre allele until now.

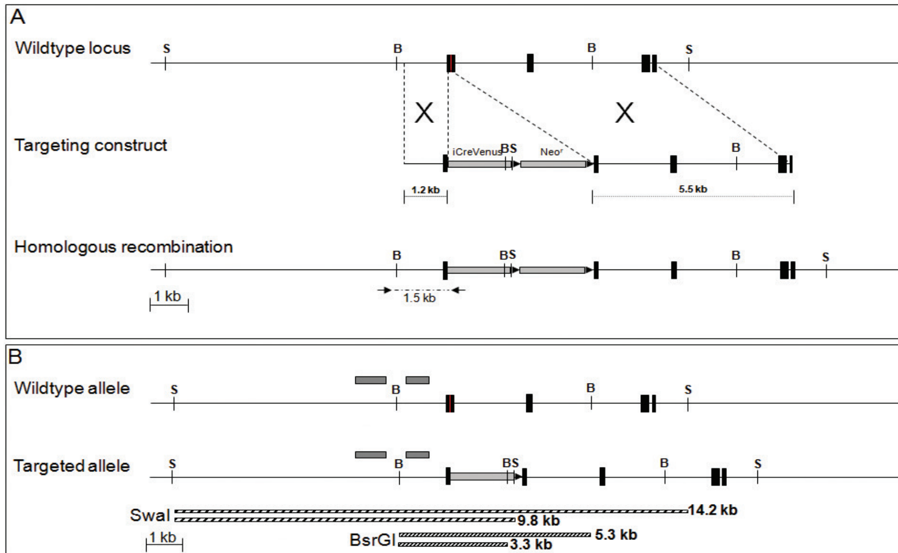

Figure 1. Tph2-Cre knockin approach
(A) The targeting construct was created to replace the start codon of the first exon of Tph2 with a iCre cassette and a neomycin resistance cassette flanked with FRT sites. The targeting construct consisted of a short arm of 1.2 kb, a long arm of 5.5 kb and a iCreVenus and neo cassette. In case of homologous recombination only 53 bp of endogenous DNA is deleted and replaced for the iCreVenus cassette and the neomycin resistance cassette. To identify ES cell clones in which homologous recombination occurred, a PCR screening approach was used. A forward primer (arrow) outside the short arm of the targeting construct and a reverse primer (arrow) in the iCre cassette were used to amplify a 1.5 kb fragment of DNA in case of homologous recombination (dotted line). (B) Approach for Southern blot screening of ES cell clones. An inside and an outside probe were used (grey boxes). For the inside probe, genomic DNA is digested with BsrGI. This will result in a 5.3 kb fragment in WT DNA, but in a 3.3 kb fragment in clones in which homologous recombination occurred due to the presence of an additional BsrGI site in the targeting construct. For the outside probe, genomic DNA is digested with SwaI, which will result in a 14.2 kb fragment in WT DNA, but in a 9.8 kb fragment in clones in which homologous recombination occurred, due to the presence of an additional SwaI site in the targeting construct.

Generation of LSL transgene lines

For construction of LSL transgene lines we used several marker genes tagged with either EGFP or mCherry, which is an improved variant of mRFP that matures more completely and is more photostable (Shaner et al., 2004). As marker genes we used SynaptophysinpHluorin (SypHy), TMSyxEGFP and TMSyxmCherry, which all specifically label axons and presynaptic terminals, and VenusKras4b which is a general membrane marker.

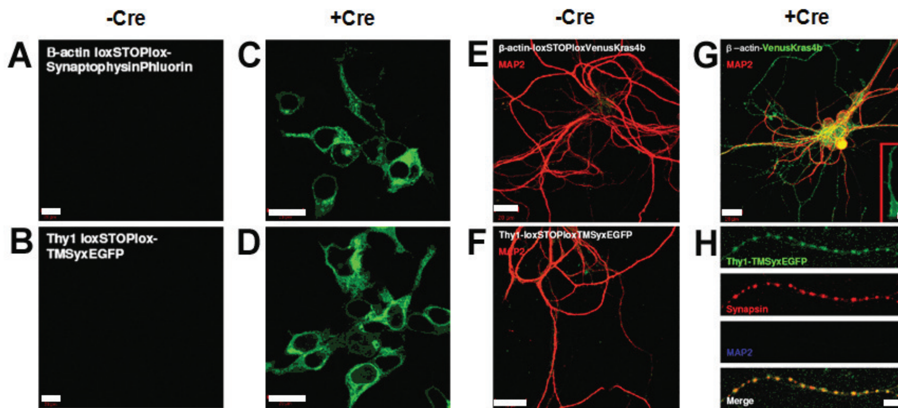

Figure 2. Validation of transgene constructs
To investigate whether the LSL approach worked, we expressed LSL transgene construct with our without iCre cDNA in HEK293 cells. (A,B) No Expression of SypHy or TMSyxEGFP in HEK293 cells after transfection of LSL-SypHy or LSL-TMSyxEGFP without iCre. (C,D) After transfection of LSL-SypHy or LSL-TMSyxEGFP with iCre, SypHy and TMSyxEGFP were expressed in HEK293 cells respectively. (E,F) In neurons, VenusKras4b or TMSyxEGFP are not expressed when transfected without iCre. (G) Upon transfection of LSL-VenusKras4b with iCre, VenusKras4b is expressed in neurons, and the membrane is labeled, also in growth cones (inset). (H) Upon transfection of LSL-TMSyxEGFP with iCre, TMSyxEGFP is expressed in axons, as shown by the absence of MAP2 staining. Immunocytochemistry for the synaptic marker Synapsin shows that there is colocalization between TMSyxEGFP and Synapsin, showing that TMSyxEGFP is targeted to synapses. Scale bars: A-G 10 μm, inset in G 2 μm, H 5 μm.

SypHy is the improved variant of SynaptopHluorin and detects secretion events due to the increase in pH in synaptic vesicles after exposure to the extracellular medium (Miesenbock et al., 1998; Granseth et al., 2006). TMSyxEGFP contains part of the transmembrane domain of Syntaxin1A coupled to EGFP; TMSyxmCherry is the same but with a red fluorophore. VenusKras4b is the EYFP derived Venus fused with the 20 amino acid C-terminal plasma membrane targeting domain of Kras4b (Welman et al., 2000). As ubiquitous promoter either the chicken β-actin promoter or the Thy1 promoter was used (Aigner et al., 1995; Ludin et al., 1996). In between the promoter and the transgene, we inserted a floxed stop sequence in order to acquire iCre mediated transgene expression (Lakso et al., 1992). Some of the constructs were expressed in heterologous cells or neurons to test whether the floxed stop sequence prevented expression and whether co-transfection of iCre resulted in expression of the transgene. Transfection of β-actin-LSL-SypHy or Thy1-LSL-TMSyxEGFP alone in heterologous cells did not result in expression of the transgene (Fig. 2A,B). In contrast, after co-transfection with iCre, there was strong expression of

both transgenes (Fig. 2C,D). Also in neurons, expression of β-actin-LSL-VenusKras4b or Thy1-LSL-TMSyxEGFP alone did not result in detectable transgene expression (Fig. 2E,F). However, after co-expression with iCre, VenusKras4b expression was detected (Fig. 2G). After co-expression of iCre and Thy1-LSL-TMSyxEGFP neurons were found which expressed TMSyxEGFP and this localized exclusively to axons and synapses as shown by the absence of staining for the dendritic marker MAP2 and co-localization with the synaptic marker Synapsin (Fig. 2H).
These transgenic constructs were linearized and injected in oocytes to generate seven founder lines of β-actin-LSL-SypHy, five founder lines of β-actin-LSL-VenusKras4b, five founder lines of Thy1-LSL-TMSyxEGFP and eleven founder lines of Thy1-LSL-TMSyxmCherry.

Screening of expression of transgenes in founder lines
In order to screen the expression of the transgenes per founder line, and to find out whether there are lines with expression of the transgene in the raphe nuclei, we first removed the LSL cassette by crossing the founder lines with the CMV-Cre mouse line, a line in which Cre is ubiquitously expressed (Schwenk et al., 1995).
This breeding strategy resulted in mice in which the LSL cassette was removed in all cells (Fig. 3A). This in contrast to crossing with Tph2-iCre mice, which is expected to result in removal of the LSL cassette only and specifically in 5-HT neurons. Thus, in 5-HT neurons the transgene will be expressed, whereas in all other cells the LSL is still present and the transgene is not expressed (Fig. 3B).
After screening all transgenic founder lines, we found several lines in which the transgene was very low and mosaically expressed. For β-actin-LSL-SypHy we found one founder line with low expression in the Purkinje cell layer of the cerebellum, and two founder lines with expression in the hippocampal CA1-CA3 region and dentate gyrus (data not shown). For β-actin-LSL-VenusKras4b we only found very low and mosaic expression of the transgene (data not shown). For Thy1-LSL-TMSyxEGFP and TMSyxmCherry, we found one line with TMSyxEGFP ubiquitously expressed in neocortex, and one line with TMSyxmCherry mosaically expressed in cortex and hippocampus (data not shown). Unfortunately, not one founder line did show expression of the transgene in midbrain or raphe nuclei areas.

Discussion

Here we have described the generation of Tph2-iCre chimaeric mice and several LSL transgenic mouse lines. We would like to eventually create mice with expression of a marker gene specifically in 5-HT neurons. This would allow us to

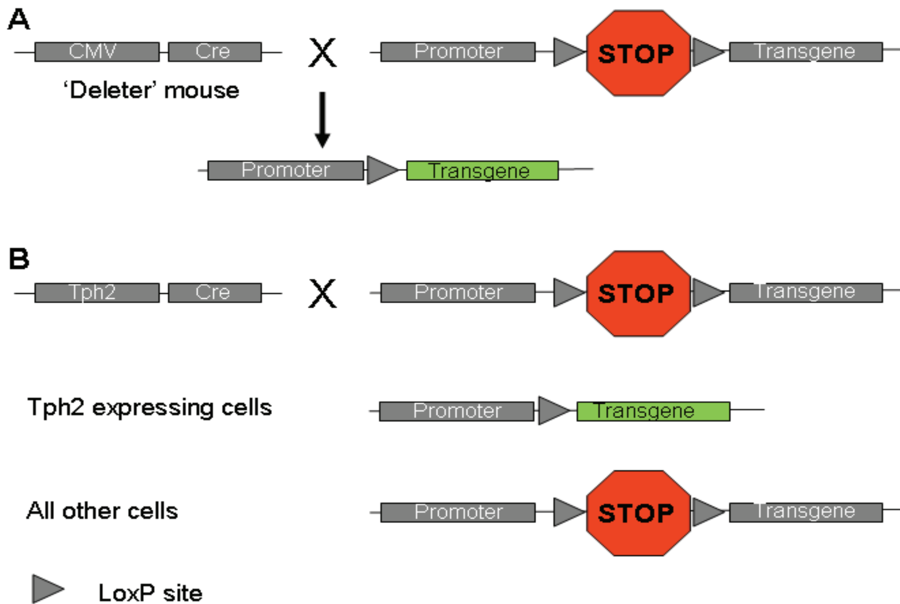

Figure 3. Approach for screening expression pattern of transgenic mice
(A) To screen the expression pattern of LSL transgenic mice, these mice were crossed with a CMV-Cre mouse, which ubiquitously expressed Cre recombinase. This results in mice in which the LSL is removed in all cells, thus allowing an analysis of transgene expression pattern. (B) Once LSL mice are identified with expression in the 5-HT system, they can be used to cross with Tph2-Cre mice to drive transgene expression specifically in the 5-HT system.

label fibers or presynaptic terminals of 5-HT neurons, and to image 5-HT fiber outgrowth and connectivity changes in living mouse brain using 2-photon in vivo microscopy (Kerr and Denk, 2008). Since one of the hypotheses for major depressive disorder is that the connectivity of the 5-HT system might be affected, such a mouse model would be valuable to study this aspect.
We choose to target iCreVenus to the first exon of Tph2, so that the endogenous start codon and small part of the first exon of Tph2 is replaced for iCreVenus. In this way we anticipated that Tph2 expression relies on endogenous Tph2 promoter activity and is specifically expressed in 5-HT neurons in which Tph2 is expressed. In contrast to this approach we could also have chosen to target iCreVenus to the 3' side of the Tph2 open reading frame using an internal ribosomal entry site (IRES). We did, however, not use this approach because the expression of a reporter gene behind an IRES is often rather low. Although using our approach we sacrifice one functional Tph2 allele, we expect that this will not significantly affect brain development and behaviour in Tph2 heterozygotes. Deletion of both Tyrosine hydroxylase alleles results in mid-gestational lethality.

However, TH heterozygotes showed no obvious anatomical or behavioural defects (Zhou et al., 1995). Therefore, we reason that our approach will not significantly affect brain development or behaviour.
Using a transgenic approach with a LSL cassette to allow transgene expression only in cells in which iCre is also present, we aimed to express these marker genes specifically in 5-HT neurons. However, none of the founder lines we screened showed expression of the transgene construct in the midbrain or raphe nuclei. It has been shown for both the chicken β-actin and mouse Thy1 promoter that these are able to induce expression of transgenes in the cortex and hippocampus (Feng et al., 2000; Roelandse et al., 2003). However, it appears more difficult to also induce expression in midbrain and raphe nuclei for these promoters, since none of our transgenic lines expressed the transgene in these regions. Recently, it was shown that a 1.8 kb region immediately upstream the Pet-1 gene is sufficient to acquire 5-HT neuron specific expression of a transgene (Scott et al., 2005a; Scott et al., 2005b). Therefore, in new experiments we could try to rely on the Pet-1 promoter to express transgenes in the 5-HT neurons.
Possibly, future experiments with novel blastocyst injections of Tph2-iCre targeted ES clones and transgene injections using the Pet-1 promoter will result in a Tph2-iCre mouse line and transgenic mouse lines with expression of reporter genes in 5-HT neurons. These mouse lines can then be used to create mouse lines with expression of reporter genes specifically in 5-HT neurons to possibly resolve the longstanding question whether psychopathological processes are a result of altered 5-HT network connectivity.

EXPERIMENTAL PROCEDURES

Generation of Tph2-iCre targeting construct

The Tph2-iCre targeting construct was designed in such a way that in the wildtype Tph2 locus, 53 bp in exon 1 are deleted, including the start codon, and replaced for a iCreVenus cassette. The Tph2-iCre targeting construct was generated as follows. The 1.2 kb short arm upstream the endogenous start codon of Tph2 was generated with PCR primers forward 5'CATGCATAGAAAGTGTCCAG3' reverse 5'TTCTTTCTCAGCAGCAGGGG3'. The 5.5 kb long arm starting 34 bp behind the Tph2 start codon was generated with PCR primers forward 5'GGGCCAGGAGAGGGTTGTCCTTGGATTCTG3' and reverse 5'CCGTACATGAGGACTCGGTGAGAGCATCTG3'. Both arms were subcloned in the pGEM-T easy vector (Promega). In the piCreVenus vector the NotI site behind Venus was removed and a linker containing SwaI, BsiWI, MluI and NotI sites was placed in the AflII site behind the polyA tail. The short arm was cloned sticky using EcoRI in front of iCreVenus in the modified iCreVenus vector. A neomycin resistance cassette flanked by FRT sites was removed from

the ploxPFRTNEOFRTloxP vector (a kind gift from Jeroen Pasterkamp, Utrecht) using partial digestion with BamHI and cloned blunt in the unique SpeI site behind the long arm in the pGEM-T easy vector. Finally, the FRTNEOFRT long arm Tph2 was cloned sticky behind the short arm iCreVenus using NotI. The construct was verified by sequence analysis. The construct was linearized with KspI for targeting.

Targeting ES cells

In order to target ES cells with the Tph2-iCre targeting construct, approximately 1 x 10^9 ES cells (line E14.1) were electroporated with 100 μg of linearized Tph2-Cre targeting construct and plated on murine embryonic feeders. The subsequent day, neomycin selection was started by adding 400 μg/ml G418. After 10 days, non targeted clones had died and 700 surviving clones were picked and individually grown. DNA of all these clones was screened to distinguish between clones in which the targeting construct was randomly inserted and clones in which homologous recombination had occurred.

To select for clones in which homologous recombination occurred, the clones were screened with a nested pcr approach using the following primers: 1st pcr forward 5'CACTTTTCTTCACACCCTTCTC3' and reverse 5' CCTGACTTCATCAGAGGTGGCATCC3' nested pcr forward 5' TTATTTGTGTGAGCACGTGA3' and reverse 5' TTTTGGTGCACAGTCAGCAG3' which results in a 1.6 kb pcr fragment in positively targeted clones. From the 700 targeted clones, eventually 5 clones were selected in which homologous recombination occurred designated 2F11, 1A1, 1C11, 4E1 and 2E11. Homologous recombination was verified using a 5' primer outside the forbidden construct and sequence analysis of this region. ES cell clones 1C11 and 4E1 were injected in blastocysts to generate chimaeric mice.

Generation of transgenic mice

In order to generate transgenic mice for conditional expression of reporter genes, the LSL cassette derived from the Pbs302 vector was used (Sauer, 1993). As promoter, the chicken β-actin and regulatory elements of the mouse Thy1 gene were used. As reporter genes, SypHy (a kind gift from Andreas Jeromin), VenusKras4b, TMSyx (transmembrane Syntaxin1A) EGFP and TMSyxmCherry were used. The constructs were cloned as follows. For β-actin-LSL-SypHy, the LSL cassette was excised from the Pbs302 vector using EcoRI and SpeI and cloned blunt in the unique XbaI site in the pβ-actin-SypHy vector. For β-actin-LSL-VenusKras4b, the LSL cassette was excised from the Pbs302 vector using EcoRI and SpeI and cloned blunt in the BglII site in the pVenusKras4b vector. Subsequently, LSL-VenusKras4b was excised using NheI and XbaI and cloned blunt in BlnI and XbaI in pβ-actin-EGFP to replace EGFP. Both β-actin-LSL-

SypHy and β-actin-LSL-VenusKras4b were linearized with AatII for transgene injection. For Thy1-LSL-TMSyxEGFP, TMSyxEGFP was excised with NheI and SspI and cloned blunt in the unique NotI site in pThy1-LSL (a kind gift from Christiaan Levelt, Amsterdam). To clone TMSyxmCherry, TMSyx was excised from pTMSyxEGFP using NheI and EcoRI and cloned blunt in pmCherry-N3 in the EclXI site. Then TMSyxmCherry was excised using NheI and SspI and cloned blunt in the NotI site the in pThy1-LSL vector. Both Thy1-LSL-TMSyxEGFP and Thy1-LSL-TMSyxmCherry were linearized with AatII for transgene injection. To create transgenic mice, linearized DNA constructs were injected in the pronucleus of fertilized C57BL/6 oocytes.

Transfection of transgene plasmids in heterologous cells and neurons
Transgene plasmids were transfected in HEK293 cells or neurons using Lipofectamine 2000 reagent according to the manufacturer's protocol. HEK293 cells and neurons were cultured as has been published before (Groffen et al., 2004; de Wit et al., 2006). In HEK293 cells, one day post-transfection expression of transgene constructs was visualized using an inverted microscope fitted with fluorescence filters. In neurons and HEK293 cells, expression of transgene constructs was analyzed using a LSM510 confocal laser scanning microscope (Zeiss, B.V. The Netherlands). Immunocytochemical analysis of neurons was performed as has been described before using monoclonal MAP2 (Chemicon) and polyclonal Synapsin I (E028) as primary antibodies and goat-anti-mouse Alexa 543/644 and goat-anti-rabbit Alexa 543 (Molecular Probes) (de Wit et al., 2006).

Screening of expression pattern
In order to investigate the expression pattern of the transgene construct per transgenic line, the founder lines were crossed with the 129-Cre mouse line, which has ubiquitous expression of Cre (Schwenk et al., 1995). Pups positive for Cre and the transgene were used for in vivo paraformaldehyde (PFA) perfusion between two and four months age. For in vivo PFA fixation, mice were anaesthetized and transcardially perfused with 4% PFA dissolved in phosphate buffered saline (PBS) (pH 7.4). After fixation, brains were removed and postfixed in 4% PFA for 24 hours at 4°C. Subsequently, brains were cryoprotected in increasing concentrations of sucrose dissolved in PBS at 4°C. For screening of expression pattern of the transgenes, the fixed and cryoprotected brains were sagitally cut in 30 µm slices using a microtome. In case the EGFP fluorescence was too low, Immunohistochemistry was performed using the free floating method. To this end, slices were first blocked using blocking solution which contained 0.1% Triton X-100 and 10% normal goat serum for two hours under gentle agitation. Slices were incubated overnight at 4°C using primary antibody,

washed three times two hours the next day with PBS 0.1% Triton X-100 and incubated with the secondary antibody for two hours. After washing for three times two hours in PBS, slices were positioned on a glass slide, allowed to dry and overlaid with a glass coverslip using Dabco Mowiol. All reactions were carried out at room temperature unless otherwise stated.

Analysis of transgene expression

To analyze the expression pattern of the transgene constructs, brain slices were analyzed using an inverted microscope equipped with fluorescence filters and a Sony camera. A LSM510 confocal laser scanning microscope was used to investigate the expression of the transgene constructs in individual neurons.

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Chapter

8

General discussion

General discussion

The aim of the studies described in this thesis was to investigate different cellular aspects of the outgrowth and connectivity of the brain 5-HT system. The 5-HT system has the remarkable feature that although there are only few 5-HT neurons, localized in the midbrain raphe nuclei, virtually every brain area receives dense 5-HT innervations. These innervations contain several 5-HT varicosities, where 5-HT is released both synaptically and as volume transmission. Due to several 5-HT receptors which are present in the brain, 5-HT has many functions and influences a wide variety of processes. Moreover, the 5-HT system is implicated in several psychopathological processes such as anxiety and depression. Despite the high social and economical importance of these conditions, there is a lot of uncertainty about the involved mechanisms. Therefore, a further cellular characterization of the outgrowth and connectivity of the 5-HT system is of high interest. We used several experimental approaches to study different cellular aspects of the outgrowth and connectivity of the 5-HT system.

In **chapter 2** we used organotypic slice cultures to study 5-HT outgrowth and connectivity and the effect of pharmacological manipulations in vitro. This revealed that the 5-HT neurite density was not affected by blocking the SERT or application of a 5-HT_{1A} agonist, but the density was reduced by application of a 5-HT_2 agonist. This was presumably due to inhibited outgrowth of the 5-HT system.

In **chapter 3**, we investigated the trafficking of SERT. We expressed SERT tagged with the fluorescent protein mCherry in hippocampal neurons. We showed that mCherry-SERT is transported in vesicles which displayed a dynamic trafficking.

In **chapter 4** we used the same approach as in chapter 3, but now we studied the localization and trafficking of Tph2, the rate limiting enzyme in 5-HT synthesis. We tagged Tph2 with EGFP and studied the localization of Tph2-EGFP in hippocampal neurons. In axons Tph2-EGFP displayed a punctate distribution and co-localized with Synaptophysin-mCherry, a marker for synaptic terminals, suggesting that Tph2-EGFP localized to synaptic terminals.

In **chapter 5** we studied the effect of a SNP in the presynaptic gene Piccolo on the trafficking and localization of SERT. Knockdown of Piccolo in neurons did not affect mCherry-SERT trafficking. The SNP in Piccolo possibly affects the localization of SERT, although future research is needed to further resolve this issue.

In **chapter 6** we crossed SERT-Cre mice with floxed Munc18-1 mice to specifically abolish Munc18-1 in SERT expressing neurons. This resulted in early postnatal lethality and a rapid degeneration of the 5-HT system. We used heterozygous mice to study the effect of removal of one Munc18-1 allele in SERT expressing neurons on behaviour using a phenotyping experiment. Basal behaviour, conditioning and anxiety related behaviour were not affected.
In **chapter 7** we describe our approach to create mice which have Cre expression specifically in 5-HT neurons. Moreover, we show that we have generated transgenic lines with a floxed stop cassette which can be used for spatial regulation of transgene expression upon Cre mediated recombination. In the future, these mice can be used to specifically label 5-HT neurons, or to specifically remove a gene in 5-HT neurons.

Roles of 5-HT in outgrowth, connectivity and behaviour

One of the current hypotheses for psychopathological processes such as depression is that altered 5-HT network connectivity might be involved in these processes. Furthermore, it is hypothesized that antidepressants such as fluoxetine might restore aberrant 5-HT network connectivity (Castren, 2005). We used organotypic slices to study these processes in an in vitro model system. Our data showed that fluoxetine did not affect 5-HT innervation density.
In adult rats, chronic fluoxetine application did result in an increased density of 5-HT projections in the forebrain (Zhou et al., 2006). Interestingly, an in vivo study using chronic fluoxetine treatment either during development or during adulthood, showed that when applied during adulthood, fluoxetine did not affect behaviour. In contrast, fluoxetine treatment during development resulted in increased anxiety-related behaviour during adulthood (Ansorge et al., 2004; Ansorge et al., 2008). Our data suggests, although it is in vitro, that this effect of fluoxetine on behaviour does not result from an altered innervation of 5-HT projections. However, in our model system we were unable to investigate whether the number of 5-HT varicosities was affected by fluoxetine application. Thus, although 5-HT projection density is unaffected, connectivity of the 5-HT system might be affected due to an altered number of 5-HT varicosities or an altered rate of 5-HT release.
At least in the snail Helisoma, 5-HT acts as an autotrophic factor on its own outgrowth; 5-HT applied at μM concentrations to the growth cones of 5-HT neurons resulted in an abrupt cessation of growth cone motility and outgrowth (Haydon et al., 1984; Haydon et al., 1987).

The 5-HT_{1A} and 5-HT_{2C} receptor might mediate this effect of 5-HT, as these receptors are expressed in the DRN (Wright et al., 1995; Clemett et al., 2000). Our data showed that 5-HT_{1A} receptor activation slightly increased 5-HT neurite

density whereas chronic activation of the $5\text{-}HT_2$ receptor decreased 5-HT neurite density. It is tempting to speculate that these receptors act in concert during outgrowth to regulate a proper development of 5-HT projections towards target areas mediated by 5-HT. Interestingly, $5\text{-}HT_{1A}$ and $5\text{-}HT_2$ receptors have different affinities for 5-HT; $5\text{-}HT_{1A}$ receptors are high affinity 5-HT receptors which have an EC50 value in the nM range, whereas $5\text{-}HT_2$ receptors are low affinity 5-HT receptors, which have an EC50 value in the µM range (Fargin et al., 1989; Marek and Aghajanian, 1994; Azmitia, 2001). The $5\text{-}HT_{2C}$ receptor is present on GABA-ergic cells in the DRN, and it is unknown whether this receptor is present on 5-HT neurons (Serrats et al., 2005). This suggests that the inhibitory effect of $5\text{-}HT_{2C}$ receptor activation is not a direct effect on 5-HT neurons, but might be an indirect effect via GABA-ergic neurons. Indeed, in vivo data supports that $5\text{-}HT_{2C}$ receptor activation inhibits neuronal firing of 5-HT neurons via GABA-ergic neurons (Boothman et al., 2006). Thus, in the DRN µM levels of 5-HT might inhibit outgrowth, whereas nM levels might stimulate outgrowth of 5-HT innervations.

Mice in which the $5\text{-}HT_{1A}$ receptor is deleted have increased anxiety-related behaviour, as they spend less time in the center in the open field test and avoid the open arms in the elevated plus maze (Parks et al., 1998; Ramboz et al., 1998). Overexpression of the $5\text{-}HT_{1A}$ receptor, on the other hand, results in reduced anxiety-related behaviour (Deng et al., 2007). A very elegant study was performed by Gross and colleagues, who showed that restoring $5\text{-}HT_{1A}$ receptor expression in the forebrain during development, but not during adulthood rescued the behavioural phenotype of $5\text{-}HT_{1A}$ KO mice. (Gross et al., 2002). Clearly, the $5\text{-}HT_{1A}$ receptor is important during development to establish normal anxiety-like behaviour. Unfortunately, no studies were performed to resolve whether 5-HT connectivity is affected in these mice.

Deletion of the $5\text{-}HT_{2C}$ receptor, on the other hand, results in compulsive-like behaviour, and these mice are obese and suffer from seizures (Tecott et al., 1995; Chou-Green et al., 2003). Unfortunately, also for these mice it is not known whether 5-HT connectivity is affected.

Trafficking of SERT and Tph2 in neurons

As the 5-HT system has long projections to several brain areas, proteins involved in 5-HT synthesis and reuptake are transported over large distances towards axonal growth cones and varicosities. In chapter 3 and 4 we focused on SERT and Tph2 transport in hippocampal neurons and observed that SERT and Tph2 displayed a distinct distribution. SERT was predominantly localized extra-synaptically and some SERT puncta localized in the vicinity of presynaptic terminals. In contrast, Tph2 accumulated in puncta which colocalized with

synaptic markers. Obviously, Tph2 is required at presynaptic terminals and varicosities for local synthesis of 5-HT. Indeed in 5-HT neurons Tph is enriched in presynaptic terminals (Pickel et al., 1976; Pickel et al., 1977). On the other hand, SERT is localized extra-synaptically to transport extracellular 5-HT which has diffused away from its release sites back into the neuron. An electron microscopy study showed that the majority of SERT in 5-HT neurons was located outside synapses (Zhou et al., 1998). Thus, the distribution of Tph2 and SERT which we found in hippocampal neurons seems to mimic the distribution of endogenous SERT and Tph2 in 5-HT neurons.

We also showed that SERT and Tph2 are transported in distinct ways through the neuron. In contrast to Tph2, SERT displayed a vesicular trafficking. Apparently, SERT contains a signal peptides which mediates the transport in vesicles. Alternatively, SERT contains transmembrane domains, which requires that SERT is transported in vesicles as these lipophilic domains are insoluble.

As SERT is transported in secretory vesicles, there should be one or more motor proteins responsible for this transport. There are several families of motor proteins which are involved in the transport of several different proteins through neurites (for a review see (Schlager and Hoogenraad, 2009). The average velocity of SERT transport vesicles and the fact that these do not display a preference for direction of movement is in accordance with the average velocity of transport vesicles containing glycine (Maas et al., 2006). Also in this study, it was shown that dynein motors are responsible for the retrograde transport of glycine (Maas et al., 2006). Dynein motors are involved in transporting other cargo molecules through neurons, such as the Trkb receptor and Bassoon (Yano et al., 2001; Fejtova et al., 2009). The trafficking dynamics of Trkb and Bassoon are comparable with the trafficking dynamics of SERT, strongly suggesting that the dynein motor protein is responsible for SERT transport (Ha et al., 2008; Fejtova et al., 2009).

A SNP in Piccolo associates with depression

Recently, a SNP in Piccolo was found which was associated with depression (Sullivan et al., 2008; Bochdanovits et al., 2009). Piccolo is a structural component of the presynaptic cytoskeletal cytomatrix (PCM) and structurally related to Bassoon, another component of the PCM (Cases-Langhoff et al., 1996; tom Dieck et al., 1998; Fenster et al., 2000). Piccolo is a multidomain zinc finger protein which contains several coiled coil domains and a PDZ domain (Fenster and Garner, 2002). Piccolo also contains two C2 domains, C2A and C2B, of which C2A functions as a low-affinity calcium sensor in presynaptic terminals (Gerber et al., 2001). The SNP in Piccolo was found to be closely localized to the C2A domain (Sullivan et al., 2008).

In the presynaptic terminal, Piccolo is involved in different processes. First of all, Piccolo, together with Bassoon, is involved in presynaptic terminal formation. Piccolo and Bassoon are transported in Piccolo-Bassoon transport vesicles together with other constituents of presynaptic terminals, and two or three of these vesicles are sufficient to form an active synapse (Zhai et al., 2001; Shapira et al., 2003; Tao-Cheng, 2007). In chapter 5 we have shown that 5-HT neurons contain Piccolo. Thus, in 5-HT varicosities Piccolo might be involved in formation of varicosities, regulating 5-HT secretion and regulation of SERT internalization. We have shown that knockdown of Piccolo did not affect SERT trafficking dynamics, suggesting that Piccolo is not involved in SERT transport.
Secondly, since Piccolo is present in the presynaptic terminal, Piccolo might be involved in the modulation of synaptic vesicle exocytosis. Indeed, Piccolo influences synaptic function by negatively regulating synaptic vesicle exocytosis, possibly via a calmodulin kinase II-dependent mechanism, mediated through the modulation of Synapsin1A dynamics (Leal-Ortiz et al., 2008). Thus, Piccolo might be involved in modulating 5-HT release from synaptic vesicles. However, the majority of 5-HT is secreted as bulk release from large dense core vesicles (LDCVs) (Bruns and Jahn, 1995). Therefore, although it might be that a SNP in Piccolo could modulate 5-HT release from synaptic vesicles, this would probably only have minor effects on extracellular 5-HT levels.

Thirdly, it was shown in heterologous cells that Piccolo is involved in the regulation of DAT internalization. Specifically, the C2A domain was involved in this process (Cen et al., 2008). As DAT and SERT belong to the same gene family and are structurally related it is conceivable that Piccolo could be involved in SERT internalization. In chapter 5, we have investigated in heterologous cells whether the SNP in the C2A domain of Piccolo alters the localization of mCherry-SERT. This showed that expression of the C2A domain of Piccolo containing the SNP seemed to alter the membrane distribution of SERT compared to the control C2A variant. It would be very interesting to further investigate whether the SNP in the C2A domain affects varicosity density of 5-HT projections or regulation of SERT membrane localization. If so, this would be the first cellular evidence that altered 5-HT network connectivity might be involved in the etiology of depression.

Deletion of Munc18-1 in SERT expressing neurons results in early postnatal lethality

Our data in chapter 6 showed that deletion of Munc18-1 in SERT expressing neurons resulted in early postnatal lethality. Although neurons are initially formed and there is normal brain development, deletion of Munc18-1 eventually results in degeneration (Verhage et al., 2000; Heeroma et al., 2003; Korteweg et

al., 2004). Our data showed that also 5-HT neurons in which Munc18-1 is deleted initially develop and grow out projections which innervated the cortex. However, after the initial generation, the 5-HT neurons undergo rapid degeneration, and at P2 only very few 5-HT neurons were left. This is the first mouse model which has a complete removal of the 5-HT system. This in contrast to the Tph2 KO mouse, which has no 5-HT synthesis in the central nervous system, but still 5-HT neurons and projections are unaffected.

Deletion of Tph2 resulted in ~50% lethality in the first four weeks (Alenina et al., 2009). Surprisingly, however, another research group did not find any increased lethality in $Tph2^{-/-}$ mice ((Savelieva et al., 2008) and personal communication). Deletion of Pet1 and Lmx1b results in mice in which ~70% and >99% of 5-HT neurons fail to survive respectively. Concerning the marked reduction in 5-HT neurons and projections in these mice, it is surprising that these mice only display subtle phenotypes (Ding et al., 2003; Hendricks et al., 2003; Zhao et al., 2006)

Unfortunately, as also thalamocortical neurons and some hippocampal neurons briefly express SERT, and thus Cre recombinase, during development, it is difficult to assess whether the observed phenotype is attributable to a degenerating 5-HT system. Therefore, crossing floxed Munc18-1 mice with Pet-Cre or Tph2-Cre mice which express Cre exclusively in 5-HT neurons should finally resolve whether complete removal of the 5-HT system affects brain development and survival.

Altered connectivity of the 5-HT system in human psychopathological processes?

Descriptive studies on the effect of psychopathological processes on human 5-HT connectivity rely on postmortem brain tissue. The last decade, however, more and more human imaging studies are performed using functional magnetic resonance imaging (fMRI) and positron emission tomographic (PET) imaging to resolve whether brain circuitry is affected in psychopathological processes associated with the 5-HT system.

Research on postmortem brain material from depressive people who committed suicide predominantly focused on mRNA expression levels or binding of radiolabeled 5-HT to 5-HT receptors. Several studies showed that there is an elevated 5-HT_{2A} and 5-HT_{2C} receptor abundance in the frontal cortex of depressed suicide victims (Arango et al., 1990; Xu and Pandey, 2000; Prasad et al., 2005). Other studies reported either reduced or increased binding or availability of 5-HT_{1A} receptors, depending on the brain region studied (Drevets et al., 2007). From these data, however, it cannot be deduced whether the altered

levels of mRNA expression or altered levels of receptor binding are the result of an alteration in expression levels or of an altered 5-HT projection density.
PET imaging is used to measure cerebral blood flow and rate of glucose metabolism as a measure for brain activity. Functional MRI is used to measure blood oxygen level dependent (BOLD) fluctuations which are a measure for activity and connectivity of brain regions. PET and fMRI studies have reported a decreased activation of cortical areas such as the dorsolateral prefrontal cortex and the anterior cingulate cortex in depressed subjects (Drevets et al., 1997; Mayberg et al., 1999). On the other hand, other studies reported an increased activation of limbic areas such as the amygdala in depression (Sheline et al., 2001; Siegle et al., 2002). Several studies have explored whether antidepressants restore aberrant activation patterns observed in depression. Antidepressant treatment increases glucose metabolism in prefrontal cortex and reduces activity in limbic areas (Sheline et al., 2001; Harvey et al., 2004b).

Although it is difficult to conclude from these imaging studies if altered brain network connectivity is a cause or consequence of the depression, one could at least speculate that connectivity changes occur at some stage. Clearly, more research is needed to further elucidate whether network connectivity alterations underlie psychopathological processes associated with the 5-HT system.

Future perspectives

There are a number of approaches which could be taken to further elucidate the connectivity of the 5-HT system, the relation between the 5-HT system and behaviour, and the involvement of the 5-HT system in psychopathology. First of all, it would be of great interest to label all 5-HT neurons and neurites with fluorescent markers. This could be achieved by creating knockin mice using the Tph2 promoter to drive expression of Cre and to cross these mice with floxed stop fluorescent reporter mice. Additionally, it has already been shown that Pet-Cre mice express Cre specifically in 5-HT neurons, and crossing with reporter mice showed that >99% of 5-HT neurons were labeled (Scott et al., 2005). These mice could then be used to reconstruct the connectivity of the 5-HT system.
Secondly, there are several subpopulations of 5-HT neurons that can be distinguished based upon their precursor genes (Jensen et al., 2008). If Cre mouse lines could be made for these specific subpopulations, these could be crossed with reporter mice or with the so-called brainbow mice (Livet et al., 2007). It would be very interesting to find out whether different subpopulations of 5-HT neurons innervate different brain regions, and are involved in different aspects of behaviour.

Additionally, these mouse lines could be used to specifically inhibit neurotransmission from these populations of 5-HT neurons, or to inhibit neurotransmission from all 5-HT neurons using Tph2-Cre mice. Expressing tetanus toxin in neurons blocks synaptic activity since tetanus toxin cleaves VAMP2 which is a protein necessary for vesicle fusion. It has been shown that this approach works in vivo, e.g. in the retina, but also for 5-HT neurons (Kerschensteiner et al., 2009; Kim et al., 2009). The work of Kim et al showed that silencing a subset of 5-HT neurons resulted in mice with behavioural alterations (Kim et al., 2009). These mice could be used to further explore the role of the 5-HT system and subsets of the 5-HT system on behaviour and to what extend these contribute to pathophysiological processes such as anxiety and depression. The other way around, these mice could be used to study whether silencing of 5-HT neurons affects 5-HT connectivity, i.e. 5-HT projection and varicosity density.

A third interesting future direction is to use mice which express Cre specifically in 5-HT neurons to inactivate 5-HT autoreceptors only in 5-HT neurons. A lot of research has been conducted in elucidating the role of 5-HT autoreceptors in determining 5-HT transmission and behaviour. The 5-HT_{1A} and 5-HT_{1B} knockout mice display altered anxiety-related and aggression-related behaviours. However, in these mice it is difficult to assess whether these phenotypes observed are due to deletion of 5-HT autoreceptors, or deletion of these receptors from non-5-HT neurons. A far more elegant approach, however, would be to generate floxed 5-HT_{1A} and 5-HT_{1B} mice and cross these with Pet-Cre or Tph2-Cre mice to selectively inactivate these receptors only in 5-HT neurons. This will allow to distinguish between the role of 5-HT autoreceptors and heteroreceptors, and to investigate to what extend the deletion of 5-HT autoreceptors affects mouse behaviour.

Recently, also a tamoxifen inducible Tph2-Cre mouse line has been generated, which allows to temporally regulate Cre expression in 5-HT neurons (Weber et al., 2009). This mouse line allows to investigate whether deleting a gene in the 5-HT system either during development or during adulthood affects 5-HT outgrowth, connectivity or mouse behaviour.

Finally, it would be interesting to further investigate whether connectivity is affected in anxiety and depression in human subjects. Novel brain imaging techniques, such as pharmaco MRI are being developed to further explore this relationship (Windischberger et al.)

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Nederlandse samenvatting

Cellulaire karakterisatie van de uitgroei en connectiviteit van het serotonine systeem in het brein

Nederlandse samenvatting

De menselijke hersenen bestaan uit ongeveer 100 miljard zenuwcellen (neuronen). Neuronen zijn de basis elementen die bijvoorbeeld gedrag en geheugen mogelijk maken. Neuronen hebben een aantal uitlopers waarmee ze met elkaar kunnen communiceren. Een neuron heeft een axon, waarmee het signalen naar een ander neuron kan versturen, en meerdere dendrieten, waarmee een neuron signalen van andere neuronen kan ontvangen. Neuronen communiceren met elkaar door het uitscheiden van signaalstoffen, neurotransmitters genaamd. Deze neurotransmitters kunnen binden aan bepaalde receptoren in een ontvangende cel, waardoor een signaal doorgegeven kan worden. De punten waar twee cellen met elkaar communiceren worden synapsen genoemd. Hier wordt door de zendende cel een presynaptische terminal gevormd op de ontvangende cel. Vanuit deze presynaptische terminal worden de neurotransmitters uitgescheiden, en deze worden opgevangen door receptoren in de postsynaptische terminal in de ontvangende cel.

Er zijn veel verschillende soorten neuronen, deze worden onder andere in groepen ingedeeld door de neurotransmitter die ze uitscheiden. Een van de groepen neuronen zijn de zogenoemde serotonerge neuronen, neuronen die de neurotransmitter serotonine uitscheiden. Deze neuronen zijn in een aantal clusters gelokaliseerd in de hersenstam. In tegenstelling tot vele andere groepen neuronen, hebben de serotonerge neuronen een aantal opvallende kenmerken. Ten eerste is het een erg kleine groep neuronen, in de menselijke hersenen gaat het ongeveer om een half miljoen cellen. Ten tweede zijn ondanks dit kleine aantal in bijna alle andere hersengedeelten uitlopers van deze serotonerge neuronen aanwezig. Ten derde wordt deze neurotransmitter in deze hersengebieden in grote hoeveelheden uitgescheiden, waardoor grote groepen andere neuronen door deze serotonerge neuronen beïnvloed kunnen worden. Ten vierde zijn deze serotonerge neuronen, het zogenaamde serotonerge systeem, betrokken bij het beïnvloeden van verschillende soorten gedrag. Als laatste is het serotonerge systeem betrokken bij aandoeningen zoals depressiviteit, angststoornissen, autisme en obsessief compulsief gedrag. Een welbekend antidepressivum zoals Prozac grijpt bijvoorbeeld aan op de serotonerge neuronen, door de heropname van uitgescheiden serotonine terug het neuron in te blokkeren.

Het idee achter aandoeningen zoals depressie is dat er een verandering ontstaat in de uitlopers van deze serotonerge neuronen naar andere hersengebieden en dat antidepressiva de uitlopers weer in de oude, gezonde staat terugbrengen. In dit proefschrift hebben we op een aantal verschillende

manieren geprobeerd te kijken naar de dynamiek van de uitgroei van deze serotonerge neuronen.

In **hoofdstuk 2** hebben we uit muizenhersenen, die we als modelsysteem hebben gebruikt, de serotonerge neuronen gesneden in een hersenplakje, een zogenaamde organotypische slice. Dit plakje hebben we naast een plakje van een ander hersengebied, de hippocampus, gelegd om te kijken of in dit kweeksysteem de serotonerge neuronen uitlopers vormden het hippocampale plakje in. Inderdaad bleken de uitlopers van de serotonerge neuronen binnen vier dagen het andere plakje in te groeien. Vervolgens hebben we onderzocht of door toediening van stoffen zoals het hierboven genoemde Prozac de uitgroei van de serotonerge neuronen geremd of juist gestimuleerd werd. Voor de stof Prozac bleek dat het toedienen geen effect had op de uitgroei van de serotonerge neuronen. Echter, toediening van een andere stof die aan een bepaalde serotonerge receptor bindt, bleek een effect te hebben op de serotonerge uitlopers. Na zeven dagen waren er namelijk minder serotonerge uitlopers in het hippocampale plakje dan zonder de toediening van deze stof. Hieruit blijkt dat chronische activatie van deze serotonerge receptor leidt tot een remming in de uitgroei van de serotonerge neuronen.

In **hoofdstuk 3** hebben we een belangrijk eiwit in serotonerge neuronen, het eiwit dat zorgt voor heropname van serotonine in het neuron na afgifte (de serotonine heropname transporter, SERT) voorzien van een rood fluorescerend eiwit (mCherry). Dit zogenaamde fusie-eiwit, mCherry-SERT genaamd, hebben we tot expressie gebracht in neuronen om te onderzoeken hoe de verdeling van dit eiwit er in neuronen uitziet. Het bleek dat dit eiwit in het neuron aanwezig was in vesicles. Dit zijn pakketjes waarmee eiwitten in een neuron getransporteerd worden. Vervolgens hebben we door middel van microscopie 'live' naar de beweging van pakketjes mCherry-SERT gekeken. Uit de analyse van de snelheid en de richting van de beweging bleek dat de pakketjes zich voortbewogen met een gemiddelde snelheid van 1.2 µm/s (=4.32 mm/uur) en dat de pakketjes geen voorkeur hadden voor de richting waarin ze zich voortbewogen in de uitlopers.

In **hoofdstuk 4** hebben we een soortgelijke aanpak gekozen als in hoofdstuk 3. Echter, nu hebben we gekeken naar het eiwit dat belangrijk is voor de aanmaak van serotonine, tryptophan hydroxylase 2 (Tph2). Dit eiwit hebben we voorzien van een groen fluorescerend eiwit, (Enhanced Green Fluorescent Protein, EGFP). Dit fusie-eiwit hebben we tot expressie gebracht in neuronen om de verdeling van dit eiwit, ook in vergelijking met mCherry-SERT, te onderzoeken. Hieruit bleek dat, in tegenstelling tot mCherry-SERT, Tph2-EGFP zich door de hele cel bevond, en niet in vesicles zat. In jonge neuronen had Tph2-EGFP geen voorkeur voor een bepaald deel van de cel. In oudere neuronen echter, bleek dat Tph2-EGFP voornamelijk aanwezig was in presynaptische

terminals, maar tegelijkertijd ook in de dendrieten. Ook in deze cellen hebben we door middel van microscopie live naar de beweging van Tph2-EGFP gekeken. Hieruit bleek dat Tph2-EGFP, in tegenstelling tot mCherry-SERT, zich niet in vesicles bevond en daardoor ook geen beweging liet zien.

In **hoofdstuk 5** hebben we onderzocht of een variatie in een gen dat codeert voor het eiwit Piccolo, effect heeft op de verdeling en dynamiek van het eiwit SERT. Deze variatie in het gen wordt geassocieerd met depressie, waardoor het erg interessant is om te onderzoeken of de variatie in dit gen verantwoordelijk is voor een verandering in de dynamiek van het serotonine systeem.

Hiertoe hebben we eerst onderzocht of het eiwit Piccolo voorkwam in de serotonerge neuronen en uitlopers, en dit bleek inderdaad het geval. Vervolgens hebben we een zogenaamde knockdown construct gemaakt waarmee de hoeveelheid Piccolo in een cel grotendeels gereduceerd kan worden. Na het tot expressie brengen van dit construct in neuronen bleek inderdaad dat de hoeveelheid Piccolo in een cel met meer dan 80% gereduceerd was. Vervolgens hebben we onderzocht of een vermindering in de hoeveelheid Piccolo van invloed is op de beweging en dynamiek van het eiwit SERT. Om dit te onderzoeken hebben we mCherry-SERT tot expressie gebracht in neuronen waarin de hoeveelheid Piccolo gereduceerd was en hebben we door middel van live microscopie naar de dynamiek van het eiwit mCherry-SERT gekeken. Dit hebben we vergeleken met neuronen waarin de hoeveelheid Piccolo niet gereduceerd was. We concludeerden uit deze vergelijking dat een vermindering in de hoeveelheid Piccolo geen effect heeft op de beweging en snelheid van bewegen van mCherry-SERT. Als laatste hebben we gekeken, in een ander cel type, of de mutante variant van het eiwit Piccolo, in vergelijking met de normale variant de verdeling van het eiwit SERT beïnvloedt. Om dit te onderzoeken hebben we de mutante variant van Piccolo en mCherry-SERT in cellen tot expressie gebracht en gekeken of de verdeling van mCherry-SERT veranderd was. In cellen waarin mCherry-SERT tot expressie gebracht was met de normale variant van Piccolo was mCherry-SERT, zoals verwacht, gelokaliseerd aan het membraan. Na expressie van mCherry-SERT met de mutante variant van Piccolo bleek echter dat de hoeveelheid mCherry-SERT aan het membraan vermindert was. Hieruit concludeerden wij dat mogelijk in serotonerge neuronen de mutante variant van het eiwit Piccolo ervoor zorgt dat er een verandering is in de hoeveelheid SERT aan het membraan. Hierdoor zou de signaaldoorgifte van serotonerge neuronen naar andere neuronen verstoord kunnen zijn.

In **hoofdstuk 6** hebben we onderzocht wat het effect is van het uitschakelen van het serotonine systeem in de muis. Om dit te onderzoeken hebben we door muizen te kruisen een muis verkregen waarbij een gen in het serotonine systeem uitgeschakeld is. Uit onderzoek naar de embryonale ontwikkeling van het serotonerge systeem in deze muizen bleek dat het serotonerge systeem in eerste

instantie tot ontwikkeling kwam. De serotonerge neuronen waren aanwezig, en serotonerge vezels groeiden uit naar andere hersengebieden. We hebben het serotonerge systeem ook bestudeerd iets later in de hersenontwikkeling van deze muizen. Dit toonde aan dat de serotonerge neuronen een veranderde morfologie vertoonden, waaruit afgeleid kan worden dat deze neuronen degenereren. Uiteindelijk zijn bijna al de serotonerge neuronen verdwenen. Ook de projecties van deze neuronen verdwenen later tijdens de hersenontwikkeling. Hieruit concludeerden wij dat het uitschakelen van dit gen uiteindelijk leidt tot een verdwijning van de serotonerge neuronen. Vervolgens hebben we gekeken wat het effect hiervan is op de ontwikkeling en het overleven van deze muizen. Dit toonde aan dat de meeste muizen vlak na de geboorte doodgingen. Enkele muizen die wel overleefden waren kleiner dan controle muizen die dit gen nog wel hadden, en gingen uiteindelijk ook binnen drie weken dood. We waren ook geïnteresseerd of het gedrag van de muizen veranderd was. Echter, aangezien de meeste muizen al snel na de geboorte dood gingen, was dit niet mogelijk. Daarom hebben we het gedrag geanalyseerd van muizen die nog een kopie van dit gen hadden, en dit vergeleken met controle muizen. Om het gedrag van deze muizen te analyseren hebben we deze muizen zeven dagen in een speciale kooi gehouden. Deze kooien bevatten een videocamera zodat later een groot aantal parameters geanalyseerd kan worden, zoals de hoeveelheid beweging, hoe vaak deze muizen drinken of hoeveel deze muizen eten. Verder konden we ook nog het angst gerelateerde gedrag en het leergedrag van deze muizen analyseren. Uit analyses van muizen met een kopie van het gen bleek dat deze geen veranderd gedrag vertoonden ten opzichte van controle muizen.

Het doel in **hoofdstuk 7** was om een muis te maken waarbij alle serotonerge neuronen en uitlopers naar andere hersengebieden gelabeld waren. Om dit doel te bereiken hebben we een gen gebruikt dat specifiek tot expressie komt in serotonerge neuronen, Tph2. Een stuk DNA voor een gen, een promoter genoemd, is verantwoordelijk voor waar en wanneer het gen afgeschreven wordt. Achter de promoter van Tph2 hebben een eiwit genaamd Cre recombinase gezet, zodat de expressie van Cre recombinase wordt gereguleerd door de Tph2 promoter en zodoende alleen tot expressie komt in serotonerge neuronen. In dit hoofdstuk hebben we beschreven hoe we embryonale stamcellen gemanipuleerd hebben en hoe we deze hebben geïnjecteerd in muizen blastocysten. Helaas is het nog niet gelukt om deze muis te genereren. Om specifiek serotonerge neuronen te labelen hebben we nog een aantal andere muizen gemaakt, waarbij het eiwit alleen aangemaakt wordt als in de cell Cre recombinase tot expressie komt. Door deze muizen met elkaar te kruisen zou Cre recombinase alleen in serotonerge neuronen ‘aan’ staan, en zou alleen in deze neuronen een zogenaamd reporter eiwit aangezet worden. Op deze manier hopen we uiteindelijk muizen te hebben waarbij specifiek in serotonerge neuronen reporter genen tot expressie komen,

waardoor we deze uiteindelijk kunnen gebruiken om in levende muizen naar de dynamische uitgroei en connectiviteit van het serotonerge systeem te kijken. Verder kunnen we dan onderzoeken of manipulatie aan dit systeem leidt tot een verandering in uitgroei van serotonerge neuronen en tot muizen die bepaalde aspecten van humane depressiviteit nabootsen. Aan de andere kant kunnen we symptomen van humane depressiviteit in muizen induceren, en onderzoeken of dit leidt tot veranderingen in het serotonerge systeem. Uiteindelijk zouden we op deze manier ook kunnen onderzoeken of medicatie tegen depressiviteit leidt tot een verandering in de connectiviteit van het serotonerge systeem en deze weer terugbrengt in de oude staat.

List of abbreviations

5-HIAA	5-hydroxyindolacetic acid
5-HT	serotonin
5-HT	5-hydroxytryptamine
8-OH-DPAT	8-hydroxy-2-(di-*n*-propylamino)tetraline
BOLD	blood oxygen level dependent
cAMP	cyclic adenosine monophosphate
CSF	cerebrospinal fluid
DAT	dopamine transporter
dGBSS	dissection Gey's balanced salt solution
DIV	days in vitro
DOI hydrochloride	(+-)-2,5-dimethoxy-4-iodoamphetamine hydrochloride
DRN	dorsal raphe nucleus
E	embryonic day
EGFP	enhanced green fluorescent protein
ES	embryonic stem
fMRI	functional magnetic resonance imaging
GIRK	G protein-activated inwardly rectifying K^+
HBSS	Hanks Balanced Salt Solution
iCre	improved Cre recombinase
IP3	inositol 3-phosphate
IRES	internal ribosomal entry site
KO	knockout
l	long
LAT1	large neutral amino acid transporter
LDCV	large dense core vesicle
Lmx1	LIM homeobox transcription factor 1
LSD	lysergic acid diethylamide
LSL	loxSTOPlox
MAO-A	monoamine Oxidase-A
MAP2	microtubule associated protein 2
MDMA	3,4-methylenedioxy-N-methylamphetamine
MHO	midbrain-hindbrain organizing centre
MRN	median raphe nucleus
neo	neomycin phosphotransferase cassette
OCD	obsessive-compulsive disorder
P	postnatal day
PBS	phosphate buffered saline
PCM	presynaptic cytoskeletal cytomatrix

PCPA	p-chlorophenylalanine
PET	positron emission tomography
Pet1	PC12 Ets factor
PFA	paraformaldehyde
PKA	protein kinase A
PKC	Protein kinase C
PKG	protein kinase G
β-PMA	β-phorbol 12-myristate 13-acetate
RRP	readily releasable pool
s	short
SEM	standard error of mean
SERT	5-HT reuptake transporter
SMB	short movement bouts
SNP	short nucleotide polymorphism
SSRI	selective serotonin reuptake inhibitor
SSV	small synaptic vesicle
Tetrahydrobiopterin BH4	(6R)-L-erythro-5,6,7,8-Tetrahydrobiopterin
TH	tyrosine hydroxylase
tPA-GFP	tissue plasminogen activator-GFP
Tph	tryptophan hydroxylase
Tph1	tryptophan hydroxylase 1
Tph2	tryptophan hydroxylase 2
VAMP2	synaptobrevin-2
VMAT2	Vesicular Monoamine Transporter 2

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Jeroen

Hoofddorp, Juli 2010

Curriculum vitae

Jacobus Johannis Dudok werd geboren op 13 juni 1981 te Zierikzee. In 1993 begon hij aan zijn VWO opleiding aan het Buys Ballot College te Goes, waar hij in 1999 zijn Gymnasium Beta diploma haalde. In augustus 1999 begon hij aan de Bachelor Biomedische Wetenschappen aan de Vrije Universiteit te Amsterdam. In deze opleiding liep hij zijn Bachelor stage van februari 2002-juni 2002 aan de vakgroep pathologie in het VU medisch centrum. Hier bestudeerde hij, begeleidt door Dr. R. Veerhuis en Ing. E. van Elk, de verschillende aggregatie vormen van Aβ1-42, het eiwit betrokken bij de ziekte van Alzheimer. In september 2002 begon hij aan de Master of Neurosciences aan de Vrije Universiteit te Amsterdam. Tijdens deze master deed hij twee stages bij de afdeling Functionele Genoomanalyse. Tijdens de eerste masterstage deed hij onderzoek naar de calcium afhankelijke translocatie van het presynaptische eiwit Doc2B, begeleidt door Dr. S. Groffen. Tijdens zijn tweede masterstage, begeleidt door Ing. R. Zalm, cloneerde hij een targeting construct en gebruikte deze om muis embryonale stamcellen mee te targetten. Zijn masterscriptie schreef hij onder leiding van Dr. J. Pronk en ging over de in silico clonering en karakterisatie van het eiwit Tph2. In augustus 2004 studeerde hij af aan de Vrije Universiteit te Amsterdam. Van oktober 2004 tot april 2009 deed hij zijn promotieonderzoek naar de uitgroei en connectiviteit van het serotonerge systeem. Dit onderzoek deed hij onder begeleiding van professor Verhage aan de vakgroep Functionele Genoomanalyse aan de Vrije Universiteit te Amsterdam. De resultaten van dit promotieonderzoek zijn in dit proefschrift beschreven. Vanaf april 2009 werkt hij als postdoctoraal onderzoeker op het Nederlands Instituut voor Neurowetenschappen (NIN) bij de vakgroep Neuromedische Genetica onder leiding van Dr. J. Wijnholds. Hier doet hij onderzoek naar de functionele rol van het eiwit MPP3 in het Crumbs complex en in neuronale cellen.

English version

Jacobus Johannis Dudok was born on the 13th of June 1981 in Zierikzee, the Netherlands. In 1993 he started his secondary education at the Buys Ballot College in Goes, where he graduated in 1999. In august 1999 he started the Bachelor Medical Biology at the Vrije Universiteit Amsterdam. During this Bachelor he carried out a research project from February 2002 to June 2002 at the research department of pathology at the VU medical center. Here he studied the different aggregation states of Aβ1-42, the peptide involved in Alzheimer's disease, supervised by Dr. R. Veerhuis and Ing. E. van Elk. In September 2002 he started the Master of Neuroscience at the Vrije Universiteit Amsterdam. During this master he carried out two research projects at the department of Functional Genomics. During his first research project he studied the calcium dependent translocation of the presynaptic protein Doc2B, supervised by Dr. S. Groffen. During his second research project he constructed a targeting construct and used this to target mouse embryonic stem cells, supervised by Ing. R. Zalm. He wrote his Master Thesis under supervision of Dr. J. Pronk about the in silico cloning and characterization of the protein Tph2. In august 2004 he graduated at the Vrije Universiteit Amsterdam. From October 2004 until April 2009 he did his PhD project on the outgrowth and connectivity of the serotonin system. This PhD project was supervised by professor Verhage and carried out at the department of Functional Genomics at the Vrije Universiteit Amsterdam. The results of that study are described in this thesis. He is currently working as a postdoctoral fellow at the Netherlands Institute for Neuroscience (NIN) in the department of Neuromedical Genetics headed by Dr. J. Wijnholds. Here he studies the functional role of the protein MPP3 in the Crumbs complex and in neuronal cells.