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EVALUATION OF MOLECULAR, CELLULAR AND BEHAVIORAL CONSEQUENCES OF SEROTONIN 1A RECEPTOR DELETION

Fülling Christine

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Faculté de biologie
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Département des Neurosciences Fondamentales

EVALUATION OF MOLECULAR, CELLULAR AND BEHAVIORAL CONSEQUENCES OF SEROTONIN 1A RECEPTOR DELETION

Thèse de doctorat en Neurosciences

présentée à la

Faculté de Biologie et de Médecine
de l'Université de Lausanne

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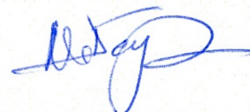

pour le Doyen
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Summary

In 1998, three different research groups simultaneously reported increased anxiety-related behavior in tests of conflict in their serotonin 1a (5-HT_{1a}) receptor knockout (KO) line with male mice being more severely affected by 5-HT_{1a} receptor deletion than female KO. Similarly, in the hippocampus, we observed increased dendritic complexity in the *stratum radiatum* of CA1 pyramidal neurons in male but not in female 5-HT_{1a} receptor KO mice. These observations prompted us to investigate gender-dependent differences of 5-HT_{1a} receptor deletion in hippocampal-related behavioral tasks. Testing our mice in anxiety-related paradigms, we reproduced the original studies showing increased anxiety-related behavior in male 5-HT_{1a} receptor KO mice when compared to male WT mice, but no difference between female 5-HT_{1a} receptor KO and WT mice. Similarly, male 5-HT_{1a} receptor KO mice were impaired in association of aversive stimuli fear conditioning paradigms. We argue that increased dendritic complexity and increased synaptic strength of CA3-CA1 synapses in the *stratum radiatum* impaired proper signal propagation attributed to overactivation of CA1 pyramidal neurons leading to impaired fear memory of male 5-HT_{1a} receptor KO mice. Similar mechanisms in the ventral hippocampus are likely to have contributed to gender-dependent differences in anxiety-related behavior in our and the original studies from 1998. In this study, we started to shed light on the 5-HT_{1a} receptor downstream signaling pathways involved in dendritogenesis of pyramidal neurons during early postnatal development. We could show that NR2B-containing NMDA receptor during development acts downstream of 5-HT_{1a} receptor and is responsible for increased amount of branching in male 5-HT_{1a} receptor KO mice. Conversely, protein and NR2B mRNA expression was increased in 5-HT_{1a} receptor KO mice at P15. Although the exact signaling cascade of 5-HT_{1a} receptor regulating NR2B-containing NMDA receptor has not been determined, CaMKII is a potential downstream effector to influence transportation and removal of NR2B-containing NMDA receptors to and from the synapse. In contrast, Erk1/2 likely acts downstream of NR2B-containing NMDA receptors and was shown to be sufficient to regulate dendritic branching. Moreover, increased NR2B-containing NMDA receptor mediated cell death via excitotoxicity during development and is likely to be involved in reduced survival of adult born neurons in the hippocampus of 5-HT_{1a} receptor KO male. The convergence of 5-HT_{1a} receptor signaling onto NR2B-containing NMDA receptor signaling enables estrogen to interfere with its downstream pathway via G-protein coupled estrogen receptor 1 activation resulting in normalization of branching and behavior in female 5-HT_{1a} receptor mice. In conclusion, our data strongly suggests a hormone-regulated mechanism that by converging on NR2B-containing NMDA receptor signaling is able to normalize morphology of pyramidal neurons and behavior of female 5-HT_{1a} receptor KO mice. Our findings provide a possible explanation for gender-dependent differences in the occurrence of mental disorders with 5-HT_{1a} receptor abnormalities as a strong predisposing factor.

Résumé

En 1998, trois équipes de recherche ont décrit un comportement de type anxieux dans des tests de conflit pour leur souris transgéniques avec une délétion du gène pour le récepteur $5\text{-}HT_{1a}$ de la sérotonine. De plus, les trois groupes rapportent un phénotype plus sévère pour le comportement anxieux chez les souris transgéniques mâles que femelles. Dans l'hippocampe, la région avec la densité de récepteur $5\text{-}HT_{1a}$ la plus élevée dans le télencéphale, nous avons observé dans le *stratum radiatum* une complexité accrue des arborisations dendritiques des neurones pyramidaux du secteur CA1 chez les souris transgénique mâles mais pas chez les femelles. Cette observation nous a encouragés à initier cette étude sur les différences en fonction du genre utilisant les tests comportementaux en rapport avec les fonctions de l'hippocampe chez les souris déficientes pour le récepteur $5\text{-}HT_{1a}$.Testant nos souris avec des paradigmes associés à l'anxiété, nous avons reproduit les données originales montrant que les souris transgéniques mâles ont un phénotype plus sévère que les souris mâles sauvages, mais qu'aucune différence n'est observée entre les femelles sauvages et transgéniques. De même, les souris mâles déficientes pour le récepteur $5\text{-}HT_{1a}$ sont handicapées dans les tests de conditionnement au stress avec des stimuli aversifs. Nous faisons l'hypothèse que l'augmentation de la complexité de l'arborisation dendritique et l'augmentation de la force du signal synaptique entres les régions CA3 et CA1 de l'hippocampe dans le *stratum radiatum* perturbe la propagation du signal nerveux qui conduit à l'hyperactivation des neurones du secteur CA1. Ceci conduit à une mémoire de stress altérée chez les souris mâles déficientes pour le récepteur $5\text{-}HT_{1a}$. Un mécanisme similaire dans l'hippocampe ventral contribue probablement aux différences en fonction du genre dans les tests pour le comportement de type anxieux qui ont été rapportés dans les études originales de 1998. Les mesures de protéine et de mRNA ont mis en évidence une augmentation de l'expression du récepteur NMDA contenant la sous-unité NR2B dans les souris déficientes pour le récepteur $5\text{-}HT_{1a}$ à P15. Dans les cultures organotypiques d'hippocampe, nous avons commencé à disséquer les messagers secondaires à l'activation du récepteur $5\text{-}HT_{1a}$ qui sont impliqués dans la régulation de la croissance dendritique des neurones pyramidaux pendant la période postnatale précoce. Nous avons démontré que les récepteurs NR2B sont en aval de l'activation du récepteur $5\text{-}HT_{1a}$ et qu'ils sont impliqués dans l'accroissement du nombre de dendrites chez la souris mâle déficiente pour le récepteur $5\text{-}HT_{1a}$. Bien que la cascade de signalisation du récepteur $5\text{-}HT_{1a}$ pour réguler les récepteurs NMDA contenant le NR2B ne soit pas établie, CaMKII est identifié comme un effecteur potentiel pour altérer le transport du récepteur NMDA à la synapse. D'autre part, Erk1/2 est probablement un messager en aval du NR2B du récepteur NMDA, et a été documenté comme suffisant pour réguler l'arborisation dendritique. L'augmentation de NR2B à la synapse des souris déficientes pour le récepteur $5\text{-}HT_{1a}$ peut conduire à une augmentation de l'excitotoxicité dans les cellules. Nous avons observé une augmentation chez la souris déficiente pour le récepteur $5\text{-}HT_{1a}$ de la

mort cellulaire dans des tranches d'hippocampe stimulées, ce qui peut être en relation avec la réduction de la survie des neurones générés dans l'hippocampe de la souris mâle transgénique adulte par rapport à la souris mâle sauvage. De plus, la convergence de la signalisation du récepteur 5-HT_{1a} sur la signalisation de la sous-unité NR2B du récepteur NMDA permet à l'œstrogène d'interférer avec sa voie de signalisation du récepteur de l'œstrogène couplé à une protéine G (GPER-1), ceci permettant à l'œstrogène de réduire la taille de l'arborisation des neurones pyramidaux de CA1 chez la femelle de la souris déficiente pour le récepteur 5-HT_{1a}.

En conclusion, nos observations suggèrent fortement qu'un mécanisme hormonal convergeant sur la voie de signalisation de la sous-unité NR2B du récepteur NMDA permet la normalisation de l'exubérance des dendrites des neurones CA1 de l'hippocampe et du comportement des souris femelles déficientes pour le récepteur 5-HT_{1a}. Ceci donne une explication possible pour la différence en fonction du genre dans l'apparition de troubles mentaux avec les variations du récepteur 5-HT_{1a} comme facteur de prédisposition important.

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List of abbreviations

4,6 diamidino-2-phenylindole	DAPI/Höchst
5-HT	5-hydroxytryptamine (serotonin)
5-HT_{1a}	serotonin 1a receptor
bp	base pair
BrdU	Bromodeoxyuridine
CA1	cornus ammonis 1
CA3	cornus ammonis 3
CaMKII	calcium-calmodulin-dependent-kinase II
cAMP	cyclic adenosine monophosphate
CK2	casein-kinase 2
Ct	cycle threshold
DAPK1	death associated protein kinase 1
DG	dentate gyrus
e	embryonic day
Erk1/2	extracellular signal-regulate kinase 1/2
fEPSP	field excitatory postsynaptic potential
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GIRK	G-protein coupled inwardly-rectifying potassium channel
IFEN	Ifenprodil
i.p.	intraperitoneal
ISH	*in situ* hybridization
KO	knockout
lx	lux
MAP2	microtubule associated protein 2
MEK	MAPK/Erk kinase
NHS	normal horse serum
NMDA	N-methyl D-aspartate
NR2A	N-methyl D-aspartate receptor subtype 2A
NR2B	N-methyl D-aspartate receptor subtype 2B
OF	open field
p	postnatal day
PBS	phosphate buffered saline
PCR	polymerase-chain-reaction
PFA	paraformaldehyde
PI	propidium iodid
PKA	protein kinase A
RasGRF1	RAS protein-specific guanine nucleotide-releasing factor 1
REST	RE1-Silencing Transcription factor
rpm	rounds per minute
SIH	stress induced hyperthermia
WT	wild-type

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I. Introduction

I.1 SEROTONERGIC SYSTEM

Serotonin (5-hydroxytryptamine, 5-HT) is an important neurotransmitter in mammals. It is released in both the peripheral and central nervous system and, hence, is involved in several biological processes. In the periphery, 5-HT is involved in cardiovascular function, bladder control, bowel motility and in many more physiological functions (Berger *et al*, 2009). In the central nervous system, 5-HT is implicated in the regulation of a variety of autonomic functions, such as energy balance, food intake and body temperature. Motor activity, sleep wake cycle and sexual behavior also depend on serotonergic drive. Moreover, 5-HT has a strong impact on higher cognitive functions, such as learning and memory, and dysregulation of 5-HT signaling is linked to anxiety and mood disorders (Nordquist and Oreland, 2010).

5-HT is synthesized from its precursor, amino acid L-tryptophan, by the enzymes tryptophan hydroxylase (Tph) and amino acid decarboxylase, whereby Tph is the rate-limiting enzyme of 5-HT synthesis. Tph exists in two different isoforms, Tph1 and Tph2, which are found in different locations of the body. Tph2 is responsible for 5-HT synthesis in the adult brain, whereas Tph1 is present in diverse tissues throughout the body. In the brain, 5-HT is produced in the raphe nuclei located along the midline of the brainstem. The raphe nuclei are classified in 9 nuclei (B1-9), and give rise to ascending and descending projections, thereby providing modulatory input to numerous networks (Muzerelle *et al*, 2014). The more caudal nuclei (B1-3) in the medulla send descending projections to the spinal cord, where among others they influence pain sensitivity (Viguier *et al*, 2013). The more rostrally located nuclei in the pons and midbrain (B5-B9) send serotonergic projections to the forebrain. The degree to which each of these nuclei innervates subregions of the forebrain differs greatly, with some brain regions receiving inputs from multiple nuclei. The hippocampus, for example, is innervated by afferent serotonergic projections from the median raphe nucleus and dorsal raphe nucleus. The raphe nuclei themselves receive inputs among others from the lateral habenula (Behzadi *et al*, 1990; Peyron *et al*, 1998), the medial septum and diagonal band nuclei (Kalen and Wiklund, 1989) as well as from several hypothalamic nuclei (Behzadi *et al*, 1990) and the medial prefrontal cortex (mPFC) (Celada *et al*, 2001).

In the adult brain, the activity of neurons in the raphe nuclei, and thus the release of 5-HT in the target areas, is self-regulated through 5-HT_{1a} and 5-HT_{1b} autoreceptors expressed in somatodendritic compartments of serotonergic cells, which downregulate release of 5-HT from nerve terminals upon activation. A further control is provided by an indirect negative feedback loop from the mPFC. More specifically, stimulation of serotonergic raphe neurons by afferents from the mPFC leads to 5-HT release, followed by inhibition of dorsal raphe neurons via the somatodendritic 5-HT_{1a} autoreceptors.

Furthermore, activation of GABAergic (gamma-aminobutyric acid, GABA) interneurons reinforces inhibition of serotonergic raphe neurons and decreases the amount of 5-HT release from the raphe (Figure 1). Likewise, activation and 5-HT release from the dorsal raphe nucleus can partially control mPFC activity by acting on cortical postsynaptic 5-HT$_{1a}$ receptors (Altieri *et al*, 2013; Celada *et al*, 2001).

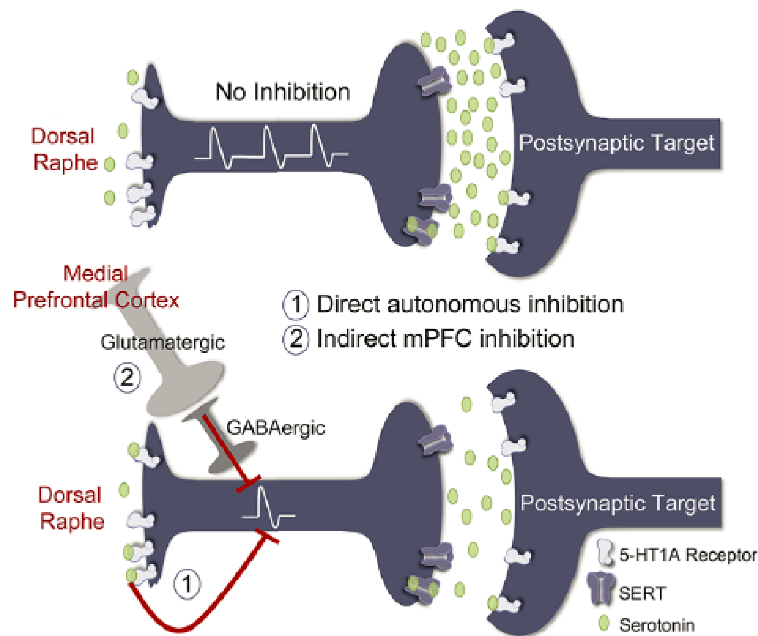

Figure 1 Synapse of serotonergic neurons releasing 5-HT in the forebrain.
Top, activation of serotonergic neurons releases 5-HT to the extrasynaptic cleft. Bottom, autoinhibitory feedback mechanism of 5-HT release via 5-HT$_{1a}$ autoreceptor stimulation and through descending glutamatergic projections from the mPFC inhibiting serotonergic firing by activating GABAergic interneurons. Figure from Altieri *et al* (2013)

5-HT RECEPTORS

The widespread serotonergic innervation of the brain allows the serotonergic system to modulate diverse biological functions. The versatile functions are mediated by at least 14 different receptor types (Barnes and Sharp, 1999). These are divided into 7 subgroups (5-HT$_1$ - 5-HT$_7$) based on their primary signaling pathway, structural features and pharmacological profile (Naumenko *et al*, 2014). Apart from 5-HT$_3$ receptors, which are ligand-gated ion channels, all other 5-HT receptors selectively couple to specific guanine nucleotide-binding proteins (G-protein). 5-HT$_1$ receptors, for example, classically bind inhibitory G-proteins, whereas receptors from the 5-HT$_2$ subfamily in general couple to G$_q$ proteins activating phospholipase C (PLC). 5-HT$_4$, 5-HT$_6$ and 5-HT$_7$ receptors couple to stimulatory G-proteins and, in contrast to 5-HT$_1$ receptors, activate adenylate cyclase. The diversity of 5-HT receptors and their different locations enable the serotonergic system to influence physiological functions in a very precise manner. Among all the 14 different receptors, the 5-HT$_{1a}$ receptor has been intensively studied, as it was the first cloned 5-HT receptor (Kobilka *et al*, 1987) and because of its implication in

the mechanism of action of antidepressants. Already in the 1970s, data from human studies on buspirone suggested the implication of this receptor subtype in anxiety and depression (Olivier *et al*, 2001).

5-HT$_{1a}$ receptor

Expression

The 5-HT$_{1a}$ receptor is highly abundant in the brain and shows similar patterns of distribution across mammals (Olivier *et al*, 2001). Nevertheless, there might be differences in the laminar distribution concerning the level of expression when comparing rodents to humans (Burnet *et al*, 1995). 5-HT$_{1a}$ receptors can be divided into two distinct classes based on their location: the auto- and heteroreceptor population. Autoreceptors can be found on serotonergic neurons in the dorsal and median raphe nuclei of the brainstem controlling the excitability of serotonergic cells, and thus the release of serotonin from their nerve terminals in the forebrain. For this reason, these autoreceptors are referred to as presynaptic 5-HT$_{1a}$ receptors. Postsynaptic heteroreceptors are expressed on excitatory and inhibitory neurons in the forebrain, where they modulate neuronal excitation and firing in response to 5HT release

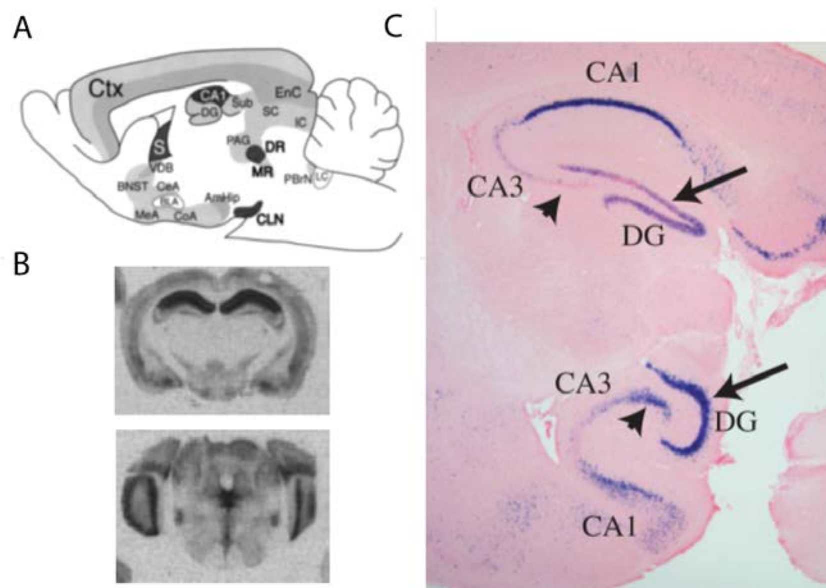

Figure 2 Expression of 5-HT$_{1a}$ receptors in the brain.
Schematic drawing of 5-HT$_{1a}$ receptor expression in the mouse brain (A). Images of ^{125}I-MPPI receptor autoradiography depicting ligand binding of 5-HT$_{1a}$ receptor in the hippocampus and the raphe nuclei (B). *In situ* hybridization of 5-HT$_{1a}$ receptor mRNA in the dorsal and ventral hippocampus shows differences in receptor expression patters (C). Figures taken from Gross *et al* (2000) (A), Gross *et al* (2002) (B) and Tanaka *et al* (2012) (C).

from serotonergic neurons (Polter and Li, 2010). Therefore, 5-HT_{1a} receptors have the dual ability to regulate the global 5-HT levels and to impact local excitability of postsynaptic cells in response to the local 5-HT release. In the forebrain, 5-HT_{1a} receptors show highest density in the hippocampus with high expression in CA1 and granule cell layer of the dentate gyrus compared to relatively weak expression in the CA3 (Zhou *et al*, 1999). Within the CA1 region, high abundance of 5-HT_{1a} receptors can be found in the *stratum radiatum* and *lacunosum moleculare*, while low expression levels are found in the pyramidal layer (Beck *et al*, 1992; Zhou *et al*, 1999). However, the expression along the dorsoventral axis of the hippocampus is not homogeneous. 5-HT_{1a} receptor expression is stronger in the dorsal CA1 region than in the ventral part and shows opposite patterns for the dentate gyrus, with higher expression in the ventral vs. dorsal part (Figure 2, C) (Tanaka *et al*, 2012). In addition to the hippocampal expression, high levels of 5-HT_{1a} receptor expression are seen in the entorhinal and frontal cortex as well as in the lateral septum (Barnes *et al*, 1999; Santana *et al*, 2004). Moderate receptor densities have been observed in some hypothalamic nuclei as well as very low levels of expression in some thalamic nuclei (Polter *et al*, 2010). Mild 5-HT_{1a} receptor expression can also be found in the olfactory bulb and amygdala (Chalmers and Watson, 1991; Polter *et al*, 2010; Pompeiano *et al*, 1992). However, receptor expression in the central and basolateral nuclei of the amygdala as identified by mRNA expression in the rat brain is very low (Chalmers *et al*, 1991; Pompeiano *et al*, 1992), and barely detectable in mice of 129Sv and C57BL/6 background when applying 125I-MPPI receptor autoradiography (Figure 2, A & B) (Gross *et al*, 2000; Li *et al*, 2012). Studies combining *in situ* hybridization and receptor autoradiography showed strong correlations between the 5-HT binding sites and mRNA receptor expression. This data suggested that 5-HT_{1a} receptor is predominantly expressed somatodendritically (Chalmers *et al*, 1991; Pompeiano *et al*, 1992). Subsequent studies on receptor membrane targeting and subcellular localization of the 5-HT_{1a} receptor confirmed these findings (Carrel *et al*, 2006; Carrel *et al*, 2008; Darmon *et al*, 1998; Langlois *et al*, 1996).

SIGNAL TRANSDUCTION

The 5-HT_{1a} receptor is a seven-transmembrane $G_{i/o}$-protein coupled receptor primarily interacting with adenylyl cyclase. When activated, GDP is exchanged with GTP at the α-subunit of the G_i-protein, which results in decreased amounts of cyclic adenosine monophosphate (cAMP) and reduced protein kinase A (PKA) activity (De Vivo and Maayani, 1986). The βγ-subunits of the G_i-protein-isoform, on the other hand, mediate activation of G-protein-coupled inwardly-rectifying potassium (GIRK) channels via direct interaction with the ion channel (Andrade and Nicoll, 1987). In turn, GIRK activation results in quick hyperpolarization and decreased firing of the neuron. In combination with 5-HT_{1a} receptor-dependent reduction of calcium currents and evoked calcium influx (Cheng *et al*, 1998), these two mechanisms

enable 5-HT to rapidly limit neuronal excitability and firing via 5-HT_{1a} receptors (Polter *et al*, 2010). Another major signaling pathway associated with 5-HT_{1a} receptor is the mitogen-activated protein kinase (MAPK) signaling pathway, normally regulated by growth factors. Of the various MAPK known, the extracellular signal-regulated protein kinase (Erk) is most influenced by 5-HT_{1a} receptor signaling. Other growth factor-regulated signaling pathways, such as the phosphatidylinositol-4,5-bisphosphate 3-kinase/protein kinase B (PI3K/Akt) pathway, can also be regulated by 5-HT_{1a} receptor. Furthermore 5-HT_{1a} receptor has been shown to regulate Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) via PKA (Cai *et al*, 2002). Figure 3 shows an overview of 5-HT_{1a} receptor signaling.

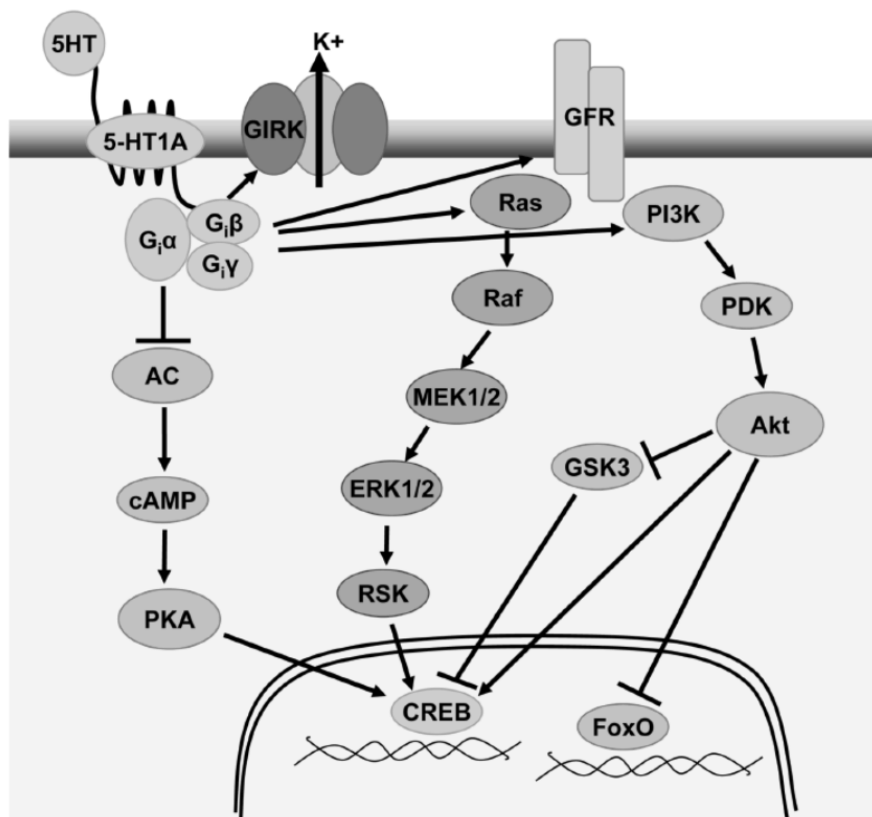

Figure 3 Signal transduction pathways of 5-HT_{1a} receptor.
Many different G-protein-dependent pathways can be activated by 5-HT_{1a} receptor. Depending on the brain region, the age of the animal and the availability of substrates, either of the downstream pathways will be preferentially activated. Gi, inhibitory Guanine nucleotide binding protein; AC, adenylate cyclase; cAMP, response element binding protein; PKA, protein kinase A; GIRK, G-protein coupled inward rectifying potassium channel; GFR, growth factor receptor; MEK1/2, MAPK/Erk kinase 1/2; Erk1/2, extracellular signal-regulated kinase 1/2; RSK, ribosomal S6 kinase; PI3K, phosphotidylinositol-3 kinase; CREB, cAMP response element binding protein; PDK, phosphoinositide dependent kinase; GSK3, glycogen synthase kinase-3; FoxO, forkhead box O transcription factors. Figure from Polter *et al* (2010)

Although the molecular organization of 5-HT_{1a} receptor transduction does not differ between brain regions, pharmacological studies reported brain region-specific effects of agonist and antagonists (Lanfumey and Hamon, 2000). These differences probably result from brain region specific interaction with different G-proteins. For example, 5-HT_{1a} autoreceptors in the anterior raphe preferentially couple

with $G_{\alpha i3}$-proteins, whereas heteroreceptors in the hippocampus mostly interact with $G_{\alpha o}$-proteins (Mannoury la Cour *et al*, 2006), explaining the differences in functional and regulatory properties of 5-HT_{1a} receptors in these brain regions. Furthermore, availability of downstream effectors such as PKA and Erk1/2 plays an important role for the signal transduction pathway. As described by Mehta *et al* (2007), 5-HT_{1a} receptor signaling can change during development, due to alterations in the enzymes involved in the downstream cascade. While protein kinase C ε (PKC ε) is recruited for activation of Erk1/2 signaling at postnatal day 6 (P6), a different PKC isoform (PKCα) is needed downstream of Erk1/2 from P15 onwards. Furthermore, coupling of 5-HT_{1a} receptor with GIRK, at least in the rat prefrontal cortex, does not occur before P14 (Beique *et al*, 2004), indicating that MAPK signaling pathways are a major target of 5-HT during the first postnatal weeks. Thus, the expression of 5-HT_{1a} receptors is not the only decisive factor for serotonergic signaling, but also the developmental profile of the effector proteins in different brain areas determines the extent of the postsynaptic effect of serotonin.

SIGNAL TRANSDUCTION IN THE HIPPOCAMPUS

In this study, we will focus on the hippocampus, as one of the regions in the forebrain with the highest expressions of 5-HT_{1a} receptors. In the hippocampus, the 5-HT_{1a} receptor can be found in excitatory pyramidal neurons as well as inhibitory interneurons (Olivier *et al*, 2001), adding another level of complexity to 5-HT_{1a} receptor signaling. As described above, different pathways can be activated due to the location of the receptor and the substrate availability. In the hippocampus, 5-HT_{1a} receptor couples mainly to $G_{\alpha 0}$ isoform, responsible for inhibition of adenylate cyclase and subsequent decrease in PKA activity. The latter is likely to modulate CaMKIIα in the hippocampal CA1 region, which is consistent with the increased CaMKIIα phosphorylation in 5-HT_{1a} receptor knockout (KO) exposed to novel environments (Lo Iacono and Gross, 2008).

The G-protein βγ complex activation results in GIRK stimulation and has been shown to interact with the Erk1/2 pathway. Although 5-HT_{1a} receptor has been described to activate Erk1/2 in differentiated hippocampal HN2-5 cells via phospholipase Cβ (PLC β), small GTPases Ras and Raf as well as active MAPK/Erk kinase (MEK) (Adayev *et al*, 1999; Adayev *et al*, 2003), comparison with *in vivo* data has revealed some discrepancies. While 5-HT_{1a} receptor activation via 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) in HN2-5 cells increased Erk1/2 phosphorylation, intra-peritoneal (i.p.) injection of 8-OH-DPAT dose-dependently decreased Erk1/2 activation in the rat hippocampus 30 min after administration (Chen *et al*, 2002). Meanwhile, intrahippocampal infusion of 8-OH-DPAT of P15 mice resulted in stimulation of Erk1/2 pathway, as demonstrated by increased pErk/Erk ratio (Mogha *et al*, 2012). These differences might be attributed to the activation of the two receptor populations due to i.p. and intrahippocampal injection. Whereas intrahippocampal injections target postsynaptic heteroreceptors, i.p. injection is more likely to induce activation of autoreceptors in the raphe (Chen *et*

al, 2002), which on short term reduces 5-HT transmission resulting in decreased activation of 5-HT_{1a} heteroreceptors and hence reduced Erk1/2 phosphorylation. Opposing effects have also been reported for cell survival and proliferation. While Erk1/2 activation is usually implicated in cell proliferation, growth and survival 5-HT_{1a} receptor activation in dissociated cultures leads to suppression of growth and branching of dendrites in hippocampal pyramidal neurons (Ferreira *et al*, 2010). In contrast, 5-HT_{1a} receptor activation of differentiated HN2-5 cells lead to increased cell survival after anoxia (Adayev *et al*, 1999; Adayev *et al*, 2003), and intrahippocampal injections of 8-OH-DPAT resulted in increased synaptogenesis, following Erk1/2 activation (Mogha *et al*, 2012). Despite contradictory results, the above mentioned studies suggest a strong link between 5-HT_{1a} receptor activation and the Erk1/2 signaling cascade, placing the receptor in a prominent position to influence cell growth, proliferation and survival.

5-HT_{1A} RECEPTOR SIGNALING TARGETS NR2B-CONTAINING NMDA RECEPTORS

As described by Ferreira *et al* (2010), constitutive deletion of the 5-HT_{1a} receptor results in exuberant dendritic arborization of CA1 pyramidal neurons in the *stratum radiatum* (Figure 4, A). Increased levels of dendritic branching of hippocampal pyramidal neurons of male 5-HT_{1a} receptor KO mice can be observed as early as P15. Most prominent differences in arborization between male 5-HT_{1a} receptor KO and wild-type (WT) mice, however, were observed in adulthood, as dendrites of the *stratum radiatum* primarily mature starting from P15 onwards (Pokorny and Yamamoto, 1981). Similar effects on dendritic arborization were evoked by culturing organotypic hippocampal slices of 4 day-old WT pups, for 15 days under 5-HT depleted conditions. In comparison to slices cultured in the presence of 5-HT, 5-HT depleted cultures display heightened dendritic branches of pyramidal neurons in the *stratum radiatum* (Figure 4, B).

The *stratum radiatum* receives its major input from the CA3 via Schaffer collaterals. Projections from the CA3 were shown to be relevant for branching of pyramidal neurons in the *stratum radiatum.* We showed that blocking the excitatory, glutamatergic input from the CA3 by cutting the Schaffer collaterals in organotypic cultures during early development results in normal amount of branching in 5-HT_{1a} receptor KO slices (Figure 4, C). Follow-up studies showed that blocking of N-Methyl-D-aspartate (NMDA) receptors with 2-amino-5-phosphonopentanoic acid (AP5) had the same effect as cutting Schaffer collaterals. Furthermore, application of NMDA receptor subunit specific antagonists 4-[2-(4-nezypliperidin-1-yl)-1-hydroxypropyl]phenol (Ifenprodil, IFEN) revealed that, in particular, NR2B-containing NMDA receptors are involved in the regulation of dendritic arborization in the *stratum radiatum* (Figure 4, B). IFEN application to 5-HT_{1a} receptor KO slices was able to normalize levels of branching to WT levels, placing NR2B-containing NMDA receptors downstream of 5-HT_{1a} receptor

signaling and suggesting that NR2B subunit expression might be altered by lack of serotonergic inputs. Consistent with this hypothesis, field excitatory postsynaptic potentials (fEPSPs) in the CA1 region evoked by Schaffer collateral stimulation in P25 male 5-HT_{1a} receptor KO mice showed increased sensitivity to IFEN as compared to WT littermates.

It is important to mention that rescue of branching via blockage of NR2B-containing NMDA receptors or administration of 5-HT is time-dependent. Whereas either pharmacological intervention normalizes branching if applied between 1 and 6 days *in vitro* (DIV), drug treatment thereafter has no effect on branching in culture. This suggests the existence of a critical period for the effect of serotonin on NR2B-dependent dendritic growth.

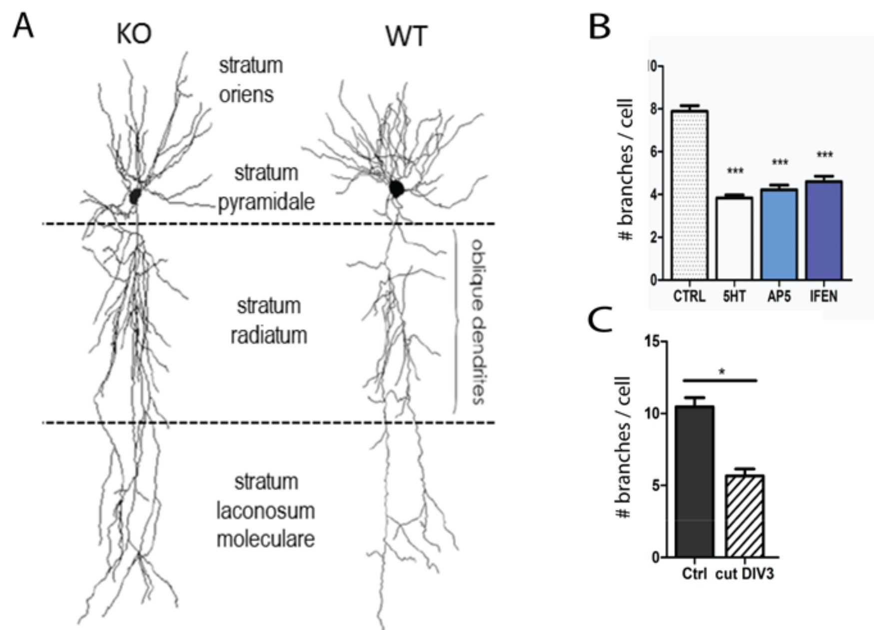

Figure 4 Impact of 5-HT_{1a} receptor deletion on dendritic branching of hippocampal pyramidal neurons.
Traces of male 5-HT_{1a} receptor KO and WT hippocampal pyramidal neurons in adulthood show an increase in branching in the *stratum radiatum* of 5-HT_{1a} receptor KO mice when compared to WT (A). In organotypic cultures branching was shown to be decreased by 5-HT (10 µM) as well as the NMDA receptor anatgonist AP5 (100 µM) and an NR2B containing NMDA receptor selective antagonist IFEN (3 µM) (B). When blocking the main excitatory input to the *stratum radia*tum by cutting Schaffer collaterals the amount of branching is normalized (C) Unpublished.laboratory data, Aouatef et al.

Results from *in vitro* hippocampal organotypic cultures revealed that NR2B-containing NMDA receptors are responsible for the increased arborization seen in 5-HT_{1a} receptor KO mice in the *stratum radiatum* of hippocamapal pyramidal neurons. These results are in line with studies implicating NR2B subunit of the NMDA receptor in branching during the early postnatal period. Adding NMDA to cultured hippocampal neurons induced filopodia formation and was implicated in the induction of secondary

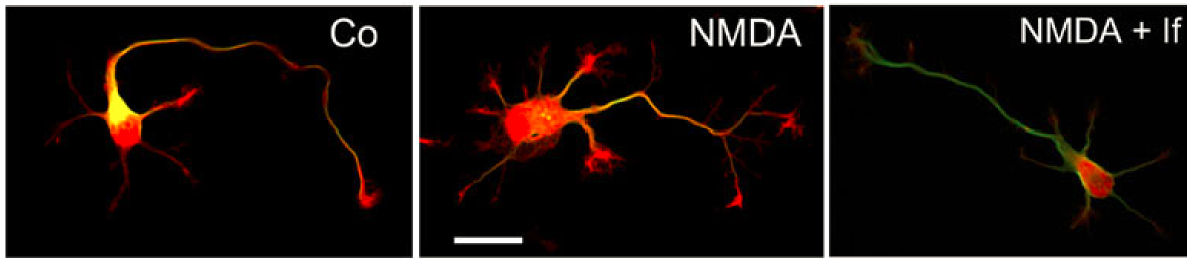

Figure 5 Influence of NR2B subunit expression on filopodia in hippocampal neurons.
Hippocampal neurons stained for F-actin and β tubulin III. NMDA (30 μM) induced filopodia formation (middle) in hippocampal neurons was increased in comparison to control (left). This increase was blocked when adding ifenprodil (6 μM) (right). NMDA (30 μM) induced filopodia formation (middle) was increased in comparison to control (left). This increase was blocked when adding ifenprodil (6 μM) (right). Figure from Henle *et al* (2012)

dendrites. In contrast, primary branches were not affected. Filopodia formation induced by NMDA application was inhibited by adding the NR2B subunit specific antagonist ifenprodil (Figure 5) (Henle et al, 2012). Conversely, exogenous NR2B-containing NMDA receptors in ventral spinal cord neurons induce increased secondary and tertiary branching, whereas NR2A-containing NMDA receptors had no effect on arborization of this cell type (Sepulveda et al, 2010). Moreover, hippocampal branching was increased in culture when NR2B-containing NMDA receptors were the dominating receptor type in hippocampal cell cultures (Sepulveda et al, 2010). The mechanism by which 5-HT_{1a} receptor influences the expression of NR2B containing NMDA receptors at the synapse, however, is not fully understood.

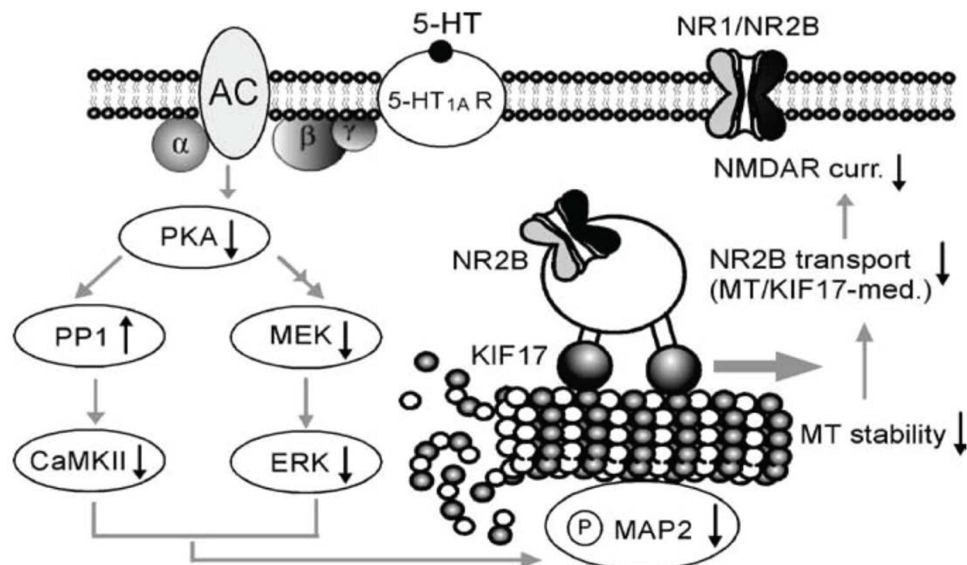

Figure 6 Downstream signaling pathway of 5-HT_{1a} receptor regulating transportation of NR2B-containing NMDA receptors to the synapse.
In cortical neurons 5-HT_{1a} receptor activation results in decreased transportation of NR2B-containg NMDA receptor to the synapse by interfering with microtubule stability. AC, adenylate cyclase; PKA, protein kinase A; PP1, protein phosphateas 1; CamKII, Ca2+/calmodulin-dependent kinase II; MEK, MAPK/Erk kinase; MAP2, microtubule-associated protein 2; MT, microtubule. Figure from Yuen *et al* (2005).

In cultured cortical neurons, 5-HT_{1a} receptor activation leads to decreased phosphorylation of microtubuli associated protein 2 (MAP2) via inhibition of Erk1/2 and CamKII (Figure 6). In turn MAP2 dissociates from microtubule resulting in decreased microtubule stability and disruption of transport of NR2B-containing NMDA receptor vesicles to the synapse (Yuen *et al*, 2005). Downregulation of Erk1/2 is in contrast to the downstream effects of 5-HT_{1a} receptor activation in the hippocampus, where 5-HT_{1a} receptor activation has been shown to result in increased phosphorylation and thus activation of Erk1/2 (Adayev *et al*, 1999; Adayev *et al*, 2005; Adayev *et al*, 2003). Therefore, it seems unlikely that 5-HT_{1a} receptor regulates the transportation of NR2B-contaning NMDA receptors to the synapse via the Erk1/2 pathway in hippocampal neurons. As 5-HT_{1a} receptor interacts with various downstream pathways it is therefore likely that the receptor influences NR2B-containing NMDA receptor via a different pathway. Another way to influence the amount of NR2B-containing NMDA receptors at the synapse is to alter the expression of the protein itself. One way to alter NR2B subunit expression has been suggested to involve the transcriptional repressor element-1 (RE1) silencing transcription factor (REST), also called neuron-restrictive silencer factor (Rodenas-Ruano *et al*, 2012). REST is a gene silencing transcription factor that is widely expressed during embryogenesis and is critical for the expression of a neuronal phenotype. In rats, REST expression is increased between P13 and P16. This increase has been shown to be responsible for the downregulation of NR2B mRNA (Figure 7). If REST upregulation is prevented by shRNA, NR2B subunit expression stays elevated (Rodenas-Ruano *et al*, 2012).

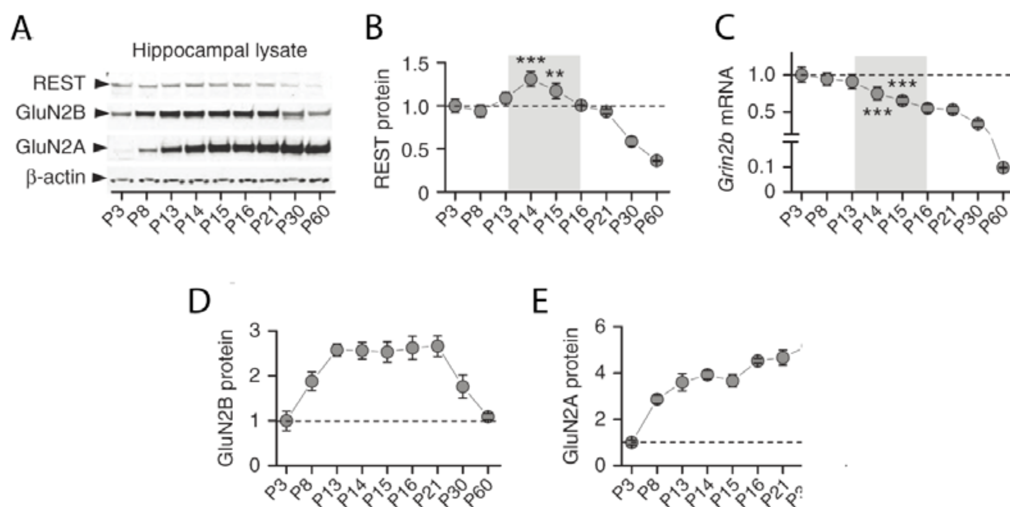

Figure 7 Regulation of NR2B subunit expression by REST.
A) Western blot showing the expression of NR2A (GluN2A), NR2B (GluN2B) and REST expression in the hippocampus during development. NR2B subunit expression is downregulated by REST increase during P13-P16 (B), which induces the downregulation of NR2B mRNA (*Grin2b*, C) resulting in decreased NR2B protein expression at P30 (D). NR2A protein expression on the other hand is not influenced by the peak of REST as its expression increases steadily from P3 onwards (E). Figure from Rodenas-Ruano *et al* (2012).

As described above, different downstream signaling pathways can be activated by 5-HT_{1a} receptor giving various possibilities by which 5-HT_{1a} receptor can influence NR2B-containing NMDA receptors and thereby dendritic branching in the *stratum radiatum* of pyramidal neurons. We, thus, sought to investigate the downstream mechanisms of 5-HT_{1a} receptor that impact NR2B- containing NMDA receptors.

I.2 MOUSE MODEL OF 5-HT_{1A} RECEPTOR KO

Constitutive 5-HT_{1a} receptor knockout (KO) mice were simultaneously introduced by three independent research groups in 1998 (Heisler *et al*, 1998; Parks *et al*, 1998; Ramboz *et al*, 1998). Despite coming from different laboratories and being backcrossed onto different genetic background strains (C57BL/6, Swiss Webster, 129SV) all knockout lines showed increased anxiety-like behavior in the commonly used conflict tests, such as the open field and elevated plus maze (Toth, 2003). Heisler *et al* (1998) and Ramboz *et al* (1998) demonstrated an anxiety-like phenotype using the elevated zero maze, and the elevated plus maze. Parks *et al* (1998) and colleagues demonstrated an increase in anxiety-related behavior in their 5-HT_{1a} receptor KO animals using the open field.

Following the initial studies in 1998, an extensive body of research has been devoted to unravel the functional role of the 5-HT_{1a} receptor in animal models and its involvement in psychiatric disorders. Studies have focused amongst others on the:

- impact of auto- vs heteroreceptor populations on the behavioral outcome
- critical time window of 5-HT_{1a} receptor expression during development
- detailed behavioral phenotyping of 5-HT_{1a} receptor KO animals

AUTO- VS HETERORECEPTOR POPULATION

In 1979, a clinical study investigating the effects of the partial 5-HT_{1a} receptor agonist buspirone, provided evidence for the receptor's involvement in anxiety and depression. Anxiolytic and antidepressant effects were seen in patients after buspirone administration (Goldberg and Finnerty, 1979; Olivier *et al*, 2001). The increased anxiety-related behavior expressed in three 5-HT_{1a} receptor KO mouse models were in line with this clinical data. As anxiety and mood disorders are highly comorbid in patients (Goes, 2015), 5-HT_{1a} receptor KO mice were also tested for depressive like behavior. However, well-established tests for depression such as the tail suspension test (Heisler *et al*, 1998) or the forced swim test (Parks et al, 1998; Ramboz et al, 1998) did not show the expected increase in depressive-like behavior, but rather anti-depressant-like effects: 5-HT_{1a} receptor KO mice showed higher levels of

mobility than WT mice in both tests, which can be interpreted as decreased behavioral despair. The lack of depressive-like symptoms after constitutive deletion of 5-HT_{1a} receptors were attributed to the inability to separately investigate the impact of auto- and heteroreceptors on this trait, which might contributed differently to the ontogeny and expression of mood disorders.

To dissect the impact of auto- and heteroreceptors, in the years following the initial description of 5-HT_{1a} receptor KO mice, conditional and tissue specific KO (loss-of-function) and rescue animals were generated (Donaldson *et al*, 2014; Garcia-Garcia *et al*, 2015; Gross *et al*, 2002; Piszczek *et al*, 2015; Piszczek *et al*, 2013; Richardson-Jones *et al*, 2010; Richardson-Jones *et al*, 2011), and siRNA injections were applied to selectively target subgroups of 5-HT_{1a} receptors (Bortolozzi et al, 2012; Ferres-Coy et al, 2013) (Table 1).

IMPACT OF AUTO- AND HETERORECEPTOR POPULATION ON ANXIETY-RELATED BEHAVIOR

In 2002, Gross and colleagues (Gross *et al*, 2002) introduced a forebrain specific inducible 5-HT_{1a} receptor rescue mouse. This mouse carried the bacterial tTA protein under the control of the CaMKIIα promoter, which leads to 5-HT_{1a} receptor expression in the cortex, hippocampus, septum and amygdala. By re-introducing 5-HT_{1a} receptor in the forebrain, but not the raphe nuclei of the brainstem, they demonstrated that forebrain expression during development is sufficient to restore normal anxiety-like behavior in the 5-HT_{1a} receptor KO mice in the open field and elevated plus maze. However, 5-HT_{1a} receptor under the CaMKIIα promoter results in heterologous expression of the receptor in brain regions such as the basal lateral amygdala (BLA, Figure 8). As the BLA is strongly involved in anxiety-related behavior, it was questioned to which extent the effects of heterologous 5-HT_{1a} receptor expression are suitable to establish causal links between endogenous 5-HT_{1a} receptor population and behavior (Richardson-Jones *et al*, 2011).

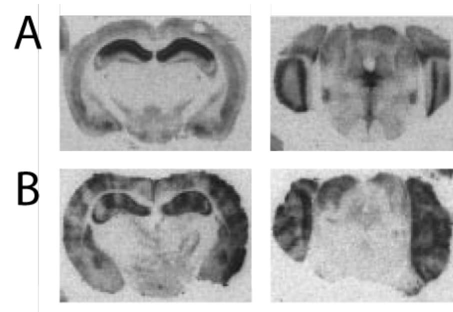

Figure 8 Heterologous expression of 5-HT_{1a} receptor under the control of CaMKIIα promoter.
^{125}I-MPPI receptor autoradiography on coronal section of WT (A) and mice expressing 5-HT_{1a} receptor under the CaMKIIα promoter (B). Low amounts of binding can be seen in the basal lateral amygdala of WT animals, high binding affinity can be seen for rescue animals in this region demonstrating the heterologous expression due to the promoter. Figure from Gross *et al* (2002).

reference	strain	breeding	mother's genotype	Method to alter 5-HT_{1a} expression	Time course of 5-HT_{1a} expression	5-HT_{1a} expression at testing	Gender of test mice	anxiety-related behavior	depression-like behavior
								in comparison to control mice	
Heisler et al. 1998	C57BL/6J	HET	HET	constitutive 5-HT_{1a} receptor KO	WT; constitutive KO	Ctx, Hpc, RN, Amg	♀ ♂	EOM: ↑ OF: ↑ Response to Novel Object: ↑	Tail suspension: ↓
Parks et al. 1998	Swiss-Webster	HET*	HET				All groups reported stronger genotype effect in ♂ than ♀	OF: ↑	FST: ↓
Ramboz et al. 1998	129/Sv	HET	HET					OF: ↑ EPM: ↑	FST: ↓
Lo Iacono et al. 2008	not clearly stated			pharmacological blockage of 5-HT_{1a} receptor with WAY-100,635	+ DOX Ctrl; WAY-100,635 treated WT	Ctx, Hpc, RN, Amg	♂	OF: ↑ EPM: = NSF: ↑	NSF: ↑
					+ DOX Ctrl; WAY-100,635 treated WT			OF: = EPM: = NSF: =	NSF: =
Vinkers et al. 2010	Swiss-Webster	HOM	HOM	pharmacological blockage of 5-HT_{1a} receptor with WAY-100,635	+ DOX Ctrl; WAY-100,635 treated WT; KO	Ctx, Hpc, RN, Amg	♂	OF: ↑ EPM: ↑	
Gracia-Gracia et al. 2015	129SvEV/Tac	HOM	HOM	pharmacological blockage of 5-HT_{1a} receptor with WAY-100,635	WT; WAY-100,635 injected WT; KO	Ctx, Hpc, RN, Amg	♂	OF: ↑ EPM: ↑	FST:=
					WT; WAY-100,635 injected WT; KO			OF: ↑ EPM: ↑	FST:=

* WT and KO mice were coming from two different lines with very similar background

reference	strain	breeding	mother's genotype	Method to alter $5\text{-}HT_{1a}$ expression	Time course of $5\text{-}HT_{1a}$ expression	$5\text{-}HT_{1a}$ expression at testing	Gender of test mice	anxiety-related behavior	depression-like behavior
								in comparison to control mice	
Gracia-Gracia et al. 2015	mixed 129/Sv; C57BL/6J; CBA/J	HET	Htr1A$^{tetO/tetO}$ not affected by DOX -> like WT	inducible $5\text{-}HT_{1a}$ KO B actin promotor	+ DOX Ctrl; KO induced during puberty; KO		♂	OF: ↑	
Gross et al. 2002	mixed 129/Sv; C57BL/6J; CBA/J	HET	not specified	inducible $5\text{-}HT_{1a}$ heteroreceptor rescue	-DOX Ctrl & WT; juvenile hetero-KO		♀ ♂	OF: ↑ EPM: ↑ NSF: ↑	NSF: ↑
					-DOX Ctrl & WT; adult hetero-KO		No sig. gender difference reported	OF: = EPM: = NSF: =	NSF: =
Piszczek et al. 2013	mixed 129S6/SV EvTac; C57BL/6J; CBA/J	HET	HET or HET auto-receptor rescue	inducible $5\text{-}HT_{1a}$ autoreceptor rescue (overexpression)	autoreceptor overexpressoin		♀ ♂	OF: = EPM: ↑	Tail suspension: =
					Ctrl		No sig. gender difference reported	OF: ↑ EPM: ↑	Tail suspension: ↑
					autoreceptor rescue; KO			OF: ↑ EPM: ↑	Tail suspension: ↑
Richardson-Jones et al. 2011	mixed 129/Sv; C57BL/6J; CBA/J	HET	Htr1A$^{tetO/tetO}$ not affected by DOX -> like WT	inducible $5\text{-}HT_{1a}$ auto- and heteroreceptor KO	+ DOX Ctrl		♂	OF: = LD Box: ↑ EPM: =	FST:=
					auto-KO			OF: = LD Box: = EPM: =	FST: ↑
					+ DOX Ctrl; adult hetero-KO; hetero-KO			OF: = LD Box: = EPM: =	FST:=

reference	strain	breeding	mother's genotype	Method to alter 5-HT$_{1a}$ expression	Time course of 5-HT$_{1a}$ expression	5-HT$_{1a}$ expression at testing	Gender of test mice	anxiety-related behavior	depression-like behavior
								in comparison to control mice	
Donaldson et al. 2014	mixed 129/Sv; C57BL/6J; CBA/J	HET	Htr1A$^{tetO/tetO}$ -> like WT	inducible 5-HT$_{1a}$ autoreceptor knockdown	auto-5-HT$_{1a}$ receptor level (ON/OFF); + DOX; behavioral testing; E0, P0, P21, P60, P140; + DOX Ctrl; autor-knockdown p14-30	Ctx, Hpc, Amg, RN	♂	OF: ↑ EPM: ↑ NSF: =	NSF: = Tail suspension: = FST: =
Richardson-Jones et al. 2010	mixed 129/Sv; C57BL/6J; CBA/J	HET	Htr1A$^{tetO/tetO}$ -> like WT	inducible 5-HT$_{1a}$ autoreceptor KO	auto-5-HT$_{1a}$ receptor level (ON/OFF); - DOX; behavioral testing; E0, P0, P21, P60, P140; + DOX Ctrl (high auto-5HT$_{1a}$-receptor); auto-KO (low auto-5HT$_{1a}$-receptor)	Ctx, Hpc, Amg, RN	♂	OF: = LD Box:=	Tail suspension: = FST: ↓ NSF after acute and chronic fluoxetine treatment: ↑
Bortolozzi et al. 2012 & Ferres-Coy et al. 2013	C57BL/6J	HOM	WT	siRNA targeting 5-HT$_{1a}$ autoreceptor	auto-5-HT$_{1a}$ receptor level (ON/OFF); siRNA infusion; behavioral testing; E0, P0, P21, P60, P140; WT; auto-KO (low auto-5HT$_{1a}$-receptor)	Ctx, Hpc, Amg, RN	♂	EPM: =	Tail suspension: ↓ FST: ↓

Table 1 Overview of methods and animal models used to study the different impact of the 5-HT$_{1a}$ auto and heteroreceptor population
HOM, homozygous; HET, heterozygous, ↑, increased anxiety-related or depressive-like behavior;↓, =, no difference between control and test animals for anxiety-related/Depressive-like behavior; decreased anxiety-related/ depressive-like behavior; EPM, elevated plus maze; LD Box, light/dark box; FST, forced swim test; NSF, novelty suppressed feeding task; ■, period of behavioral testing; DOX, doxycyclin; Ctx, cortex; Hpc hippocampus; Amg, amygdala; RN, raphe nuclei; ■, region expressing 5-HT$_{1a}$ receptor

Compelling evidence for a role of heteroreceptors in anxiety was recently provided by a study that achieved restricted rescue of 5-HT_{1a} receptor in cortical areas (Piszczek *et al*, 2015). In this report, endogenous re-expression of 5-HT_{1a} receptor was driven by the Emx-1 promoter, which is responsible for dorsal telencephalon-specific expression (Iwasato *et al*, 2004). Cortical rescue of the 5-HT_{1a} receptors resulted in decreased anxiety-related behavior in the light/dark box and the elevated plus maze in 5-HT_{1a} KO mice, supporting the link between 5-HT_{1a} heteroreceptors and anxiety.

The results described so far, however, are contradicting those gathered in loss-of-function experiments. Using the tTS/tetO, mice were generated that lacked 5-HT_{1a} heteroreceptors in the hippocampus, amygdala and prefrontal cortex (Richardson-Jones *et al*, 2011). When tested in the open field and light/dark box, no effect on anxiety-related behavior could be seen in these 5-HT_{1a} heteroreceptor deficient mice.

Contrary to 5-HT_{1a} heteroreceptor suppression, 5-HT_{1a} autoreceptor suppression resulted in increased anxiety-related behavior. Suppression of the 5-HT_{1a} autoreceptor population was achieved by breeding $Htr1a^{tetO/tetO}$ mice with a transgenic line controlling the tTS gene by the raphe-specific Pet-1 promoter fragment. Reducing 5-HT_{1a} autoreceptor expression to up to 80 % during development led to increase anxiety-related behavior as measured by the distance moved in the center of an open field (Donaldson *et al*, 2014; Richardson-Jones *et al*, 2011), the amount of entries into and distance moved in the lit compartment of the LD Box (Richardson-Jones *et al*, 2011) as well as the amount of time spent in and entries into the open arm of the elevated plus maze (Donaldson *et al*, 2014). Nonetheless, overexpressing 5-HT_{1a} autoreceptors in serotonergic neurons via the tryptophan hydroxylase 2 promoter in 5-HT_{1a} receptor KO mice was not sufficient to modulate anxiety-related behavior (Piszczek *et al*, 2013).

Clearly, contradicting results due to multiple constrains on exact expression or suppression of either of the receptor populations and differences in timing, make it difficult to draw final conclusions on which receptor population is responsible for the anxious phenotype of constitutive 5-HT_{1a} receptor KO mice. Considering that both receptor populations seem to be implicated in anxiety-related behavior, it is likely that they work in concert to regulate anxiety-related behavior rather than only one of them being fully responsible for the anxious phenotype of 5-HT_{1a} receptor KO mice (Piszczek *et al*, 2015; Piszczek *et al*, 2013).

Impact of auto- and heteroreceptor population on depressive-like symptoms

Data collected on depressive-like behavior in the above mentioned loss-of-function and rescue studies has been more consistent than for anxiety-related behavior. Re-expression of 5-HT_{1a} heteroreceptor via CaMKIIα promoter led to normalized behavior in the novelty suppressed feeding task (Gross *et al*, 2002).

This task measures hyponeophagia, which is the reduction in feeding response to a novel environment (Dulawa and Hen, 2005), whereby prolonged time needed to feed is associated with depressive-like behavior. In agreement with these findings, only mice deficient of 5-HT_{1a} heteroreceptors during development, showed increased immobility in the forced swim test (Donaldson *et al*, 2014; Richardson-Jones *et al*, 2011). In contrast, suppression of 5-HT_{1a} heteroreceptors in adulthood does not affect depressive-like behavior, suggesting that 5-HT_{1a} heteroreceptors act developmentally to establish the circuitry underlining depressive-like behavior (Chilmonczyk *et al*, 2015). Reduction of 5-HT_{1a} autoreceptor during adolescence under the control of the raphe specific Pet-1 fragment or in adulthood via siRNA injections into the dorsal raphe in adulthood resulted in antidepressant-like effects in the forced swim test and highly influenced the animals' responsiveness to antidepressant treatment (Bortolozzi *et al*, 2012; Ferres-Coy *et al*, 2013; Richardson-Jones *et al*, 2010). Contrary, depressive-like behavior could not be altered by overexpressing 5-HT_{1a} autoreceptors in KO mice (Piszczek *et al*, 2013). These findings point out that each receptor population influences depressive-like behavior in a different way. Whereas 5-HT_{1a} heteroreceptors act during development to modulate depressive-like behavior but have no direct impact in adulthood, 5-HT_{1a} autoreceptors are able to modulate depressive-like behavior in adulthood but do not alter depressive-like behavior developmentally.

DEVELOPMENTAL IMPORTANCE OF 5-HT_{1A} RECEPTOR

Over the last decades, growing evidence has linked stressful periods during early-life with the ontogeny of mental disorders (Meaney, 2001). However, genetic predispositions are determining an individual's susceptibility to develop mental disorders under certain, most likely stressful, environmental conditions (Bale and Epperson, 2015). Identification of such genetic predispositions, their critical developmental period of action as well as their susceptibility to external factors, is therefore needed to understand the developmental regulation of mental disorders. 5-HT_{1a} receptor has been implicated in anxiety and mood disorders already in the 70s. Its abundancy within the brain and its early expression during development (Figure 9) place the 5-HT_{1a} receptor in a dominant position to influence developmental mechanisms

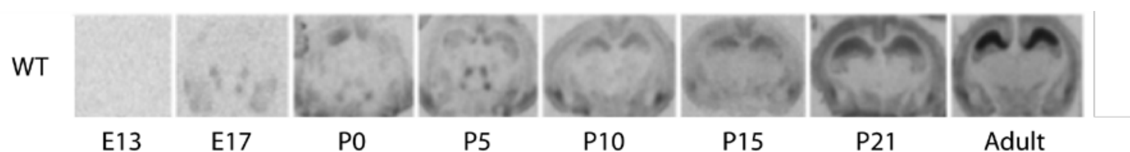

Figure 9 Expression pattern of 5-HT_{1a} receptor during early development.
The expression pattern of the receptor is well suited to influence developmental mechanisms as it starts to be expressed around E17 and already reaches adult-like expression levels around P21. Figure from Gross *et al* (2002)

and permanently alter brain circuits. Different methods have been used to identify the critical period of action for the 5-HT_{1a} receptor (Table 1). Using pharmacological approaches to deliver the 5-HT_{1a} receptor antagonist WAY-100,635 via secreting mini-pumps or by injecting mother and pup, Lo Iacono *et al* (2008) and Vinkers *et al* (2010) showed that 5-HT_{1a} receptor blockade functioning between P14-35 and p0-21, respectively, was sufficient to increase anxiety-related behavior in the open field in adult mice. In contrast, blockage of the receptor in adulthood (P60-81)(Lo Iacono *et al*, 2008) or late adolescence (P50-64)(Garcia-Garcia *et al*, 2015) did not elevate anxiety-related behavior neither in the open field nor in the novelty suppressed feeding task. Inducible KO mice that were depleted of 5-HT_{1a} receptors from P28 onwards also show increased anxiety-related behavior in parameters of the open field. Altogether, these observations suggest a developmental role of 5-HT_{1a} receptor functioning for the establishment of normal anxiety-related behavior. More refined approaches applying tissue specific inducible deletion of either 5-HT_{1a} auto- or heteroreceptors came to similar conclusion (Gross *et al*, 2002; Richardson-Jones *et al*, 2011). Although it is yet unclear which of the two receptor populations is responsible for elevated levels of anxiety, no change in anxiety-related behavior could be evoked when either of them were depleted during adulthood.

In comparison to anxiety-related behavior, depressive-like behavior can be altered by manipulating 5-HT_{1a} receptor expression during both early-life and adulthood. As mentioned above, data suggest a neurodevelopmental influence of 5-HT_{1a} heteroreceptors on depressive-like behavior. The autoreceptors population, on the other hand, seems responsible for the modulation of depressive-like behavior during adulthood.

Data gathered by Weller *et al* (2003) and Gleason *et al* (2010), however, indicates that not only the pups' but also the mothers' genotype is influencing the anxiety-related and depressive-like behavior of

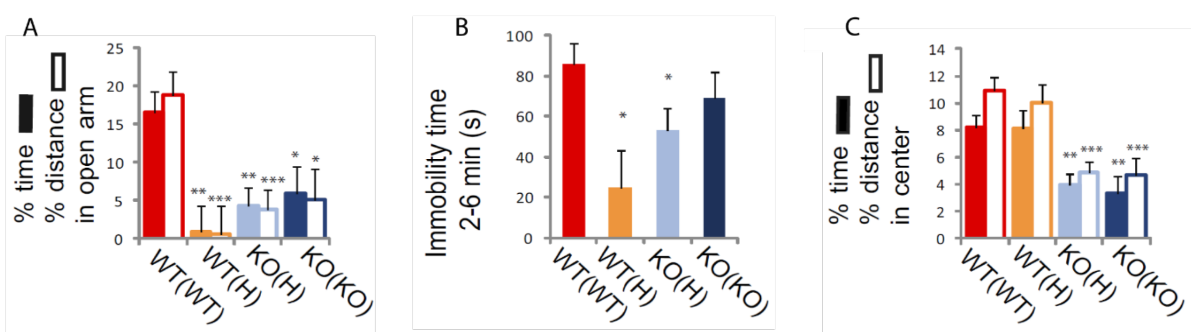

Figure 10 Impact of the mothers genotype on anxiety-related behavior of the offspring.
The mothers genotype critically influences the behavior of the offspring in tests for anxiety-related behavior and depression. Anxiety-related behavior is impaired in WT offspring of heterozygous mothers in the elevated plus maze (A), but not in the open field (C). Behavior of KO offspring is not influenced by the genotype of the mother for measures of anxiety. Depressive-like behavior is influenced by the mothers genotype in both WT and KO offspring (B). (WT(WT) = WT offspring of WT mother; WT/KO(H)= WT/KO offspring of heterozygous mother; KO(KO) = KO offspring of KO mother) Figure from Gleason *et al* (2010)

the offspring (Figure 10). Comparing heterozygous (HET) offspring raised by either a WT or 5-HT_{1a} receptor KO dam showed increased levels of ultrasonic vocalization and, hence, elevated levels of anxiety of HET pups of 5-HT_{1a} receptor KO than WT dams Weller *et al* (2003). In addition, Gleason *et al* (2010) showed that WT mice raised by HET mothers exhibited decreased behavioral despair in the forced swim test and increased anxiety-related behavior in the elevated plus maze in comparison to WT mice raised by WT mothers (Figure 10 & 11). Furthermore, homozygous 5-HT_{1a} receptor KO and WT mice (originally derived from Rene Hen (Ramboz *et al*, 1998)) obtained from heterozygous mating,

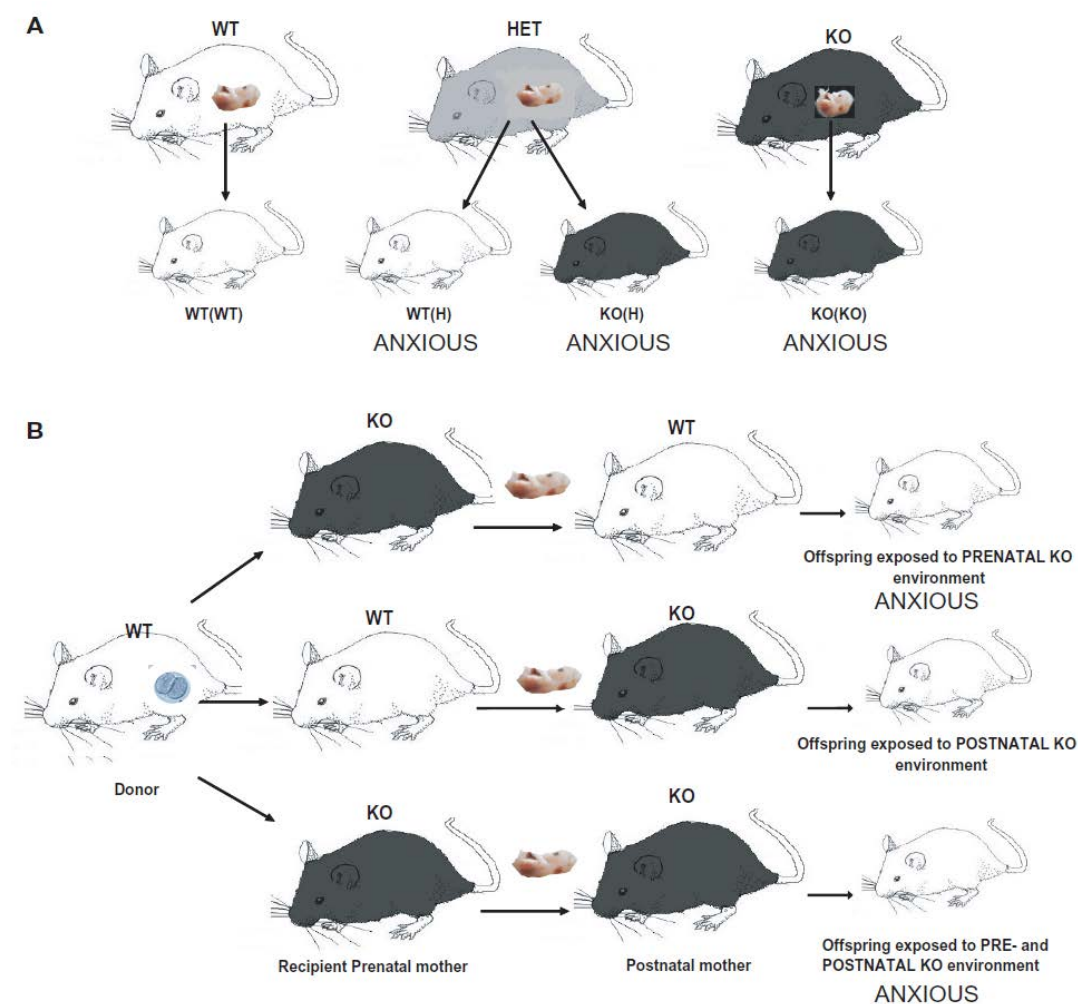

Figure 11 Impact of the mothers' genotype on severity of 5-HT_{1a} receptor deletion.
A) Breeding schemes for homozygous (WTxWT and KOxKO) and heterozygous (HETxHET) breeding. Homozygous breeding results in anxious KO offspring and WT offspring with normal anxiety-related behavior. Heterozygous breeding results in anxious WT and KO offspring. B) Embryonic cross-fostering of WT pups results in increased anxiety-related behavior when embryos are exposed to KO environment but not when cross-fostered to KO mothers after birth, suggesting that the prenatal period is essential for the impact of the mothers' genotype. Figure from Gleason *et al* (2011)

showed no deficits in anxiety-related measures in the open field, light dark box or the elevated plus maze (Groenink *et al*, 2003). Although contradicting at first sight, this data indicates that genetic alterations of the mother can at least partially be passed on to further generations by nongenetic transmission.Effects of differential maternal care of the mothers as mechanism of nongenetic transmission could be ruled out, as no differences in quality of maternal care of KO and WT mice was observed (Weller *et al*, 2003). This was supported by embryonic transfer experiments in which WT pups were transplanted to 5-HT_{1a} receptor KO, but raised by WT mothers still show increased anxiety-related behavior (Figure 11B). Similarly, WT pups transplanted to WT mice but raised by 5-HT_{1a} receptor KO mothers do not show increased anxiety-related behavior. Additionally, these experiments highlight the embryonic rather than the postnatal period as critical for nongenetic transmission (Gleason *et al*, 2010). Although increased anxiety-related behavior seems to be caused by delayed maturation of the granular cell layer in the ventral hippocampus, the exact mechanism of nongenetic transmission is unknown (Gleason *et al*, 2010). Despite the lack of explanation, results from these studies emphasize the role of 5-HT_{1a} receptor as a nongenetic factor. Moreover, 5-HT_{1a} receptor deletion itself is a genetic predisposition for increase anxiety-related behavior, the severity of 5-HT_{1a} receptor deletion on behavior, however, seems highly susceptible to external factors such as the mother’s genotype.

GENDER-DEPENDENT DIFFERENCES IN 5-HT_{1A} RECEPTOR KO MICE

In humans, anxiety and mood disorders occur more frequently in women than in men (Albert, 2015). Therefore, the observation that male constitutive 5-HT_{1a} receptor KO mice of all three different background strains were more severely impacted by receptor depletion than females was unexpected (Figure 12) (Heisler *et al*, 1998; Parks *et al*, 1998; Ramboz *et al*, 1998). Also, changes in behavior were also more consistent across tests in males than in females (2012 Altieri). For example male 5-HT_{1a} receptor KO mice differed significantly from WT male in anxiety-related paradigms in the open field as well as in the elevated plus maze, whereas female 5-HT_{1a} receptor KO mice did not differ significantly from WT females in the open field (Ramboz *et al*, 1998). Despite these indications from initial reports there is no study attempting at extensively elucidating gender differences f 5-HT_{1a} receptor deletion. Further hints are only given by Zanettini *et al* (2010): separating 5-HT_{1a} receptor KO mice from their mother during the early postnatal period resulted in increased ultrasonic vocalization of male but not female 5-HT_{1a} receptor KO mice when compared to WT mice. By contrast, other studies reported a lack of gender-dependent differences and presented pooled results from both gender (Gross *et al*, 2002; Piszczek *et al*, 2015; Piszczek *et al*, 2013), although the small number of animals per sex might have led to an underestimation of gender effects.

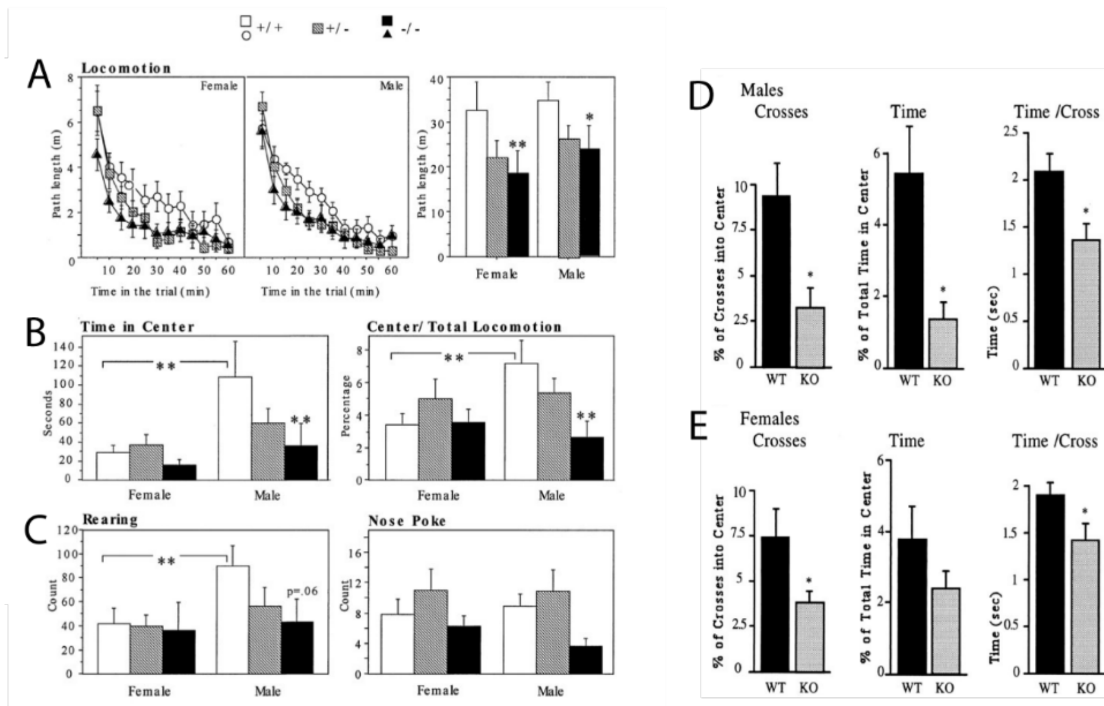

Figure 12 Gender-dependent differences in anxiety related behavior of 5-HT$_{1a}$ receptor KO mice.
Anxiety-related behavior measured in the original studies of 1998 by Ramboz *et al* (A-C) and Parks *et al* (D-E) comparing the behavior of male and female 5-HT$_{1a}$ receptor KO with WT mice in the open field. Female mice in both studies show less severe effects of 5-HT$_{1a}$ receptor depletion: for example, they are affected in the amount of crosses to the center and the time/cross, but not for the time spent in the center of the open field (E). Male 5-HT$_{1a}$ receptor mice on the other hand, show increased anxiety-related behavior for all parameters measured (D).

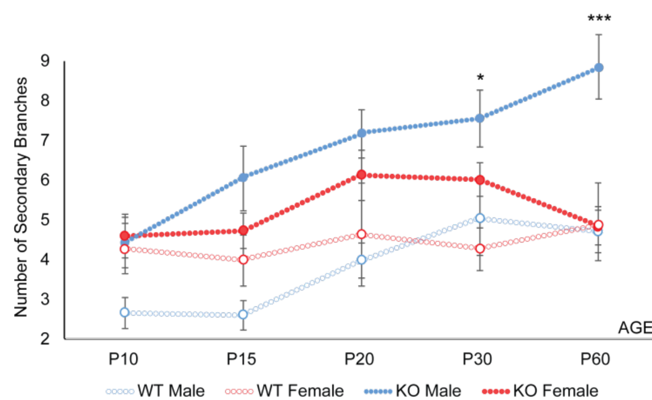

Figure 13 Dendritic branching of pyramidal neurons in the *stratum radiatum*.
Both male and female 5-HT$_{1a}$ receptor KO mice show an increase in branching during the first four weeks of development. With the onset of puberty at around P30 the amount of branches decreases in female 5-HT$_{1a}$ receptor KO mice whereas it further increases in male 5-HT$_{1a}$ receptor KO mice. Male 5-HT$_{1a}$ receptor KO mice show significantly increased amounts of branching at P60 in comparison to all other groups. Unpublished laboratory data, A. Pinault et al.

When analyzing the dendritic complexity of KO mice in our laboratory we found increased arborization of CA1 pyramidal neurons in the *stratum radiatum* in adult 5-HT_{1a} receptor KO mice when compared to WT (Pinault et al, unpublished data). Interestingly, this difference was more pronounced in male 5-HT_{1a} receptor KO mice than in females. Increased arborization of CA1 pyramidal neurons could be observed as early as P20 in both male and female 5-HT_{1a} receptor KO mice. In adulthood, dendritic complexity was normalized in female KO mice and was not anymore distinguishable from that seen in WT mice, whereas the amount of arborization in male KO increases even more (Figure 13). These results prompted us to further investigate gender-dependent differences of 5-HT_{1a} receptor KO mice in behavioral tests and to elucidate the molecular mechanisms underlying the decreased effects observed in female mice (see study aim p.37).

DETAILED PHENOTYPE OF 5-HT_{1A} RECEPTOR TRANSGENIC MICE

ANXIETY-RELATED BEHAVIOR

The original study in 1998 already described anxiety-related behavior of 5-HT_{1a} receptor KO mice in conflict tasks such as the open field, the elevated zero and the elevated plus maze. In 2006, Klemenhagen and colleagues also confirmed the anxious phenotype of 5-HT_{1a} receptor KO mice in the light/dark box.

The classical behavioral test for anxiety-related behavior (open field, light/dark box, elevated plus maze) are based on conflict in a new environment and are all highly dependent on the animal's urge to explore. Ultrasonic vocalization, stress-induced hyperthermia and the Vogel lick-suppression test on the other hand are independent of the mouse's locomotor activity, but at the same time measure a different kind of anxiety-related behavior.

Ultrasonic vocalization is particularly used to measure anxiety-related behavior in pups. Zanettini *et al* (2010) Weller *et al* (2003) and Vinkers *et al* (2010) used this test in 5-HT_{1a} receptor KO pups to measure anxiety-related behavior in pups after separation from their mother. Zanettini *et al* (2010) and Vinkers *et al* (2010) reported increased ultrasonic vocalization in 5-HT_{1a} receptor KO mice and mice in which 5-HT_{1a} receptor was blocked during the early postnatal period by WAY-100,635 injection, indicating higher levels of anxiety in 5-HT_{1a} receptor KO mice. This data was confirmed by increased anxiety-related behavior of WAY-100,635 injected mice in the elevated plus maze at P21. In contrast, Weller shows decreased vocalization of 5-HT_{1a} receptor KO pups suggesting lower levels of anxiety related behavior. Although contradicting, these differences might be due to the fact that whereas Vinkers *et al* (2010) and Zanettini *et al* (2010) tested male and female pups separately, Weller *et al* (2003) did not differentiate between genders which might have hampered the results gained in this study.

The Vogel-lick test has been introduced in 1971 to measure anxiety-related behavior and to assess the efficiency of anxiolytic drugs (Vogel *et al*, 1971). After being deprived of drinking water for 24 h mice are allowed to drink a certain amount of water before shock is delivered. The more anxious the mice the less they will approach the water bottle after the first shock administration. When exposed to the Vogel lick-suppression test 5-HT_{1a} receptor KO mice showed the same amount of lick-suppression as WT animals (Klemenhagen *et al*, 2006). Similarly, stress induced hyperthermia is normal in 5-HT_{1a} receptor KO mice (Pattij *et al*, 2002; Pattij *et al*, 2001). Stress induced hyperthermia is another test to measure anxiety-related behavior in rodents. It measures the increase in body temperature due to a stressor. Measuring basal body temperature intrarectally serves as stressor for this test. After a 10-15 min core body temperature is measured again. The increase in body temperature in response to the stressor is called stress-induced hyperthermia (Olivier *et al*, 2003). Altogether this data demonstrates that particularly anxiety as it is measured in open field, light/dark box and elevated plus maze are effected by 5-HT_{1a} receptor depletion but not tests independent of locomotor activity.

STARTLE RESPONSE AND PREPULSE INHIBITION

Prepulse inhibition and startle response are in general used to measure sensorimotor gating. Deficits in this sensorimotor gating process are associated with the schizophrenia and other mental disorders. Male and female 5-HT_{1a} receptor KO mice have been tested in these paradigms (Dirks *et al*, 2001; Dulawa *et al*, 2005). No differences for the startle response or prepulse inhibition were observed, indicating that sensorimotor gating in 5-HT_{1a} receptor mice is normal.

DEPRESSIVE-LIKE BEHAVIOR

The 5-HT_{1a} receptor has been linked in depression since studying the effects of buspirone in human patients. Furthermore, decreased 5-HT_{1a} receptor expression has been shown to influence anxiety disorders and depression (Albert and Lemonde, 2004; Lemonde *et al*, 2004a). However, 5-HT_{1a} receptor KO mice surprisingly showed increased antidepressant-like behavior in the forced swim and tail suspension test. Although both tests are extensively used to study the acute effects of antidepressants, they only test aspects of depression. Hence, further tests were needed to clearly define the impact of 5-HT_{1a} receptor deletion on depressive-like behavior in mice. The sucrose preference task applied by Donaldson *et al* (2014), however, did not show differences in anhedonia of 5-HT_{1a} receptor KO and WT mice. On the other hand, depressive-like behavior was seen in the novelty-suppressed feeding task. Mice with developmental suppression of the 5-HT_{1a} heteroreceptor, or pharmacological blockage of the receptor during early development showed longer latency to feed in an anxiogenic environment (Gross *et al*, 2000; Gross *et al*, 2002; Lo Iacono *et al*, 2008). In addition, constitutive 5-HT_{1a} receptor KO mice showed increased paradoxical sleep (Boutrel *et al*, 2002), a parameter that is often linked with depression (Asarnow *et al*, 2014; Kupfer, 1976). Moreover, the effects of 5-HT_{1a} receptor depletion on

adult neurogenesis were studied by Santarelli *et al* (2003). Adult neurogenesis has been extensively studied in the context of anxiety and mood disorders (Jacobs *et al*, 2000) and has been shown to be implicated in the response to selective serotonin reuptake inhibitors (SSRIs), which are widely used antidepressants. In 5-HT_{1a} receptor KO mice survival of adult born neurons is decreased, while proliferation levels are normal. Also, 5-HT_{1a} receptor expression of immature and mature adult born granule cells has recently been implicated in the response to SSRIs (Samuels *et al*, 2015). Altogether, further evidence has been collected for the implication of 5-HT_{1a} receptor in depressive-like behavior over the last years. However, increased depressive-like behavior was only sparsely seen in constitutive 5-HT_{1a} receptor KO mice.

LEARNING AND MEMORY

There are numerous tests measuring different aspects of learning and memory. 5-HT_{1a} receptor KO mice have been exposed to the Morris water maze, Y-maze, operant conditioning, different protocols of fear conditioning and novel object recognition. Spatial learning and memory has been measured in the Morris water maze and the delayed version of the Y-maze (Sarnyai *et al*, 2000; Wolff *et al*, 2004). In the Morris water maze 5-HT_{1a} receptor KO mice exhibited deficits during the learning phase, as they needed longer to learn the location of the hidden platform and they performed much worse in the probe trial than WT mice, showing no preference for the quadrant associated with the platform. Similar results were obtained in the Y-maze, showing that 5-HT_{1a} receptor KO mice have impaired spatial memory (Sarnyai *et al*, 2000).

Freezing to context and cue was tested in fear conditioning. Whereas Groenink *et al* (2003) observed no difference in the amount of freezing to either context or cue, Klemenhagen *et al* (2006) observed increased amount of freezing when re-exposed to the training context (Figure 14). More refined fear conditioning paradigms analyzing the ability of 5-HT_{1a} receptor KO mice to distinguish between partially and completely predictive cues for foot shock revealed impairments of 5-HT_{1a} receptor KO mice to differentiate between these types of cues. When exposed to ambiguous or partial cues, 5-HT_{1a} receptor KO mice showed increased amount of freezing in comparison to WT mice (Klemenhagen *et al*, 2006; Tsetsenis *et al*, 2007). This data shows a strong impairment of 5-HT_{1a} receptor KO mice for learning and memory processes. Especially hippocampal-dependent memory tasks were impaired whereas prefrontal cortex dependent operant conditioning was not impaired by 5-HT_{1a} receptor deletion (Pattij *et al*, 2003). Interestingly, deficits seen in hippocampal-dependent tasks resemble cognitive abnormalities seen in patients with depression and further emphasis the involvement of 5-HT_{1a} receptor in depressive-like behavior.

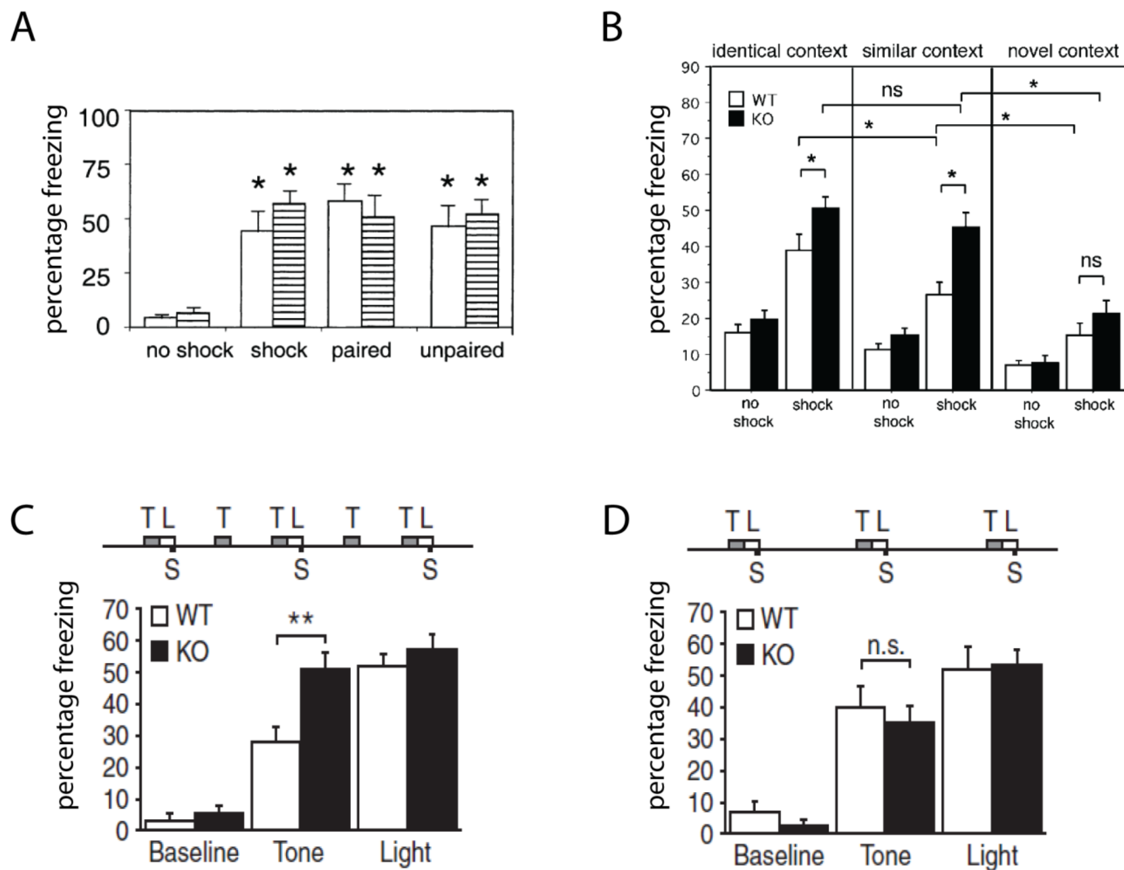

Figure 14 Fear conditioning of 5-HT$_{1a}$ receptor KO mice.
Whereas Groenink *et al* (2003) does not observe differences in % freezing between WT and 5-HT$_{1a}$ receptor KO mice (A), significant differences for contextual fear conditioning are seen by Klemenhagen *et al* (2006) (B). Furthermore, increased % freezing is observed in 5-HT$_{1a}$ receptor KO mice when exposed to a similar context equipped with a mixture of cues from the training context and unfamiliar cues (B). In the study by Tsetsenis *et al* (2007), similar difficulties to distinguish between partially predictive and completely predictive cues can be seen in C. 5-HT$_{1a}$ receptor KO mice show increased freezing to the partial cue (T=tone) in comparison to WT mice, but to not differ from WT in freezing response to the complete cue (L=light). If tone and light are equally predictive no difference is seen for the % freezing to tone of 5-HT$_{1a}$ receptor KO and WT mice (D).

Altogether data gathered within the last decade on 5-HT$_{1a}$ receptor deletion has broadened the understanding of the behavioral phenotype of 5-HT$_{1a}$ receptor KO mice. Overall, increased anxiety-related behavior has been confirmed and the involvement of 5-HT$_{1a}$ receptor deletion for depressive-like behavior extended. Particularly effects of receptor deletion on secondary symptoms of depression, such as impairments of cognitive functioning and sleep, have been highlighted.

I.3 Study aim

The impact of serotonin receptor deletion as a tool to understand its contribution to brain development and function has been extensively studied over the past two decades. In particular, this approach is continuously building a deeper understanding broad impact of the $5\text{-}HT_{1a}$ receptor on brain development and function. In this perspective, the present study was divided into a behavioral and a molecular part to elucidate new aspects of the biology of this receptor. The behavioral part focused on gender-dependent behavioral differences of $5\text{-}HT_{1a}$ receptor KO mice, whereas the molecular part was applied to study the downstream mechanism of $5\text{-}HT_{1a}$ receptor signal transduction and their effects on molecular and cellular levels

Gender-dependent behavioral differences of $5\text{-}HT_{1A}$ receptor KO mice

$5\text{-}HT_{1a}$ receptor KO mice have been shown to display elevated levels of dendritic branching of oblique dendrites of hippocampal pyramidal neurons (Ferreira *et al*, 2010). Comparing adult male and female $5\text{-}HT_{1a}$ receptor KO mice, we observed increased dendritic arborization in male but not in female $5\text{-}HT_{1a}$ receptor KO mice. Consistently, the original studies on $5\text{-}HT_{1a}$ receptor KO mice all reported more sever effects of $5\text{-}HT_{1a}$ receptor deletion on anxiety-related behavior in male than in female mice. Hence, it was our aim to further investigate the gender-dependent differences seen in f $5\text{-}HT_{1a}$ receptor KO mice. Starting with anxiety-related behavioral tasks we expanded our behavioral test battery to compare depressive-like behavior and learning and memory abilities of male and female $5\text{-}HT_{1a}$ receptor KO mice, as both are traits affected by $5\text{-}HT_{1a}$ receptor deletion in male mice. Tests were chosen based on published data that showed impairments of male $5\text{-}HT_{1a}$ receptor KO mice. Furthermore, as differences in arborization were observed in the dorsal hippocampus we added learning and memory tasks measuring hippocampal functioning to elucidate whether alterations in arborization could have impacted hippocampal-dependent memory functions. To study gender-dependent differences for depressive-like behavior we specifically focused on olfactory functioning, as it has not been implemented in studies with $5\text{-}HT_{1a}$ receptor KO mice so far. In addition, we aimed at analyzing changes in adult neurogenesis in the subventricular zone and the dentate gyrus to further the understanding of gender-dependent differences in depressive-like behavior.

$5\text{-}HT_{1A}$ receptor signaling and its downstream targets

As mentioned above $5\text{-}HT_{1a}$ receptor deletion leads to increased dendritic branching in the *stratum radiatum* of pyramidal neurons by altered NR2B-containing NMDA receptor expression among others during the early developmental period. As the downstream pathways of $5\text{-}HT_{1a}$ receptor signaling leading to changes of NR2B-containing NMDA receptor expression have not yet been studied in the hippocampus, we aimed at investigating potential $5\text{-}HT_{1a}$ receptor-dependent mechanisms of NR2B-

containing NMDA receptor regulation. We focused on the impact of 5-HT_{1a} receptor regulation of NR2B-containing trafficking to and removal from the synapse as well as possible influences of 5-HT_{1a} receptor on REST expression which in turn can result in altered NR2B-containing NMDA receptor expression. Using organotypic cultures we studied the effects of altering NR2B-containing NMDA receptor expression on branching to identify which mechanism might be influenced by 5-HT_{1a} receptor downstream signaling. Furthermore, we wondered, whether increase NR2B-containing NMDA receptors could lead to increased cell death during development.

II Material & Methods

II.1. Subjects

All experiments were carried out in compliance with institutional regulations and Swiss guidelines for animal experimentation. All procedures were approved by the Veterinary Office of the Canton De Vaud.

Backcrossing into C57BL/6J background

Observations in the open field showed that 5-HT$_{1a}$ receptor KO mice on a mixed 129SV/C57BL/6J background exhibit extremely low levels of novelty induced locomotor activity. As locomotor activity in 129SV mice is in general lower than in C57BL/6J mice (Loos *et al*, 2014; Molenhuis *et al*, 2014; Paulus *et al*, 1999), we decided to further backcross our mice on the C57BL/6J background to avoid low levels of locomotor activity under basal and novelty conditions as a confounding factor for our study (Milner and Crabbe, 2008). Successful backcrossing was determined by locomotor activity of 5-HT$_{1a}$ receptor KO and WT mice in the open field.

5-HT$_{1A}$ receptor KO mice

5-HT$_{1a}$ receptor KO mice were obtained from Prof. Rene Hen (Ramboz *et al*, 1998) and bred in our facilities. Upon arrival 5-HT$_{1a}$ receptor KO mice were crossed with Thy1-GFP-M mice (see below). This resulted in GFP labeling of sparse neuronal subsets, thus allowing morphological analysis of hippocampal pyramidal neurons. Mice from then onwards were kept on a 129SV/C57BL/6J mixed background.

Thy1-GFP-M

Thy1-GFP-M transgenic mice (C57BL/6J background) were ordered from the Jackson Laboratory (USA) and crossed with 5-HT$_{1a}$ receptor KO mice to introduce GFP labeling of neurons. To prevent loss of GFP expression over generations in our line, Thy-1-GFP-M mice were re-introduced every third year.

C57BL/6Rj

C57BL/6Rj female mice were obtained from Janvier (France). These mice were used to further backcross the 5-HT$_{1a}$ receptor KO mice on a C57BL/6 background. They were mated with 5-HT$_{1a}$ receptor KO male mice to generate a heterozygous population. Females were chosen to be C57BL/6Rj to ensure that alterations in maternal care due to the 5-HT$_{1a}$ receptor KO could not influence the offspring's behavior. Heterozygous male and female mice were mated amongst each other - but not within litters - to produce wild-type and KO mice. The offspring were again used to further backcross the mice to get closer to the C57BL/6Rj background. This procedure was repeated four times before mice were used for behavioral experiments.

Mice husbandry

All breeders were housed in groups of three to five animals in propylene cages with standard bedding (Lignocel, JRS, Germany). For breeding, one male and two females were housed in one cage and pregnant females were isolated before giving birth. Animals were maintained under a 12 h light/dark cycle (lights on at 7.00 AM) at 22 - 24°C and approx. 50 % humidity, with tap water and pellet food (*Kliba Nafag*, Provimi Kliba AG, Switzerland) available *ad libitum*. During the light phase constant background noise was provided by a radio (approx. 60 dB). Once a week, the animals were handled briefly during the exchange of bedding.

Mice assigned to behavioral experiments were transferred to a different housing room at weaning and housed in groups of three to five animals of the same gender and genotype per cage. They were kept under the same conditions, but no radio provided any background noise. Cage cleaning and handling was performed by the experimenter to facilitate habituation to handling.

Animal identification

To identify the genotype of mice before weaning, pups were toe clipped between postnatal day (P) 5-P12, and toes used for DNA isolation and PCR (see below). To reduce stress due to restraining the mice to read the toe number before or after behavioral testing, test mice were marked on their back or head using H_2O_2. Dying the fur with H_2O_2 results in a reddish stain which can be identified easily and allows the experimenter to distinguish between mice from one cage without needing to restrain and turn them.

II.2. Genotyping

Extraction solution A (100 µl, 25 mM NaOH, 0.2 mM Ethylendiaminetetraacetic acid, EDTA) were added to the tissue sample and incubated at 95 °C for 45 min. Thereafter, tubes were quickly shaken and placed on ice for 10 min. Extraction solution B (100 µl , 40 mM Tris HCL, adjusted to pH 5.0) were added, samples mixed well and centrifuged at 13000 rpm for 5 min at room temperature. The supernatant was transferred to a sterile tube and used to determine DNA concentrations. Concentrations were measured using NANOdrop 1000 (Witec AG, Switzerland). 250ng of DNA were mixed with 5' Prime Master Mix (5 Prime, Germany), $MgCl_2$ solution (25 mM, 5 Prime, Germany), primers (Table 2), and the volume adjusted to 20 µl with H2O. This mix was used for DNA amplification in the polymerase chain reaction (PCR). The applied PCR protocol was highly dependent on the primers used. Protocols for identification of GFP, 5-HT_{1a} receptor KO band (BC) and 5-HT_{1a} receptor WT (AC) band are described in Table 4. 2.0 µl loading buffer (10x Blue Juice Gel Loading Buffer, Invitrogen, Switzerland) were added to the amplified sample thereafter and 9.5 µl of each sample were loaded onto a 2 % agarose gel containing 4 µl SYBR Safe DNA gel stain (Invitrogen, Switzerland) and run at 130 V. Bands were identified by band size

comparing the height of the band with a DNA ladder (100bp peqGOLD, peqlab, Switzerland). Mice expressing either the WT or the 5-HT_{1a} receptor KO band were identified as WT and 5-HT_{1a} receptor KO mice respectively, mice having both bands were classified as heterozygous.

5-HT_{1a} receptor	GFP
A 5'-AAC TAT CTC ATC GGC TCC TTG-3' B 5'-GTT AAG GGT GAG AAC AGA-3' C 5'-CTT CTT TTC CAC CTT CTT GAC-3'	1 5'-CA GGC CAC AGA ATT GAA AGA TCT-3' 2 5'-GTA GGT GGA TCT AGC ATC ATC C-3' 3 5'-AAG TTC ATC TGC ACC ACC G-3' 4 5'-TCC TTG AAG AAG ATG GTG CG-3'

Table 2 Primer pairs for amplification of WT and 5-HT_{1a} receptor KO band and GFP

Primer	GFP		5-HT_{1a} receptor AC		5-HT_{1a} receptor BC	
	Temp. °C	Time s	Temp. °C	Time s	Temp. °C	Time s
Lid temperature	105		105		105	
Initial denaturation	94	90	94	150	94	150
denaturation	94	30	94	30	94	30
annealing	60	60	57	30	57	30
elongation	72	60	72	60	72	60
Final elongation	72	600	72	600	72	600
Cycles	35		35		40	

Table 3 PCR protocols for different primer pairs

II.3. Behavioral phenotyping

With the onset of adulthood (P60) all mice, independent of the batch, were tested in the conflict tests to measure anxiety-related behavior. Thereafter, mice were tested for depressive-like behavior and their cognitive abilities in different test batteries. Two groups (A and B) of mice of similar size (Group A: WT female n=24, male n=12; 5-HT_{1a} receptor KO female n=13, male n=12; Group B: WT female n=9, male n=8; 5-HT_{1a} receptor KO female n=8, male n=9) were tested simultaneously for different aspects of depressive-like behavior and cognitive integrity (Figure 15). A third group (C) of mice (WT female n=10, male n=12; 5-HT_{1a} receptor KO female n=11, male n=8) was used to supplement data sets gathered with group A and B.

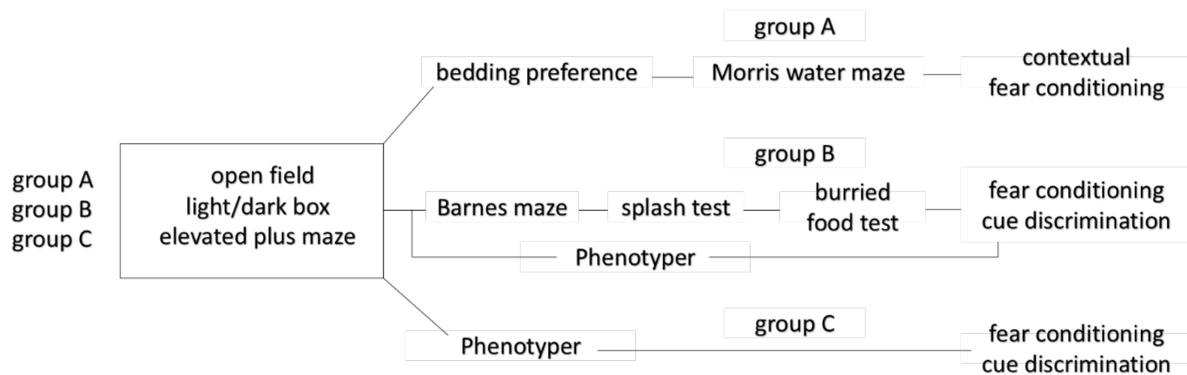

Figure 15 Order of testing of group A, B and C.
All mice were tested in the three most common conflict tests for anxiety. Thereafter, mice were divided into two groups and tested for depressive-like behavior and cognitive integrity. The order of tests was chosen according to the impact of stress. Mice were tested in less stressful tests first.

BEHAVIOR HABITUATION

Two days before testing, animals were habituated to transportation and behavioral rooms. For this, mice were transferred to the behavior rooms in their home cage and left in the waiting area for three hours before being moved to the testing area for another three hours. Light conditions were set according to the light conditions of the first behavioral test.

GENERAL TESTING CONDITIONS

At the day of testing, animals were transferred to the behavior rooms and left undisturbed in the waiting area for at least 1 h to recover from transportation. Thereafter, animals were tested one at a time (open field, light/dark box, elevated plus maze, Morris water maze, Barnes maze, splash test, tube test) or four animals simultaneously in one box each (Phenotyper, fear conditioning, bedding preference, buried food test). After testing animals were moved to a holding cage filled with bedding to avoid impact on cage mates still to be tested. After each session, all mazes were cleaned with 5 % ethanol and whipped dry. At least one day of rest was left in between tests to minimize any effects of stress caused during testing. In addition, the order of tests was set from the least stressful to the most stressful test (Figure 15), to avoid carryover effects. Mice were allowed to rest for at least one week if tested in the Morris water maze and the cue discrimination paradigm in fear conditioning.

TRACKING

Behavior was tracked or scored manually using ANY-maze 4.50 (Stoelting, USA) and EthoVision XT 11 (Noldus, The Netherlands). A camcorder (Handycam HDR-CX130, SONY Corporation, Japan) was used to record grooming during the splash test and for the tube test.

Assessment of general behavior

To measure baseline locomotor activity, food consumption and sucrose preference, mice were singly placed into the Phenotypers (Noldus, The Netherlands) at 5 pm at the day of testing. The Phenotyper is a 30 x 30 cm Plexiglas box that is provided with a fodder, two drinking bottles and an infrared translucent shelter. The Phenotyper provides a light/dark cycle with lights on at 7 am and off at 7 pm. An infrared light was active the entire time of testing, enabling the camera to record during lights off. The floor of the Phenotypers was covered with common bedding and fodders filled with regular chow (*Kliba Nafag*, Provimi Kliba AG, Switzerland). One of the bottles was filled with tap water, whereas the other one was filled with 2 % sucrose solution to measure sucrose preference (sucrose preference p.75). Food, water and sucrose solution were available *ad libitum*. Mice were kept in the Phenotypers for three consecutive nights, and the baseline activity (distance moved) was analyzed by the tracking software. Food, water and 2 % sucrose solution intake were measured before and after the testing period. Furthermore, the amount of licks per bottle was measured by two independent LickOmeters (Noldus, The Netherlands). To avoid a bias due to the location of the bottle containing the sucrose solution, the place of the bottles was switched in between mice. Mice were removed from the Phenotypers at 4 pm at the last day of testing.

Anxiety-related behavior

Anxiety-related behavior of all mice was tested in the open field, light/dark box and the elevated plus maze. Mice that had to be excluded from one of the tests for technical reasons - such as the tracking system being unable to detect the mouse in the time window relevant for the test - were also excluded from the other two tests to ensure comparability between the three tests. In total 28 5-HT_{1a} receptor KO and 32 WT male as well as 29 5-HT_{1a} receptor KO and 30 WT female mice were included in these three tests.

Open Field

The open field consisted of a circular box of light gray polypropylene (Semadeni Plastic Technology, Switzerland) with a diameter of 74 cm. Light conditions were set to 40 lx in the center of the maze. At the beginning, mice were placed at the rim of the maze, as indicated by the red X in Figure 16 and allowed to explore the maze for 10 min. The maze was divided into three virtual concentric zones (center, intermediate and wall zone), each covering 1/3 of the radius (Figure 16). The total distance moved, time spent and number of entries in the center, intermediate and wall zone were analyzed automatically by the tracking software. The location of the mouse's center point was used as a measurement for zone entries.

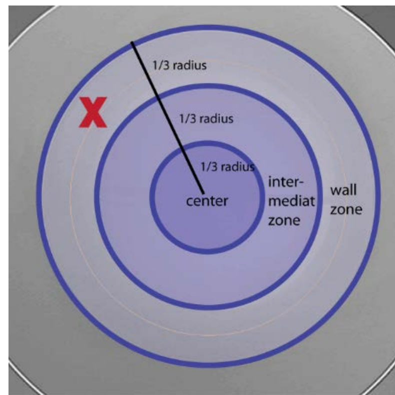

Figure 16 Virtual zones of the open field.
The picture shows the division of the open field into three different virtual zones by 1/3 of the radius. **X** indicates the start location for each trial. The amount of time spent in each zone is indicative for the level of anxiety of mice.

LIGHT/DARK BOX

The light/dark box is made of white PVC, bordered by 20 cm high walls and divided into two parts. The lager part of the box (20 x 40 cm) was brightly illuminated (165 lx) providing an aversive environment, whereas the smaller, dark compartment (20 x 20 cm) provided a safe and comfortable environment. The dark compartment was covered by a black, infrared translucent lid and could be separated from the lit compartment of the box by a door (5 x 5 cm). For testing, mice were placed in the dark compartment and allowed to habituate to the new situation for 30 s. Subsequently, the door was opened, and mice allowed to freely explore both compartments for 5 min. The distance moved during these 5 min was analyzed automatically. The number of entries (all four paws in the lit or dark compartment), latency to enter the lit compartment, rearing and risk assessment (if mouse entered the lit compartment only with parts of the body, head and forepaws) were scored manually.

ELEVATED PLUS MAZE

The elevated plus maze (EPM) consists of two open and two enclosed arms (30.5 x 5.5 cm) emanating from a central platform (5.5 x 5.5 cm) elevated 50 cm above the floor. Mice were placed in the center of the maze facing a closed arm and allowed to freely explore the maze for 5 min. Light conditions were set to 15 lx at the tip of the open arm. The latency to enter the open arm, time in the open and closed arms as well as the amount of entries were scored manually, whereas the distance moved was analyzed by the tracking software. An entry into the open or closed arm was scored when all four paws entered the arm.

Learning & memory

Morris water maze

Water maze training took place across seven consecutive days for four trials/day per animal with an inter trial interval (ITI) of 15 min. A trial was completed when the mouse reached the platform or 60 s had elapsed. Upon failure to find the target platform, mice were guided to the platform and left there for 10 s of orientation. At the fourth day before the training session started and 24 h after the last training session, spatial memory was assessed in a probe trial. Opposite starting positions were used for each probe trial. During probe trial, the platform was removed from the pool and mice were left to swim for 60 s. To investigate reversal learning the platform was moved to the quadrant opposite to the quadrant previously associated with the escape platform. Four reversal trials were run immediately after the last probe trial and four more 24 h thereafter. The escape latency, swim speed and the time spent in the quadrants of the maze were measured automatically.

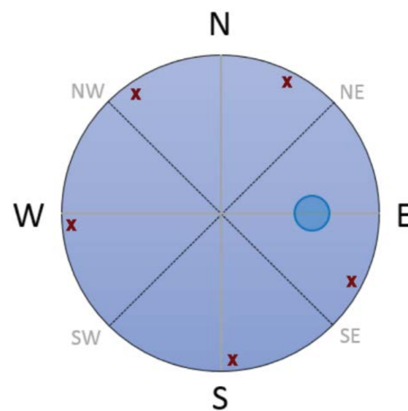

Figure 17 Scheme of Morris water maze.
X symbols depict the location of the in-maze cues, the blue circle shows the location of the hidden platform during training. For the reversal trial the platform was moved to the opposite quadrant. Starting points for each trial varied, mice were either placed in the water next to the NE, SE, SW or the NM mark.

Barnes Maze

To assess spatial learning and memory under less stressful conditions than in the Morris water maze one batch of mice was tested in the Barnes maze (Noldus, the Netherlands). The Barnes maze was placed in a brightly lit room. It consists of a circular platform (Ø 100 cm) that is elevated 50 cm above the floor. At the rim of the maze there are 20 holes of which one leads to a small escape box (Figure 18). The room itself was decorated with distinct cues to facilitate orientation. Mice were placed in a cylinder in the middle of the maze and allowed to acclimatize for 10 s. Thereafter, the cylinder was removed and the mouse allowed to freely explore the maze for 3 min. A trial was finished when the mouse climbed into the escape hole or if 3 min had elapsed. If the mouse failed to locate the target

hole, it was gently guided towards it and left in it for one minute. Four trials per day were run per mouse with an ITI of 15 min. The training lasted for four days in total. During training trails the latency to reach the target hole, velocity (cm/s) and the distance moved until reaching the target hole were analyzed automatically. The errors made (nose pokes to other holes) were scored manually. At day V a probe trial was conducted to assess spatial memory. Mice were again placed in the maze and allowed to move freely for 90 s. The time they spent in the quadrant associated with the target hole and the latency to reach the target hole were measured.

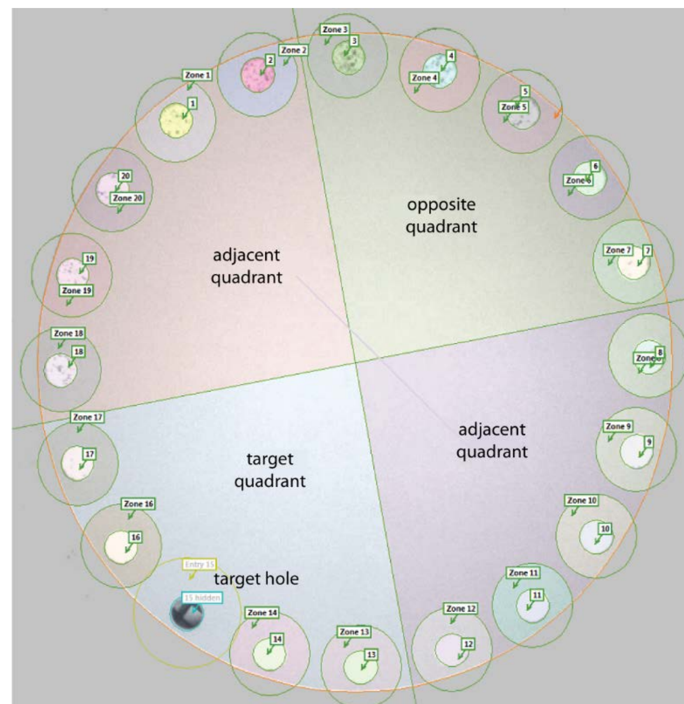

Figure 18 Virtual zones of the Barnes maze.
Screenshot of the arena set up (Ethovision). For the probe trial the maze was divided into four virtual zones (target, adjacent and opposite quadrant). The time spent in these quadrants was used to assess spatial memory.

FEAR CONDITIONING

Habituation, training and exposure were conducted in 17 x 17 x 25 cm fear conditioning boxes (46000-100 Fear Conditioning System, Ugo Basile, Italy) equipped with a grid floor able to deliver a foot shock of constant current. These boxes were placed in isolation cubicles 55 x 60 x 57cm (Ugo Basile, Italy) which were equipped with fan, speakers, light, infrared light and camera. The hardware was controlled via EthoVision XT 11 (Noldus, Netherlands) and adapted as needed for each experiment. In general, the fan speed was set to 50 % and a constant background white noise of 4 % was provided. In all experiments, freezing behavior – defined as the absence of movement except respiration - was measured using the automatic activity analysis (EthoVision XT 11 settings: activity setting was set to 0.1

% and instances shorter than 1 s were excluded) which was validated by manual scoring. Two different batches of animals were used for contextual fear conditioning to ambiguous cues (group A) and cue discrimination (group B and C).

Habituation

To ensure that the fear conditioning boxes themselves did not evoke freezing behavior, all test animals were exposed to the box one day before the training session. The animals were placed in the experimental context and allowed to explore the environment for 10 min. Thereafter, they were placed back in there home cages and left undisturbed until the next day.

Contextual fear conditioning to ambiguous cues

Training was conducted as described in Klemenhagen *et al* (2006) with slight alterations. The walls of the fear conditioning boxes were covered with stripped wall papers and lights (10 lx) turned on. The grid floor was left exposed and the animals placed in the boxes and allowed to explore. Three foot shocks of 0.2 mA lasting 1 s each were delivered. The first shock was delivered after 180 s had elapsed, the other two followed within 1 min intervals. Mice were removed from the boxes after a total duration of 8 min. 24 h after training, freezing behavior was assessed in a context similar to the training environment. The grid was covered by a white plastic sheet and two of the checkered wall papers were exchanged by white wall papers. Lights and white noise were provided as in the training context. The amount of freezing was measured for 8 min. 24 h after exposure to the ambiguous environment, mice were again exposed to the training context and the amount of freezing was assessed for 8 min.

Cue discrimination

24 h after habituation the animals were placed back in the test boxes. Training conditions were similar to the ones described in Crestani *et al* (1999). Mice were allowed to habituate to the boxes for 3 min before being exposed to a partial (tone; 10 s, 80 dB, 2500 Hz) and a complete cue (light; 10 s, 10 lx). The electric shock (0.4 mA, 1 s) presented at the offset of the complete cue only (Figure 19). The tone was presented every 3 min, twice alone and three times serially in combination with the light-shock pairing. Immediately after the last tone-light-shock pairing, animals were removed from the boxes. 24 h later, freezing was assessed automatically for 3 min with no stimulus and subsequently for 6 min in the presence of only the tone and for an additional 6 min in presence of only light. The context was not changed in between training and expression of freezing.

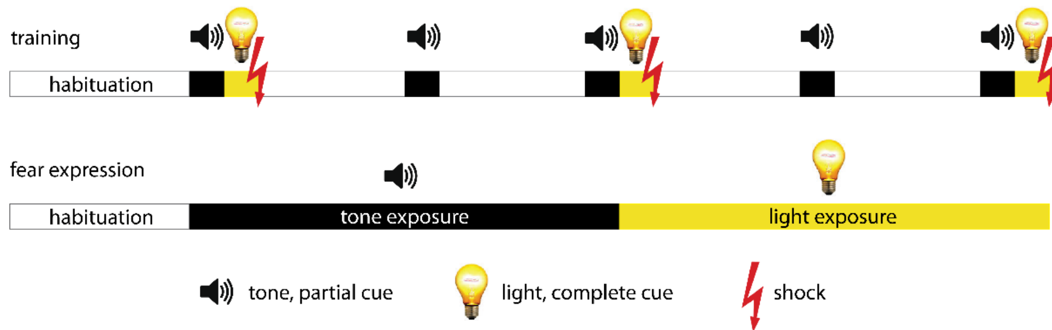

Figure 19 Scheme of cue discrimination training and exposure 24 h thereafter.
Mice were exposed to the partial cue (tone) five times during training which was only followed 3 times by the complete cue (light) - shock pairing. During the fear expression trial mice were exposed to the partial and complete cue for 6 min each.

Behavioral response to the electric shock

The response to the electric shock during training was analyzed from video tape to ensure that there were no significant differences between gender and genotype for foot shock sensitivity. The reaction of the animals was categorized into flinching, movement and jumping for each of the three shocks given during training. The behaviors were assigned scores from 1 to 3 respectively and the average of the three responses compared with the other animals.

Tube Test

The tube test was performed to determine dominance status of the animals. Male mice were habituated to the Plexiglas tube (Ø 3 cm x 30 cm, *evonik* Industries, Germany) one day before testing. For this matter mice were placed in a cardboard tube which was positioned in front of the Plexiglas tube. An identical cardboard tube was located at the opposite end of the Plexiglas tube. When the mice had walked through the Plexiglas tube, they were moved to the other ending and walked through the tube in the opposite direction. This procedure was repeated twice to ensure habituation. At the day of testing, cardboard tubes containing a WT and a 5-HT_{1a} receptor KO mouse, respectively, were simultaneously placed in front of the Plexiglas tube at opposite ends. This procedure ensured that animals entered the tube at the same time and met in the middle, thus preventing a bias caused by the meeting point (Figure 20). The test ended when one of the mice completely retreated from the tube (all paws out) or when two minutes had elapsed. Each pair was tested four times, with mice starting from alternating sides of the tube. The mouse remaining in the tube was designated the winner (score = 1), whereas the retreating mouse was the loser (score = 0). If none of the mice had retreated during 2 min period both got a score of 0.5. Matches in which the mice crossed over each other were not scored.

Figure 20 Mice interacting in the tube test.
Two mice are meeting in the middle of the Plexiglas tube at the beginning of the test. Two cardboard tubes can be seen at each end which are used to facilitate the entrance of the mice into the tube.

DEPRESSIVE-LIKE BEHAVIOR

SPLASH TEST

The splash test was performed as an indication for depressive-like behavior and executed, as described before (Ducottet *et al*, 2004) but with slight alterations. Testing was conducted during the light phase. Due to group housing, mice were removed from their home cage shortly before testing and placed into a holding cage. Each mouse was individually reintroduced in the home cage after being sprayed with 10 % sucrose solution. The total time spent in and the frequency of self-grooming were measured manually during a 5 min time period.

TESTS FOR OLFACTION

All tests for olfaction were carried out in the dark phase starting earliest one hour after lights off and ended at least four hours before lights on. The experiments were carried out under red light in at test box of 40 x 34 x 17 cm.

BURIED FOOD TEST

Mice were food deprived for 16 h before testing. At 6 am, food was removed from the cages. Mice (WT female n=10, male n=12; 5-HT_{1a} receptor KO female n=11, male n=8) were tested starting from 10 pm onwards, and food was placed back in the cages thereafter. Test boxes were filled with approximately 3 cm of fresh bedding (Lignocel, JRS, Germany). A quarter of a cookie (Oreo, Nabisco, USA) was buried and covered with 1 cm of bedding. Mice were placed in the box for 10 min or until they had found the piece of cookie (cookie needed to be visible for the experimenter). Thereafter, mice were moved back to their home cage and the same procedure was repeated the next day with the cookie in a different place. Testing took place at three consecutive days and was videotaped for further analysis. The latency to uncover the piece of cookie was scored manually.

BEDDING PREFERENCE

Two glass bowls were filled with either dirty or fresh bedding, respectively, and placed in opposite ends of the test box. Dirty bedding was taken from cages with at least 3 – 4 mice of the same gender as the

test mouse. The test was repeated twice: once the bowls containing the bedding were covered with a grid, the other time the bedding was accessible to the mice and they could dig during testing. Mice were left in the test box and allowed to explore for 10 min. The time spent exploring or digging in both bowls was measured. The preference % was calculated as follows:

$$\%\ bedding\ preference = \frac{time\ spent\ in\ proximity\ to\ dirty\ bedding}{(total\ time\ spent\ in\ proximity\ to\ dirty\ and\ clean\ bedding)} \times 100$$

In total 12 5-HT_{1a} receptor KO and WT male mice as well as 13 5-HT_{1a} receptor KO and 14 WT female mice were used for this test.

II.2 MOLECULAR ANALYSIS

WESTERN BLOT

Hippocampi from KO and WT animals of different ages were rapidly dissected on ice and sunk into 100 µl ice-cold lysis buffer (20 mM HEPES, 10 mM NaCl, 3mM $MgCl_2$, 2.5 mM EGTA, 0.1 mM DTT, 1mM Na_3VO_4, 50 mM NaF, 1 % Triton X-100 (all Sigma, USA), 1x cOmpelte protease inhibitor cocktail, 1x phosSTOP (both Roche, Switzerland). The tissue was homogenyzed using a glass tissue grind pestle (size 20, Kimble Chase, USA) and, depending on the size of the hippocampi, up to 400 µl of lysis buffer were added. 2 µl of sonicated lysate were used to determine the protein concentration applying the Bradford method. 10 µg of lysate was applied on SDS-PAGE for separation of the proteins. Transfer to the nitrocellulose membrane (0.45 µm, Biorad, Switzerland) was conducted using the Biorad system. After transfer, Ponceau staining was conducted and the membrane scanned to determine the total protein concentration for normalization. The membrane was washed in 1x phosphate buffered saline (PBS) to remove Ponceau staining and blocked (130 mM NaCl, 5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 3mM NaOH, 5g/l Casein (all Sigma, USA)) for one hour. Primary antibodies (mouse anti-NR2B 1:5000; UC Davis/NIH NeuroMab Facility, USA, rabbit anti-NR2A 1:5000, Merck Millipore, USA) were diluted in blocking buffer containing 1 % Tween20, and membrane incubated at 4 °C overnight. After washing steps in 1x PBS, the secondary antibody diluted in blocking buffer was applied (1:15000 IRDye® 800 CW (polyclonal) anti-rabbit IgG or anti-mouse IgG, LI-COR, Germany), and membrane was incubated for one hour. Membranes were scanned using LI-COR Odyssey imaging system using infrared technology to detect the bands and to measure intensities.

ISOLATION OF SYNAPTONEUROSOMES

Synaptoneurosomes were isolated as described by Hollingsworth *et al* (1985). Briefly, tissue samples were homogenized as described above. The volume of the samples was filled up to 400 µl with lysate buffer and 10 µl were kept aside for total protein fraction. Lysate was pressed through a 100 µm pore nylon mesh filter (Millipore, Switzerland) and remaining, followed by 2 filtration steps in which the lysate was pressed through 5 µm pore size filters (Millipore, Switzerland). Following 20 min of centrifugation at 3000 rpm at 4 °C the supernatant was resuspended in 100 µl of lysate buffer. Western blot procedure was carried out as described above.

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (QRT-PCR)

PRIMER DESIGN AND EFFICIENCY

Primer pairs for GAPDH, NR2B, REST, REST/4, 5-HT$_{1a}$ receptor were designed using Primer-Blast and generated by Microsynth (Switzerland). PCR products of 149-313 bp length were generated by applying specific primers (Table 4) to a cDNA sample of a WT pP10 animal and run at a gradient ranging from 55 to 60°C in 8 steps. The temperature that produced a single band of the correct size with the highest yield (Figure 21) was used to produce samples for sequencing and as annealing temperature for qRT-PCR. Amplified DNA products were loaded on 2 % agarose gels for separation, bands cut and DNA extracted with the QIAquick Gel Extraction Kit (Qiagen, Basel, Switzerland). DNA samples were send to Fasteris SA (Switzerland) for sequencing. After sequence verification with Primer-Blast, primer efficiency was determined by running serial dilutions of a WT P10 sample.

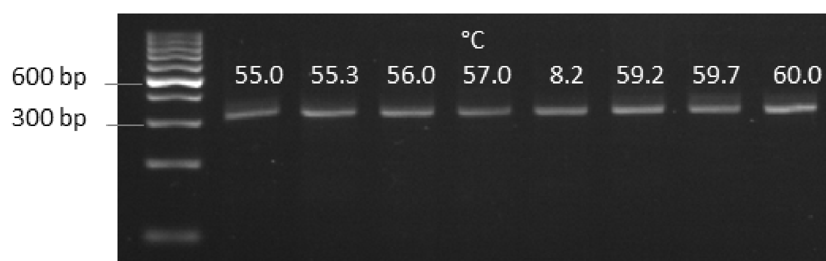

Figure 21 Example of cDNA amplification with different annealing temperatures.
cDNA amplification using the primer pairs for REST. 58.2 °C was chosen as annealing temperature for qPCR.

RT-PCR

Total RNA was extracted from freshly dissected hippocampus at different postnatal ages (P5, P10, P15, P20, P30, P60) using the RNeasy® Plus Mini Kit (Qiagen, Switzerland). RNA concentration was measured

with NANOdrop 1000 (Witec AG, Switzerland). 1 µg of total RNA was used for reverse transcription. For this purpose, the RNA was incubated with oligo(dT) (0.5 µg/µl, Microsynth, Switzerland) at 85 °C for 3 min. Afterwards, the reverse transcriptase (Moloney murine leukemia virus, MMLV, Promega, Switzerland) and master mix (5x RT-Buffer, 10 mM dNTP Mix, Promega, Switzerland) were added and cDNA synthetized at 44°C for 1 h followed by an heat-inactivation step at 95 °C for 10 min.

qPCR

qPCR amplification of cDNA was performed on the BioRadMyIQ Single-Color Real-Time PCR detection system using BioRad SsoAdvancedTM Universal SYBR ® Green Supermix (Biorad, Switzerland). cDNA samples were amplified using the following protocol: 3 min of heat activation at 95 °C were followed by a total of 45 cycles of 30 s at 94°C, 1 min at the primer specific annealing temperature (see Table 4) and 1 min at 72°C. Following a melting curve from 65-95°C (increment 0.5°C) was run.

All samples were run in triplicates and normalized to GAPDH that was run on the same plate. Relative gene expression was calculated using an efficiency corrected ddCT method (Pfaffl, 2001).

Transcript	Primers (5' → 3')	temp. during qPCR	product size (bp)	primer efficiency (%)
5-HT_{1a} receptor NM_008308.4	F: AAGTGGACTCTGGGTCAGGT R: GGCGTCCTCTTGTTCACGTA	59.6	149	79.48
NR2B NM_008171.3	F: GTCATGCTCAACATCATGGAAG R: GCTTCTTCAGCTGATTCTGGAT	55.3	202	90.49
REST NM_011263.2	F: AGCGAGTACCACTGGAGGAAACA R: AATTAAGAGGTTTAGGCCCGTTG	58	313	90.58
REST (& REST4) NM_011263.2	F: GAACTGGGAAGTTTGGAGCTAA R: TGCACAAACTGCTCTTCAGATT	59	200	94.02
GAPDH NM_008084.2	F: AGACCCCACTAACATCAAATGG R: ATGCATTGCTGACAATCTTGAG	same as gene of interest	212	dependent on gene of interest

Table 4 Primer sequences for qRT-PCR

DIG *IN SITU* HYBRIDIZATION

Probe generation

A 15 day old WT male mouse was used for probe generation. The pup was decapitated and the brain removed quickly. The entire brain was homogenized in lysis buffer (RTL buffer, RNeasy® Plus Mini Kit, Qiagen, Switzerland) and a fraction of the homogenate was used for RNA extraction. RNA was extracted using the RNeasy® Plus Mini Kit (Qiagen, Switzerland). 1-2 µg of RNA was used for reverse transcription which was conducted according to manufacturer's instructions (Omniscript RT Kit, Qiagen, The Netherlands). 500 ng of cDNA were used to amplify the sequence of interest. After purification, the PCR

products were amplified using sense and antisense primers of the region of interest containing the T7 RNA polymerase promoter sequence. Amplicons were separated on a 2 % agarose gel and purified using MinElute PCR Kit (Qiagen, The Netherlands). A fraction of the amplicon was send to Fasteris (Switzerland) to verify sequence specificity.

5’ and 3’ Digoxygenin (DIG) labeled cRNA probes were synthesized during four hours of incubation of 1μg DNA with DIG RNA labling mix (Roche, Siwtzerland), 10 mM DTT, 5x RNA polymerase buffer (Promega, USA), RNase inhibitors (Promega, USA) and T7 RNA polymerase (Promega, USA). Thereafter, probes were purified with the RNeasy® Plus Mini Kit (Qiagen, The Neherlands) and their concentration determined using NANOdrop 1000 (Witec AG, Switzerland). Probes stored at -80 °C until usage.

IN SITU HYBRIDIZATION

WT and 5-HT_{1a} receptor KO mice of 15 days of age were injected with a lethal dose of pentobarbital (50 mg/kg) i.p. and were perfused with 4 % paraformaldehyde (PFA) solution (Sigma, Switzerland). Brains were removed, post-fixed for one hour, transferred to 0.1 M phosphate buffer buffer (PB, 2.64 mM NaH_2PO_4, 0.92 mM Na_2HPO_4, pH 7.4) and stored at 4 °C until the next day. Brains were sunk in agarose and cut into 50 µm sections with the Leica VT1000 S vibrating blade microtome (VT1000S, Leica Microsystems, Germany). Free floating sections were fixed in 4 % PFA solution for 3 min and then washed with PBS. After 30 min of incubation with proteinase K solution (Roche, Switzerland) at 37 °C, sections were fixed again in 4 % PFA. Acetylation solution (0.5 % acetic anhydride, 1.35 % triethanolamine, 0.067 % HCl in DEPC-treated water (all Sigma, USA)) was applied for 10 min to reduce electrostatic attraction between hybridization probes and basic proteins in the tissue. A washing step with 1x PBS followed. Subsequently, hybridization buffer (50 % deionized formamide (Merck, Germany), 10 % dextran sulfate (Fluka, Germany), 25 % SSC, 0.3 mg/ml Yeast tRNA, 1x Denhardts (all Sigma, USA), 0.1 % Teewn20 (Fluka, Germany), 5 mM EDTA (Sigma, USA)) was added, and sections incubated for 3 h to decrease unspecific binding of the probes. The probes were heat-denaturated at 65 °C for 15 min and kept on ice applied thereafter. Probes (200ng) were applied and sections incubated at 60 °C overnight in a wet chamber (humidified with 50 % formamide, 25 % SSC solution). The day after, sections were subjected to multiple rinses with decreasing % of SSC (from 25-0.5 %), and a final washing step in 1x PBS. Sections were incubated with alkaline phosphatase conjugated anti-Digoxigenin (1/300, Roche, Switzerland) in blocking solution (1x PBS, 0.1 % Tween-20, 0.1 % bovine serum albumin) overnight. A washing step in 1x PBS was followed by rinses in Tris-buffer (0.1 M Tris pH7.5, 0.15 M NaCl). Subsequently, immunostaining was revealed using the alkaline phosphatase color reaction applying 4.5 µl/ml nitro blue tetrazolium and 3.5 µl/ml 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Roche, Germany). The reaction was stopped by washing sections in 1x PBS. After staining with 4’6-Diamidin-2-phenylindol (1:1000, DAPI, Invitrogen, Switzerland) sections were mounted and coverslipped with

Mowiol mounting solution (0.2 M Tris (Sigma,USA), 25 % glycerol (Merck, Germany), 10 % Mowiol 4-88 (Calbiochem, Germany)).

II.3 Morphological Analysis

Organotypic cultures

Organotypic cultures can be used as an *in vitro* model to study the effects of pharmacological manipulation on the dendritic arborization during the critical period of development. We used organotypic cultures to study the effect of 5-HT_{1a} receptor on the synaptic expression of NR2B-containing NMDA receptors.

Preparation & maintenance

Organotypic cultures were prepared according to the method of Stoppini *et al* (1991). Briefly, pups at postnatal day 4 were sprayed with 70 % EtOH and quickly decapitated. Skin and skull were cut and brains dropped rapidly into cold dissection medium (Earle's minimum essential medium, 25 mM HEPES, 10 mM Tris-base, 1 M D(+) dextrose, 3 mM Tris (all Sigma, USA), pH 7.4). Under the dissecting microscope, hemispheres were separated and hippocampi isolated carefully using two forceps. Hippocampi were cut into 350 μm thick slices using a McIlwain tissue chopper (Mickle Laboratory Engineering LTD CO, UK) and carefully transferred to a petri dish containing cold dissection medium. Slices were gently separated and individually transferred to Millicell inserts (Millipore, Germany). No more than six (propidium iodide study, see below) to eight slices (drug treatment to assess dendritic complexity) were plated on an insert. Inserts were quickly placed in six well culture plates (BD Falcon, USA) containing 1 ml of serum free culture medium (Neurobasal medium supplemented with 2 mM of L-glutamine and B27 (all Gibco, USA)) each. Slices were cultured under air-medium interface conditions at 35 °C in 5 % CO_2 until further processing. The culture medium was renewed every second day by removing 500 μl of the old and adding 520 μl of fresh and equilibrated culture medium.

Drug Treatment

To understand how 5-HT_{1a} receptor regulates NR2B-containing NMDA receptor expression at the synapse and, thereby, dendritic morphology of CA1 pyramidal neurons, two different drugs were applied to the culture medium. U0126 - an Erk1/2 inhibitor - which is expected to downregulate 5-HT_{1a} receptor-dependent delivery of NR2B-containing NMDA receptors to the synapse (Yuen *et al*, 2005) in absence of 5-HT. TBB, a casein kinase 2 inhibitor should block the removal of NR2B-containing NMDA receptors from the synapse and should result in increased branching. 5-HT was added to the culture in WT-like culture conditions and ifenprodil (IFEN), a selective antagonist of NR2B-containing NMDA

receptors, was added as a control to otherwise 5-HT free medium, to decrease the amount of branching. Concentrations of each drug are described in Table 5. If not stated differently, drugs were added to the culture medium with each change of medium.

Drug	stock concentration	final concentration	solvent
5-HT *(5-Hydroxytryptamine)*	10 mM	10 μM	H_2O
Ifenprodil *(4-[2-(4-nezypliperidin-1-yl)-1-hydroxypropyl]phenol)*	30 mM	3 μM	H_2O
U0126 *(1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene)*	10 mM	10 μM	DMSO
TBB *(4,5,6,7-Tetrabromobenzotriazole)*	10 mM	1 μM	DMSO

Table 5 Drugs applied on organotypic cultures

Fixation, staining & counting

After 15 days *in vitro* (DIV 15), the culture inserts were sunk in 4 % paraformaldehyde (pH 7.4, Sigma, USA) and left for fixation in the dark for 40 min. Subsequently, inserts were washed twice with 1x PBS before the membrane was cut and the pieces containing slices were transferred to a 24 well plate. Immunostaining was done free floating. After washing, slices were covered with blocking buffer (1x PBS, 2 % NHS, 0.3 % Triton X-100, Azide 1g/l) and left on the shaker for one hour. Chicken anti-GFP (1:500, AVES, USA) was diluted in blocking buffer and slices incubated with primary antibody in the cold room (4 °C) over three nights. Following three washing steps, the secondary antibody (1:200, fluorescein goat anti-chicken IgY coupled to Alexa 488 fluorochrome, F1005, AVES, USA) was applied and slices incubated overnight. DAPI (1:1000) staining was performed in 0.1 M PB shortly before mounting. Finally, slices were coverslipped in Mowiol mounting medium (0.2 M Tris, 25 % glycerol, 10 % Mowiol 4-88) and left to dry. Oblique dendrites of CA1 pyramidal neurons were counted live using the 20x objective of the Axio vision Zeiss microscope.

II.4 Analysis of cell survival

Propidium Iodide Study in organotypic cultures

Organotypic hippocampal slices were cultured as mentioned in section (organotypic cultuers p.54) and maintained for nine days *in vitro*. At DIV9, 100 µl of propidium iodide (PI) (1mg/ml in 1x PBS, Sigma, USA) solution was added directly to the culture medium, and cultures were incubated for 40 min in the incubator. Subsequently, all culture inserts (Millipore, USA) were transferred to fresh equilibrated

medium to stop further uptake of PI. For acquiring images, culture inserts were transferred to a petri dish containing 1 ml of fresh, equilibrated medium and pictures were taken using a LeicaMZ 16 FA binocular. To achieve 100 % cell death in the culture, the culture medium was exchanged by 1 ml 1x PBS and cultures incubated at 4 °C overnight. The next day 100 µl PI solution was added to 1x PBS, and cultures again incubated for 40 min. After transfer to a fresh petri dish containing 1x PBS, pictures were captured as described above.

Cell death was measured by fluorescence intensity of PI in the CA1 region of the hippocampus. Intensity of fluorescence was quantitated using FIJI (Schindelin *et al*, 2012). The area of the region of interest was the same between acute and at 100 % cell death for one slice but could vary between slices.

The percentage of cell death was calculated by comparing the intensities measured acutely and at 100 % of cell death.

$$\%\,cell\ death = \frac{fluorescent\ intensity\ experiment}{fluorescent\ intensity\ 100\%\ cell\ death} \times 100$$

Adult neurogenesis

Injections, Exposure to olfactory cues & perfusion

At weaning, four mice per group (male and female WT as well as male and female 5-HT_{1a} receptor KO mice) were single housed to avoid social interaction and constant exposure to olfactory cues of cage mates. At P32, mice were injected i.p. three times (ITI: 3h) with 100mg/kg Bromodeoxyuridine (BrdU, Sigma, USA), dissolved in 0.9 % sterile NaCl. BrdU is a synthetic thymidine analog that binds to newly synthesized DNA during the S phase of the cell cycle and hence marks proliferating cells. 28 days after injections, mice were placed in an open field (45 x 45 cm). After two hours in the open field, two glass bowls were introduced. One was filled with dirty bedding as described above (bedding preference p.49), the other one was filled with clean bedding. The bowls were covered with a plastic grid, to avoid digging. This step was introduced to be able to study activation on the olfactory bulb to compare the survival of adult born neurons and olfactory functioning. After 30 min, mice were anesthetized with pentobarbital (50 mg/kg) i.p. and were perfusion-fixed intracardially with 4 % PFA. Brains were collected and postfixed for 30 min in 4 % PFA solution before being rinsed three times with 0.1 M PB at room temperature. Brains were stored at 4 °C until further processing.

Cutting and Immunostaining

Brains were cryprotected by immersion into 30 % sucrose solution 24 h before being snap frozen for cutting on the cryotome with dry ice. Coronal and horizontal sections were cut at 50 µm using the Microm HM440E cryotome (Histocom AG, Switzerland). After cutting, sections were rinsed in 0.1 M PB

and subsequently incubated for two hours at 65 °C in 50 % formamide 2xSSC solution. This procedure was followed by DNA denaturation in 2 M HCl for 30 min at 37 °C. Thereafter, sections were rinsed in 0.1 M borate buffer at pH 8.5 for 10 min. Following two rinses in 1x PBS and two rinses in 1x PBS + 0.3 % Triton X-100, slices were incubated for 60 min in blocking solution (3 % normal horse serum (NHS), 1x PBS + 0.3 % Triton X-100 pH 7.4). Sections were then incubated overnight at room temperature with BrdU antibody (mouse monochlonal anti-BrdU, DAKO/Agilent Technologies, USA) diluted 1/50 in blocking solution. Following washing steps in 1 x PBS, sections were incubated for 2 h with secondary antibody. Finally, nuclei were revealed using DAPI staining, sections mounted and coverslipped in mowiol. Four to six sections were used to stain for Ki67 and doublecortin (DCX) positive cells. Sections were rinsed in 1x PBS, followed by rinses in 1x PBS, 0.3 % Triton X-100 (pH 7.4) and were blocked for one hour in blocking solution (1x PBS, 2 % NHS, 0.3 % Triton X-100 (pH 7.4)). DCX (goat anti-DCX, 1/500, Santa Cruz Biotechnology, USA) and Ki67 (rabbit anti-Ki67, 1/ 250, Abcam, UK) antibodies were diluted in blocking solution and sections incubated overnight at 4 °C. Secondary antibodies were diluted in blocking solution and applied after washing sections in 1x PBS and sections incubated for 90 min. The washing buffer was switched from 1x PBS to 0.1 M PB and sections stained with DAPI to reveal nuclei. Following the last washing step, sections were mounted and coverslipped in mowiol.

Cell number quantification

The number of BrdU positive cells was counted to assess survival rates of adult born neurons in WT and 5-HT_{1a} receptor KO mice. Four to six sections per mouse were stained for BrdU, and immunopositive cells were counted in the granular cell layer and the subgranular zone of the DG. Ki67 and DCX positive stained cells were counted in the same region to compare amounts of cell proliferation and maturation.

II.5 Statistics

Data was analyzed using GraphPad Prism 5 (Graphpad Software, Inc., USA) and JMP10 software (SAS Institute Inc., USA). All data was tested for normal distribution applying the Shapiro-Wilk test. Not normally distributed data was rank-transferred. Behavioral data was analyzed using a two-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test. Repeated measures ANOVA was used for analysis of the Morris water maze, Barnes maze, fear conditioning and general locomotor activity in the Phenotypers as well as licks over time for sucrose preference. Greenhouse-Geisser correction was applied to account for violation of sphericity when necessary. Molecular data was analyzed using a one-way ANOVA followed by Bonferroni *post hoc* test, or if not normally distributed, a Kruskal-Wallis test followed by Dunn's Multiple Comparison test. In all figures and text, values are represented as mean ± standard error of the mean (SEM) with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

III Results

III.1 Backcrossing

When starting our experiments, we observed extremely low levels of novelty induced locomotor activity in male and female 5-HT_{1a} receptor KO in comparison to WT mice (WT female n=30, male WT n=29, 5-HT_{1a} receptor KO female n=30, 5-HT_{1a} receptor KO male n=22; Figure 22, OLD)). As locomotor activity is a confounding factor for anxiety-related measures in all conflict tasks (open field, light/dark box, elevated plus maze), we risked to mask possible differences between male and female 5-HT_{1a} receptor KO mice. The difference in locomotor activity was likely induced by different expression of the mixed background (129SV/C57BL/6) in 5-HT_{1a} receptor KO and WT mice. The 129SV strain expresses lower levels of baseline and novelty induced locomotor activity and might have been more prominent in 5-HT_{1a} receptor KO than WT mice. To adjust for this shift and to increase locomotor activity, we backcrossed our mice further on C57BL/6 background. We used novelty induced locomotor activity as a reference for successful backcrossing. Figure 22 shows the total distance moved in the open field of mice of the original 129SV/C57BL/6 background in comparison to mice backcrossed for five generations on C57BL/6 background (WT female n=30, male WT n=33, 5-HT_{1a} receptor KO female n=31, 5-HT_{1a} receptor KO male n=27; Figure 22, NEW)). It can be seen that locomotor activity of male and female 5-HT_{1a} receptor KO mice was increased significantly (two-way ANOVA main effect of genotype

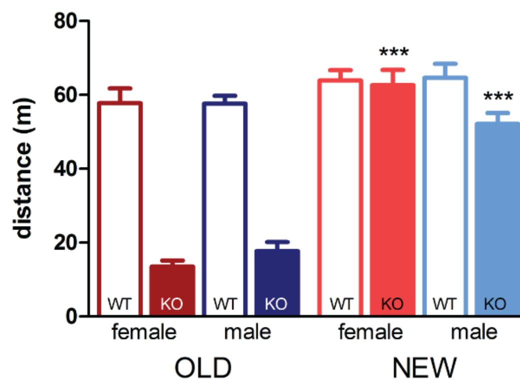

Figure 22 Increase of locomotor activity after backcrossing.
Mice on mixed 129SV/C57BL/6 background (OLD) were backcrossed with C57BL/6 (NEW) to increase novelty induced locomotor activity in 5-HT_{1a} receptor KO mice. A significant increase in locomotor activity can be observed in male and female 5-HT_{1a} receptor KO mice after backcrossing when comparing total distance moved in a 10 min session in the open field. *** indicate significant difference between OLD and NEW mice ($p<0.001$).

$F_{(1,226)}$=89.31 p<0.001;main effect of gender $F_{(1,226)}$=0.20 p=0.66)). Bonferroni *post hoc* test revealed a significant difference between male and female 5-HT_{1a} receptor KO mice on the mixed background in comparison to male and female 5-HT_{1a} receptor KO mice on the C57BL/6 background (p<0.001). These results show that backcrossing successfully increased locomotor activity of 5-HT1a receptor KO mice and thereby reduced the likelihood of locomotor activity as a confounding factor for activity based behavioral tests. All results shown in this study are based on backcrossed animals of the fourth and fifth generation.

III.2 BEHAVIORAL PHENOTYPING OF 5-HT_{1A} RECEPTOR KO MICE

The 5-HT_{1a} receptor has long been implicated in anxiety and mood disorders in humans and has been shown to influences anxiety-related and depressive-like behavior in mice (Goldberg *et al*, 1979) (Deakin, 1993). In addition, the likelihood to develop mental disorders is highly dependent on the gender (Forbes *et al*, 2004). Conversely, initial studies in 1998 showed that anxiety-related behavior of male 5-HT_{1a} receptor KO mice was more severely affected by 5-HT_{1a} receptor deletion than that of female mice (Heisler *et al*, 1998; Parks *et al*, 1998; Ramboz *et al*, 1998). However, no follow-up study has aimed at further investigating these gender-dependent differences. Our study therefore subjected male and female 5-HT_{1a} receptor KO and WT mice to a behavioral test battery to investigate potential gender-dependent differences. In general, tests included in this study were chosen based on two different criteria. Either they had already been reported to demonstrate significant differences between male 5-HT_{1a} receptor KO and WT mice, but had ignored potential gender-dependent differences (e.g. Morris water maze), or they have never been tested but added to the general understanding of the behavioral phenotype (e.g. splash test for depressive-like behavior). Mice used in this study were assigned to different groups and tested for anxiety-related and depressive like behavior as well as cognitive integrity, but were never subjected to all of the tests described below. For group size and detailed description of the test batteries see (behavioral phenotyping p.41).

GENERAL LOCOMOTOR ACTIVITY AND FOOD INTAKE

First, we investigated whether basic behavior such as food intake and locomotion differed between 5-HT_{1a} receptor KO and WT mice to identify possible confounding factors for further behavioral testing. In total 11 female and 11 male WT and 12 female and 10 male 5-HT_{1a} receptor KO mice were tested in the Phenotypers (Noldus, The Netherlands). Behavioral parameters for locomotor activity were obtained in the Phenotypers during three consecutive nights. Food intake was measured at the beginning and end

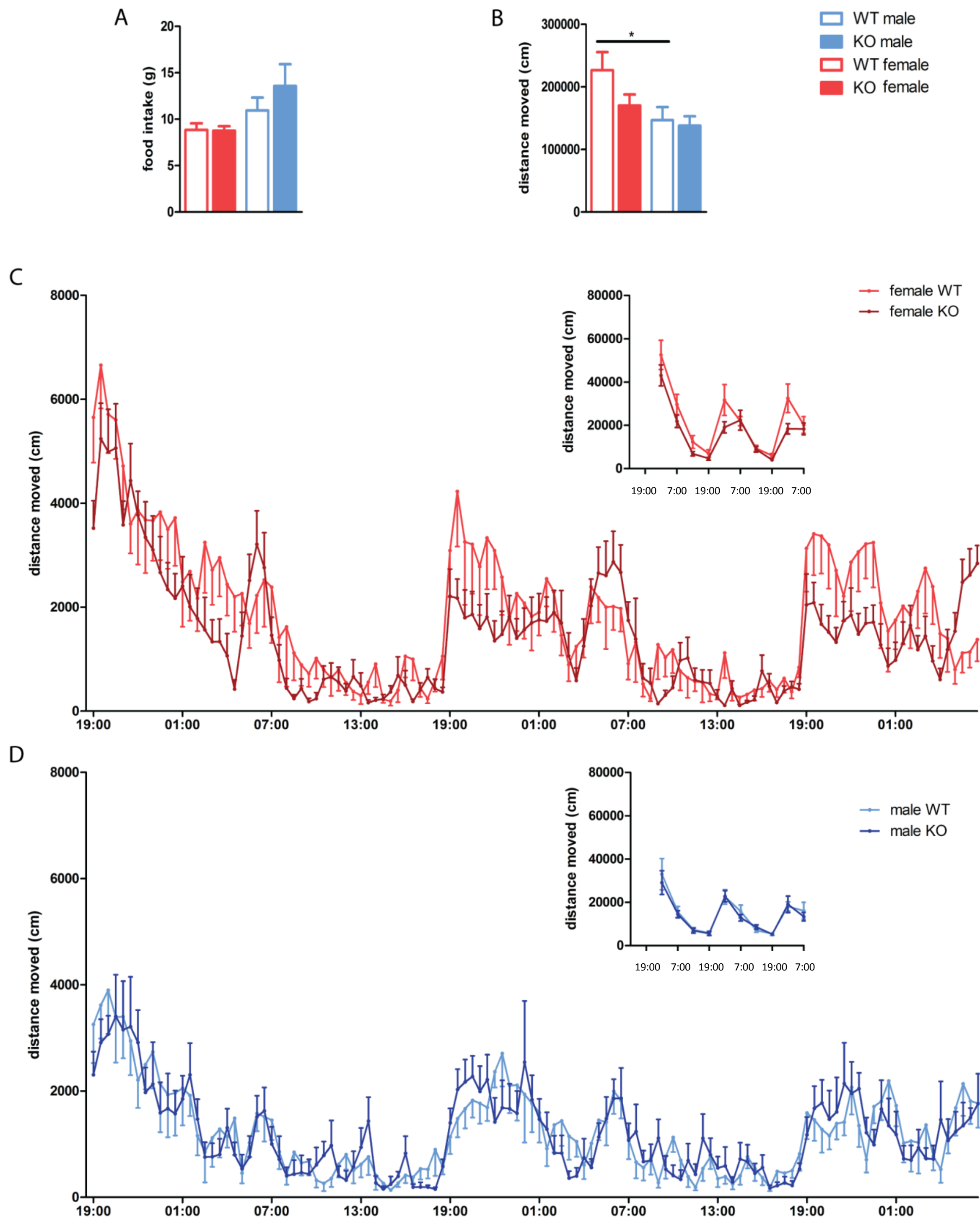

Figure 23 General behavior of 5-HT$_{1a}$ receptor KO and WT mice measured in Phenotypers.
Food intake (A) and total distance moved during the entire time of testing (B) was significantly influenced by gender with male mice eating overall more than female mice and females moving overall more than males. Significant differences were seen comparing female and male WT mice for the total distance moved over the entire time of testing. (C) and (D) depict the average distance moved in 30 min bins (and 6 h bins, inserts) of female (C) and male (D) 5-HT$_{1a}$ receptor KO and WT mice.

of testing. Differences seen in food intake were in line with general elevated food intake of male vs. female mice (Figure 23, A) (two-way ANOVA $F_{(1,41)}$= 6.75, p=0.0130). Bonferroni *post hoc* test revealed significant differences for food intake between 5-HT_{1a} receptor male and female KO mice (p<0.05). Genotype had no impact on food intake (two-way ANOVA main effect of genotype $F_{(1,41)}$=0.92 p=0.34). Figure 23 C & D depicts average locomotor activity over 30 min periods and the average of total distance moved over 6 h of female (C) and male (D) 5-HT_{1a} receptor KO and WT mice. All animals were highly active during the habituation phase within the first dark phase followed by low locomotor activity during the resting phase. Furthermore, two peaks of activity could be seen during the dark phase in the second and third night of testing. The first phase starts with light offset and ends around 3:00 am and the second one starts shortly after 5:00 am and ends approximately around 8:00 am. An overall effect of gender was seen on locomotor activity when comparing the total distance moved over 6 h time periods (two-way repeated measures ANOVA main effect of gender $F_{(3.53,\ 141.04)}$=0.39 p=0.006; main effect of genotype $F_{(3.53,\ 141.04)}$=0.74, p=0.44) and the total distance moved over the entire time of testing (two-way ANOVA main effect of gender $F_{(1,40)}$=6.73 p=0.0132; main effect of genotype $F_{(3.53,\ 141.04)}$=0.39 p=0.14). Bonferroni *post hoc* tests revealed a significant difference between male and female WT (p<0.05) mice for total activity, but no difference between male and female 5-HT_{1a} receptor KO mice (Figure 23, B). In conclusion, both male and female 5-HT_{1a} receptor KO mice as well as WT controls exhibit a normal sleep wake cycle, with low activity during the light phase and high activity during the dark phase. Furthermore, differences seen in locomotor activity of female 5-HT_{1a} receptor KO mice during the second peak of activity in the dark phase are unlikely to impact further behavioral testing, as all mice were tested between 9 am and 6 pm. Furthermore, decreased locomotor activity in conflict tasks such as the open field, light/dark box and the elevated plus maze is usually seen as a confounding factor resulting in false positives for measures of anxiety-related behavior. Having observed no differences in overall locomotor activity in the Phenotypers indicates that decreased locomotor activity in conflict tasks in this study is a result of anxiety, rather than a confounding factor.

ANXIETY-RELATED BEHAVIOR

To assess gender-dependent differences in anxiety-related behavior of 5-HT_{1a} receptor KO mice when compared to WT mice, animals were tested in three different conflict tasks: open field, light/dark box and elevated plus maze. All these tests evoke a natural conflict between the mice's innate aversion of brightly lit, open and - in case of the elevated plus maze - elevated spaces, and the spontaneous exploratory behavior induced by unfamiliar environments. In total, 30 WT female 33 WT male as well as 31 5-HT_{1a} receptor KO females and 27 5-HT_{1a} receptor KO males were included for these tests.

Open field

Anxiety-related behavior in the open field was significantly increased in male 5-HT_{1a} receptor KO mice (Figure 24, A-D). Significant effects could be seen for the amount of entries into the center of the open field (two-way ANOVA main effect of genotype $F_{(1,115)}=18.25$, $p<0.001$; main effect of gender $F_{(1,115)}=0.00$, $p=0.97$; gender by genotype interaction $F_{(1,115)}=1.65$, $p=0.20$; Bonferroni *post hoc* difference between male 5-HT_{1a} receptor KO and WT mice $p<0.001$, difference between female5-HT_{1a} receptor KO and WT $p>0.05$) and the time spent in the center of the open field (two-way ANOVA main effect of genotype $F_{(1,115)}=18.52$, $p<0.001$; main effect of gender $F_{(1,115)}= 0.12$, $p=0.73$; gender by genotype interaction $F_{(1,115)}=3.05$, $p=0.08$; Bonferroni *post hoc* difference between male 5-HT_{1a} receptor KO and WT mice $p<0.001$, difference between female5-HT_{1a} receptor KO and WT $p>0.05$), which was also reflected in the different amount of time that 5-HT_{1a} receptor KO male mice spent in the wall zone of the maze in comparison to WT males (two-way ANOVA main effect of genotype $F_{(1,115)}=19.15$, $p<0.001$; main effect of gender $F_{(1,115)}= 2.65$, $p=0.11$; gender by genotype interaction $F_{(1,115)}=2.85$, $p=0.09$; Bonferroni *post hoc* difference between male 5-HT_{1a} receptor KO and WT mice $p<0.001$, difference between female 5-HT_{1a} receptor KO and WT $p>0.05$). As total distance moved was also significantly decreased in 5-HT_{1a} receptor KO males (two-way ANOVA main effect of genotype $F_{(1,115)}=6.15$, $p=0.0146$; main effect of gender $F_{(1,115)}= 2.54$, $p=0.11$; gender by genotype interaction $F_{(1,115)}=0.9$, $p=0.34$; Bonferroni *post hoc* difference between male 5-HT_{1a} receptor KO and WT mice $p<0.05$, difference between female5-HT_{1a} receptor KO and WT $p>0.05$), it is possible that locomotor differences confounded results gathered for anxiety-related behavior. However, having observed no difference in general locomotor activity, it is likely that decreased locomotor activity is triggered by increased anxiety of male 5-HT_{1a} receptor KO and should not be treated as confounding factor.

Light/dark box

Female and male 5-HT_{1a} receptor KO mice showed increased levels of anxiety-related behavior in the light/dark box (Figure 24, E-G), but females were less affected than males. Two-way ANOVA revealed a significant difference for gender and genotype for the total distance moved in the light/dark box (main effect of gender $F_{(1,115)}=6.03$, $p=0.0156$; main effect of genotype $F_{(1,115)}=16.52$, $p<0.0001$; gender by genotype interaction $F_{(1,115)}=1.39$, $p=0.24$). Bonferroni *post hoc* tests showed an effect of distance moved between male and female 5-HT_{1a} receptor KO ($p<0.05$), but not WT mice ($p>0.05$) and a significant difference between male WT and 5-HT_{1a} receptor KO males ($p<0.0001$), but not between females ($p>0.05$). Furthermore, a decreased amount of entries to the lit compartment of male and female 5-HT_{1a} receptor KO mice was observed (main effect of gender $F_{(1,115)}=4.08$, $p=0.046$; main effect of genotype $F_{(1,115)}=21.15$, $p<0.0001$; gender by genotype interaction $F_{(1,115)}=0.37$, $p=0.54$). Bonferroni *post hoc* tests revealed significant differences for the amount of entries into the lit compartment between female ($p<0.05$) and male ($p<0.0001$) WT and 5-HT_{1a} receptor KO mice. No significant

difference was seen for gender in the *post hoc* test. The time spent in the lit compartment was not influenced by either gender or genotype, although a trend could be seen for the interaction between gender and genotype (main effect of gender $F_{(1,115)}=1.59$, $p=0.21$; main effect of genotype $F_{(1,115)}=1.34$, $p=0.25$; gender by genotype interaction $F_{(1,115)}=2.87$ $p=0.09$).

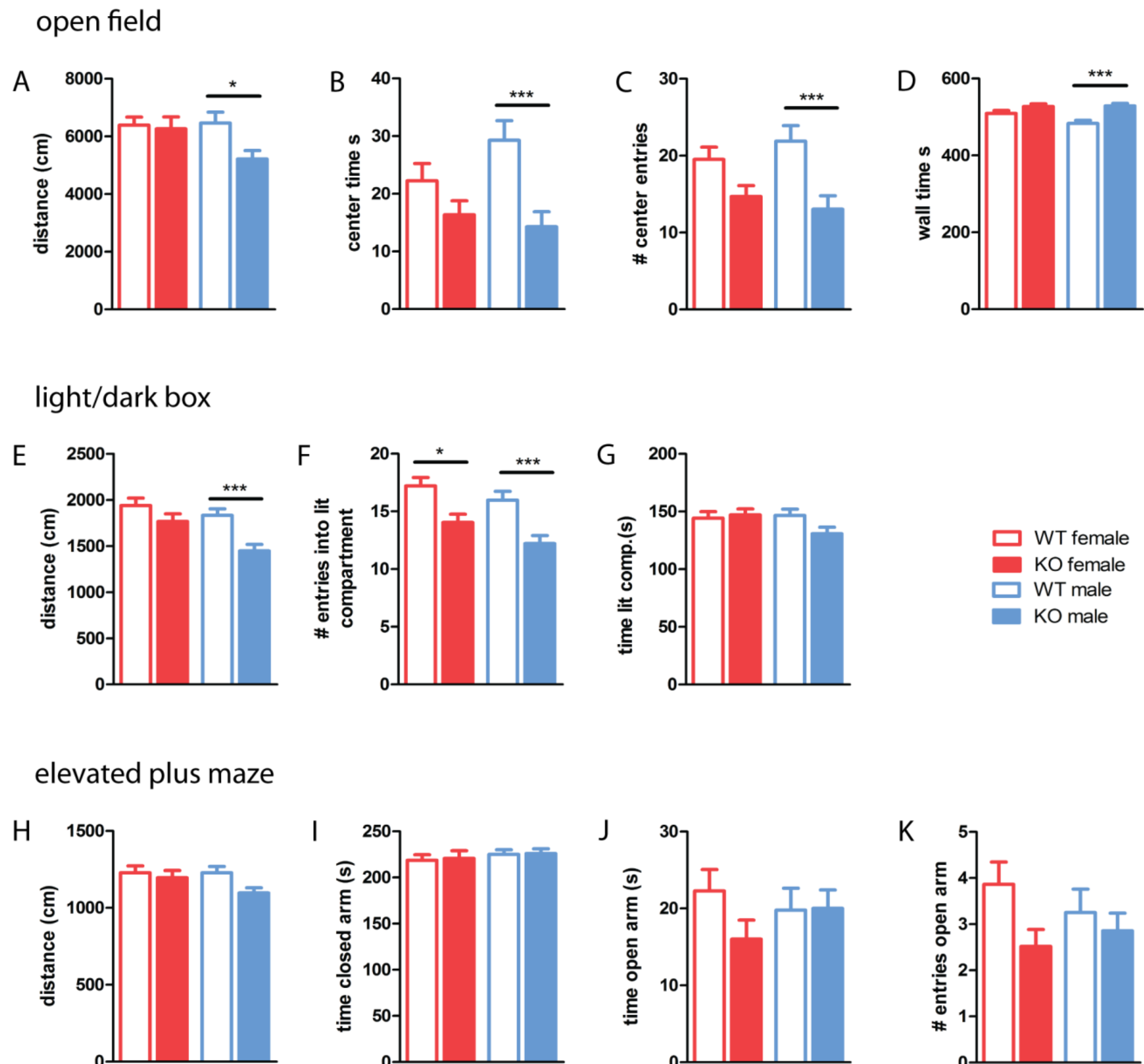

Figure 24 Anxiety-related behavior of 5-HT$_{1a}$ receptor KO and WT mice.
Anxiety-related measures of male are stronger affected than female 5-HT$_{1a}$ receptor KO mice in the open field (A-D) and the light/dark box (E-G) when compared to WT controls. No difference between groups were observed in the elevated plus maze (H-K).

Elevated plus maze

Although male 5-HT$_{1a}$ receptor KO mice showed significantly increased anxiety-related behavior in the open field and both, male and female 5-HT$_{1a}$ receptor KO mice showed elevated levels of anxious behavior in the light/dark box, no significant impact of genotype on anxiety-related measures was seen for the elevated plus maze (Figure 24, H-K). Neither male nor female 5-HT$_{1a}$ receptor KO mice differed

significantly from WT mice in the amount of entries into the open arm of the elevated plus maze (two-way ANOVA main effect of gender $F_{(1,115)}$=0.31, p=0.58; main effect of genotype $F_{(1,115)}$=0.32, p=0.77; gender by genotype interaction $F_{(1,115)}$=1.67, p=0.20), the time spent in the open arm (two-way ANOVA main effect of gender $F_{(1,115)}$=0.06, p=0.81; main effect of genotype $F_{(1,115)}$=0.63, p=0.43, gender by genotype interaction $F_{(1,115)}$=2.23, p=0.14) or the distance moved in the open arm of the elevated plus maze (two-way ANOVA main effect of gender $F_{(1,115)}$=0.17, p=0.68; main effect of genotype $F_{(1,115)}$=2.27, p=0.14; gender by genotype interaction $F_{(1,115)}$=0.82, p=0.37). A gender-dependent difference was seen for the entries into the closed arm (two-way ANOVA main effect of gender $F_{(1,115)}$=7.21, p=0.008, Bonferroni *post hoc* revealed significant difference between female and male WT mice, p<0.01; main effect of genotype $F_{(1,115)}$=0.13, p=0.72; gender by genotype interaction $F_{(1,115)}$=1.83, p=0.18) no difference was seen for the time spent in the closed arms (two-way ANOVA main effect of gender $F_{(1,115)}$=0.19, p=0.68; main effect of genotype $F_{(1,115)}$=0.18, p=0.67; gender by genotype interaction $F_{(1,115)}$=0.29, p=0.59).

In summary, anxiety-related behavior is more affected by 5-HT_{1a} receptor deletion in male than in female 5-HT_{1a} receptor KO mice, as they are influenced in their behavior in the open field and the light dark box, whereas female 5-HT_{1a} receptor KO mice only exhibit decreased amount of entries into the lit compartment in the light dark box.

LEARNING AND MEMORY

The Morris water maze, contextual fear conditioning to ambiguous cues and cue discrimination were previously used to assess cognitive functioning of 5-HT_{1a} receptor KO mice. In all tests, 5-HT_{1a} receptor KO mice differed significantly from WT controls. In the Morris water maze, male 5-HT_{1a} receptor mice were impaired in hippocampal-dependent learning and memory, as they showed delayed learning effects during training and were unable to recall the location of the platform in the probe test (Sarnyai *et al*, 2000; Wolff *et al*, 2004). Furthermore, male 5-HT_{1a} receptor KO mice showed increased freezing behavior to ambiguous cues in contextual fear conditioning and lack the ability to discriminate partial and completely predictive cues (Klemenhagen *et al*, 2006; Tsetsenis *et al*, 2007). These studies were conducted on male mice only, thus neglecting a potential gender-dependent phenotype. We exposed our mice to the same test paradigms to investigate to which extent gender could influence learning and memory. Furthermore, to understand if stress is a confounding factor for these measurements, we tested mice in the Barnes maze which is known to be less stressful than the Morris water maze (Harrison *et al*, 2009; Karabeg *et al*, 2013).

Morris water maze

11 male and 12 female 5-HT_{1a} receptor KO as well as 12 male and 12 female WT mice were tested in the Morris water maze task. Male and female 5-HT_{1a} receptor KO and WT mice learned to locate the hidden platform within given amount of time (two-way repeated measures ANOVA main effect of time $F_{(4.55, 195.7)}$=8.10, p<0.0001; Figure 25, A). No difference in learning abilities were seen in the Morris water maze comparing the latency to find the platform of 5-HT_{1a} receptor KO mice to WT controls (two-way repeated measures ANOVA main effect of genotype $F_{(4.55, 195.7)}$=0.76, p=0.40). Furthermore, no gender-dependent difference could be observed (two-way repeated measures ANOVA main effect of gender $F_{(4.55, 195.7)}$= 0.76, p=0.74). Data was not confounded by differences in swim speed as none of the groups different from the others at any given time during training (two-way repeated measures ANOVA main effect of gender $F_{(4.55, 195.7)}$=0.37, p=0.14; main effect of genotype $F_{(4.55, 195.7)}$=0.19, p=0.46; gender by genotype interaction $F_{(4.55, 195.7)}$=0.06, p=8.2) Figure 25, B).

Memory was measured during the probe trial at training day IV and 24 h after the last training session (indicated by the arrows in Figure 25, A). The first probe trial showed that all groups tested were yet unable to associate the platform location with spatial cues, as none spent significantly more than 25 % of the time in the target quadrant. Furthermore, no difference in the preference for either of the quadrants was seen for gender or genotype (two-way repeated measures ANOVA main effect of gender $F_{(1.71, 73.39)}$=0.03, p=0.63; main effect of genotype $F_{(1.71, 73.39)}$=0.001, p=0.96; main effect of quadrant $F_{(1.71, 73.39)}$=0.67, p<0.0001; Figure 25, C). 24 h after training day VII groups were again exposed to a probe trial, this time starting from a different location of the maze than the probe trial at day IV. Again no difference for the preference of the quadrants was seen comparing gender and genotype (two-way repeated measures ANOVA main effect of gender $F_{(1.32, 57.94)}$=0.02, p=0.82; main effect of genotype $F_{(1.32, 57.94)}$=0.03, p=0.62), but all groups spent significantly more than 25 % of the test time in the target quadrant (two-tailed, one sample t-test, % of time in target quadrant, for all groups tested p<0.05).

After the last probe trial, mice were tested for reversal learning. During 4 trials per day on two consecutive days, mice were supposed to locate the platform in the opposite quadrant to the original location. No effect of either gender or genotype was observed (two-way repeated measures ANOVA main effect of gender $F_{(1, 43)}$=0.01, p=0.51; main effect of genotype $F_{(1, 43)}$=0.008, p=0.56; gender by genotype interaction $F_{(1, 43)}$=0.008, p=0.58). Furthermore, as can be seen in Figure 25 E and F, neither WT nor 5- HT_{1a} receptor KO mice learned the new location of the platform. Considering, that the learning process before has taken more than four days, it might be that this batch of mice is rather slow in learning and would need one or two days longer to associate the platform with the new location.

Taken together, no difference for learning and memory was seen for either male or female 5-HT_{1a} receptor KO mice when compared with WT controls.

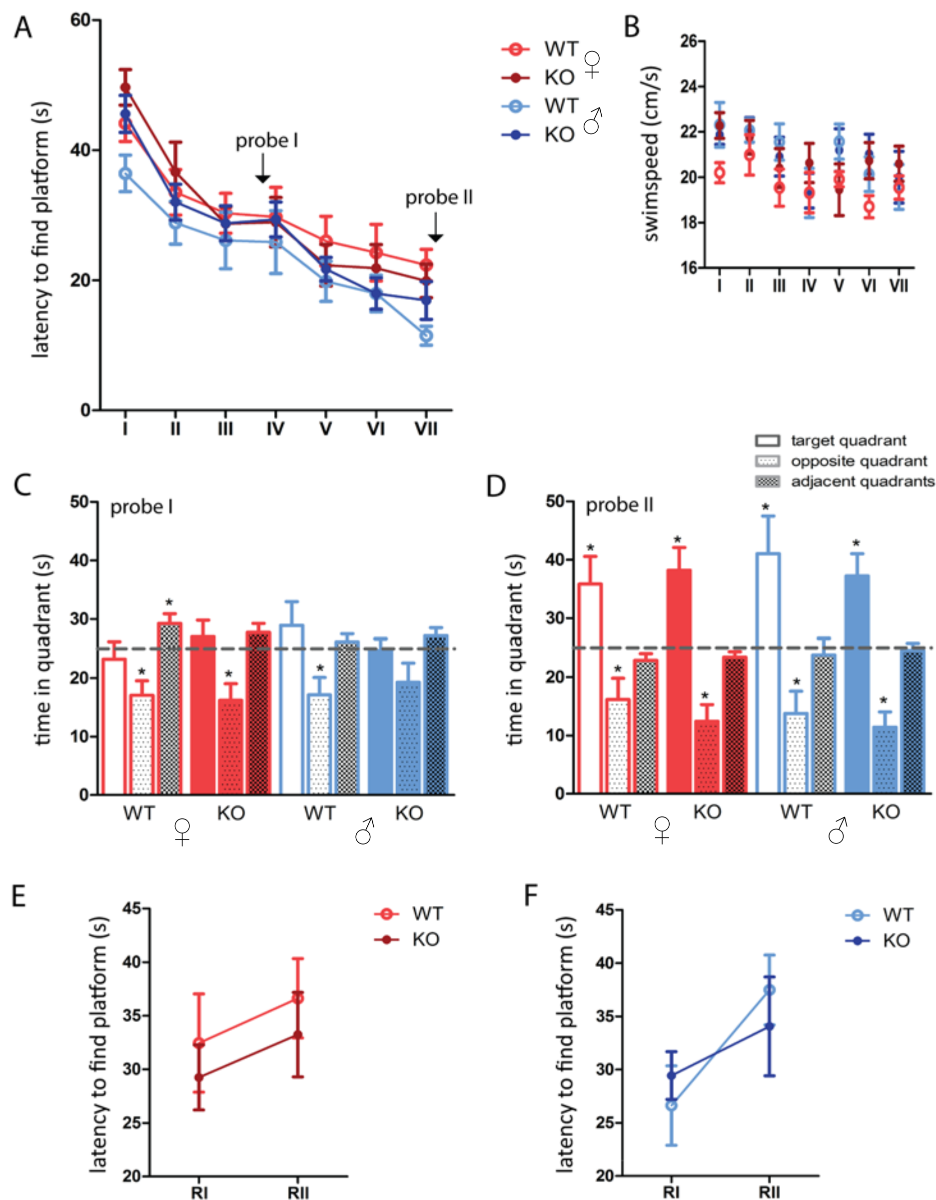

Figure 25 Cognitive abilities tested in the Morris water maze.
Learning curve of male and female 5-HT_{1a} receptor KO and WT mice shows no differences in learning abilities (A). Average swim speed of each group/day of training reveals no significant effects of activity on latency to find the hidden platform (B). Probe tests to evaluate spatial memory were conducted at training day IV (C) and 24 h after the last training day (D). Animals were unable to associate the hidden platform with a specific quadrant after three days of training, but spent significantly more than 25 % of time in the target quadrant when tested 24 h after the last training session. Asterisks in graph C and D indicate significant difference from chance level (grey dotted line). E and F show the latency to find the platform after it is placed to the opposite quadrant during two consecutive training days (RI & RII).

Barnes maze

A different batch of animals was tested in the Barnes maze to investigate learning and memory under less stressful conditions than in the Morris water maze. Two of the male WT animals fell off the maze during the training phase and were hence excluded from the test. In total, 8 female and 5 male WT, as well as 8 female and 7 male 5-HT_{1a} receptor KO mice were tested in the Barnes maze. Analysis of the training phase (day I-IV) revealed significant differences between male and female mice, but not between genotype, when comparing latencies to reach the target hole (two-way repeated measures ANOVA main effect of gender $F_{(2.07, 51.81)}=0.41$, $p=0.024$; main effect of genotype $F_{(2.07, 51.81)}=0.12$, $p=0.19$; gender by genotype interaction $F_{(2.07, 51.81)}=0.17$, $p=0.46$; main effect of time ($F_{(2.07, 51.81)}=0.96$, $p<0.0001$). Bonferroni *post hoc* tests showed significant differences for WT and 5-HT_{1a} receptor KO mice for the latency to reach the target hole (Bonferroni *post hoc* tests comparing day I and IV, WT male $p<0.001$, KO male $p<0.05$), whereas neither WT nor 5-HT_{1a} receptor KO female mice showed significantly decreased latencies to reach the target hole when comparing training day I and IV (Figure 26, A). In comparison, the amount of error visits to other holes than the target hole was significantly decreased for all groups comparing day I and IV (two-way repeated measures ANOVA main effect of time $F_{(2.65,66.28)}=0.41$, $p=0.024$, Bonferroni *post hoc* test difference between day I and IV, $p<0.001$ for all groups tested), indicating that female and male 5-HT_{1a} receptor KO and WT mice were able to decrease mistakes made, and that, despite increased latencies female mice learned to find the target hole (Figure 26, C). Although distance moved until reaching the target hole (Figure 26, B) did not differ between groups (two-way repeated measures ANOVA main effect of gender $F_{(2.45, 61.15)}=0.32$, $p=0.13$; main effect of genotype $F_{(2.45, 61.15)}=0.08$, $p=0.49$; gender by genotype interaction $F_{(2.45, 61.15)}=0.17$, $p=0.31$), a significant decrease of velocity was seen in 5-HT_{1a} receptor KO females in comparison to the other groups (two-way repeated measures ANOVA main effect of gender $F_{(2.44, 61.11)}=0.07$, $p=0.74$; main effect of genotype $F_{(2.44, 61.11)}=0.37$, $p=0.017$; gender by genotype interaction $F_{(2.44, 61.11)}=0.33$, $p=0.021$). Bonferroni *post hoc* tests revealed significant differences between WT and 5-HT_{1a} receptor KO female at testing day III ($p<0.0001$) and IV ($p<0.001$) and between WT male and 5-HT_{1a} receptor KO female at day III ($p<0.05$), implying that velocity might be a confounding factor for the time needed to reach the target hole (Figure 26, D). Results for female 5-HT_{1a} receptor KO mice have therefore been interpreted with care.

At day V, mice were exposed to the same maze but the escape box usually attached to the maze was removed, to investigate whether mice would still show a preference for the hole that was associated with escape. No significant difference was seen between groups for the latency to reach the former target hole (two-way ANOVA main effect of gender $F_{(1,24)}=1.22$, $p=0.028$; main effect of genotype

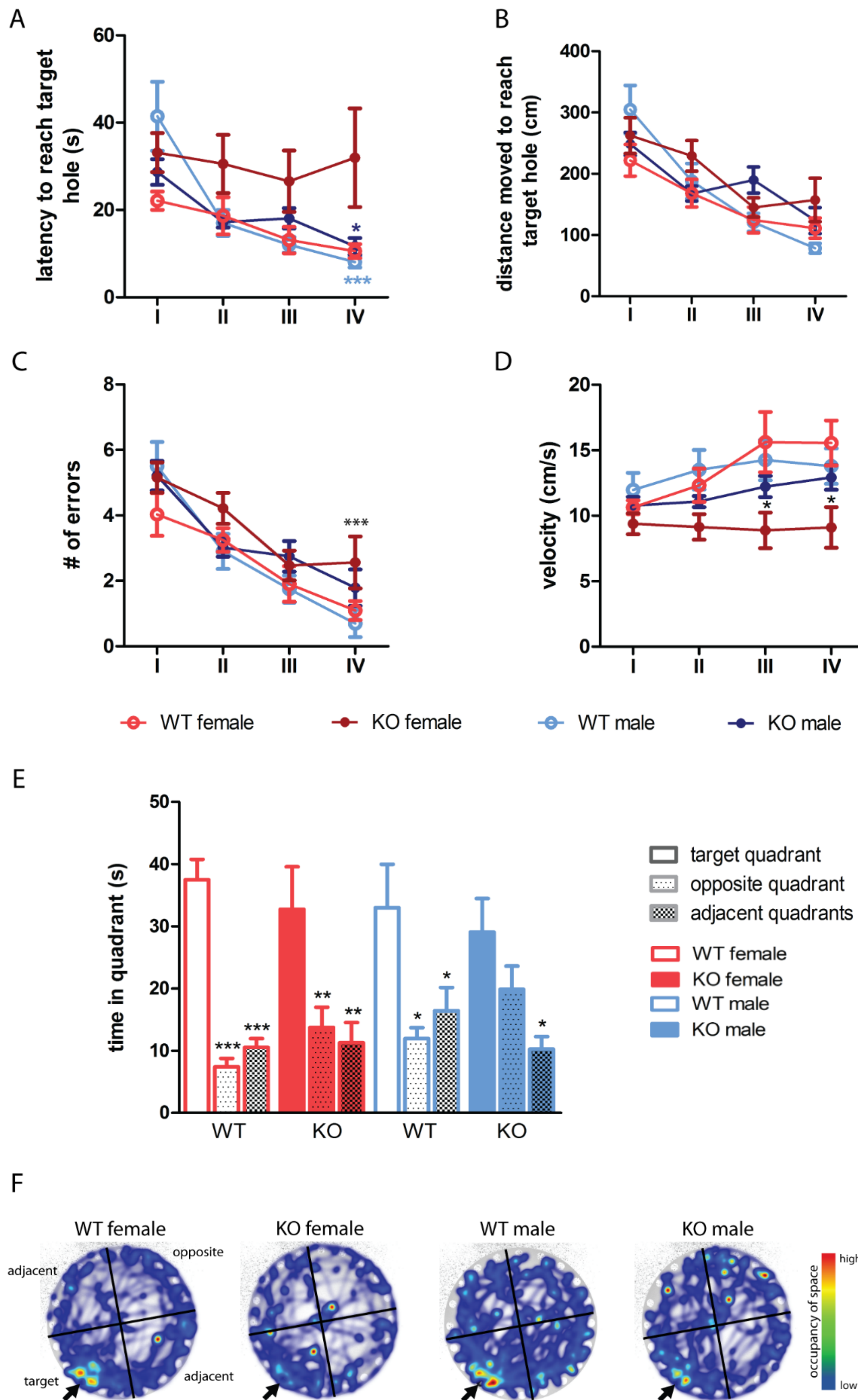

Figure 26 Spatial learning and memory abilities tested in the Barnes maze.
Learning curve showing the decreased amount of time needed to reach the target hole (A) distance moved to reach the target hole (B), errors (number of nose pokes to other holes than the target hole) (C) and velocity (D). The time spent in each of the quadrants is depicted in (E). The heatmaps in (F) show the average time spent in each location per group, red indicates high amounts of time, blue, little amount of time. Arrows indicate the location of the target hole.

$F_{(1,24)}$=3.43, p=0.077; gender by genotype interaction $F_{(1,24)}$=1.96, p=0.17) nor did the latency differ from the average latency of the last training day (data not shown). The time spent in the target quadrant did also not differ between groups (two-way repeated measures ANOVA main effect of gender $F_{(1.4, 35.09)}$=0.17, p=0.21; main effect of genotype $F_{(1.4, 35.09)}$=0,22, p=0.26; gender by genotype interaction $F_{(1.4, 35.09)}$=1.96, p=0.17), but most of the groups showed a significant increase of time spent in the target quadrant when compared to the time spent in the opposite or adjacent quadrant (two-way repeated measures ANOVA main effect of quadrant $F_{(1.4, 35.09)}$=1.38, p<0.0001, Bonferroni *post hoc* tests target vs opposite, WT male, WT female, 5-HT_{1a} receptor KO female all p<0.01; target vs adjacent WT and 5-HT_{1a} receptor KO male p<0.05, WT female p<0.001 and 5-HT_{1a} receptor KO female p<0.01 FIG X E). Male 5-HT_{1a} receptor KO mice are the only group that did not show a significant difference between the target and opposite quadrant, indicating that they did not perfectly associate the target hole with the target quadrant, but alternate between the two quadrants. This was confirmed when comparing the heatmaps displaying the position of the subject in the maze by a color code (Figure 26). This representation emphasizes how male 5-HT_{1a} receptor KO mice spent more time (indicated by the red color) at the hole opposite to the target hole.

FEAR CONDITIONING

BEHAVIORAL RESPONSE TO ELECTRIC SHOCK

Differences in foot shock sensitivity might affect fear memory evoked by this experience. To assess foot shock sensitivity, the shock response during the training session for cue discrimination was categorized into flinching, movement and jumping. Scores were given for each reaction due to its severity from 1 to 3, respectively. The average of all three shock responses per mouse was used to compare the groups. Two-way ANOVA revealed a significant difference for genotype but not gender (ANOVA main effect of gender $F_{(1, 73)}$=0.76 p=0.38; main effect of genotype $F_{(1, 73)}$= 4.08 p=0.047) and Bonferroni *post hoc* tests showed a significant difference between female WT and 5-HT_{1a} receptor KO mice (p=0.012), but not between male WT and 5-HT_{1a} receptor KO mice (p=0.64) (Figure 27). However, considering that shock sensitivity was evaluated on a scale of three degrees, the differences in sensitivity in female mice appears very small and is unlikely to have affected fear memory as female 5-HT_{1a} receptor KO mice barely differ from WT females, see below. These results exclude that fear conditioning was influenced by major difference in shock sensitivity.

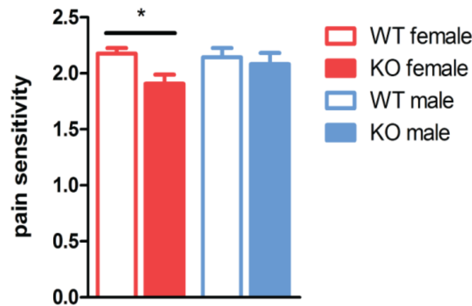

Figure 27 Sensitivity to electric shock delivered during the training session.
Reaction to shock was categorized into flinching, moving and jumping (scored 1 to 3) and the average of the three shocks compared between groups. Female 5-HT_{1a} receptor KO mice showed decreased pain sensitivity in comparison to WT female. The difference itself however, appears rather small considering that the shock sensitivity is evaluated on a scale of three degrees.

CONTEXTUAL FEAR CONDITIONING TO AMBIGUOUS CUES

5-HT_{1a} receptor KO mice have previously been exposed to contextual fear conditioning to analyze fear-memory processing. Male 5-HT_{1a} receptor KO mice showed increased freezing behavior in contextual fear conditioning when re-exposed to the training environment as well as to an ambiguous environment only partly resembling the training context (Klemenhagen *et al*, 2006). To determine whether gender has an impact on fear-related memory, we tested our mice in a similar protocol as described by Klemenhagen *et al* (2006).

Exposure of mice to the fear conditioning boxes one day before training was not able to evoke high levels of % freezing (female WT 2.5 % ± 0.05, female 5-HT_{1a} receptor KO 1.3 % ±0.07, male WT 2.8 % ± 0.08, male 5-HT_{1a} receptor KO 2.9 % ± 0.10), confirming the absence of fear inducing cues in the training environment. The training session consisted of a 3 min habituation phase, followed by three electric shock with 1 min intervals. In total the training session lasted 8 min. 24 h after training, mice were placed 8 min in the fear conditioning boxes, which were altered from the training context. A white plastic sheet was covering the metal bars that had delivered the shock the day before and two of the checkered wall papers were replaced by white wall papers (Figure 28). Male and female WT and 5-HT_{1a} receptor KO mice responded similar to the ambiguous and training environment after training, no overall effect of genotype or gender was observed (two-way repeated measures ANOVA main effect of gender $F_{(1.14, 53.42)}=0.55$ $p=0.14$; main effect of genotype $F_{(1.14, 53.42)}= 0.04$ $p=0.60$). Within group comparison, however, revealed significant differences between trials (two-way repeated measures ANOVA main effect of trials $F_{(1.14, 53.42)}=3.98$ $p<0.0001$). *Post hoc* Bonferroni revealed a significant increase of % freezing in female WT mice ($p<0.05$) in comparison to baseline conditions during habituation, but no increased fear-related behavior in any of the other groups tested (Figure 28). This suggests that female WT mice are more sensitive to ambiguous cues than female 5-HT_{1a} receptor KO or male mice. When re-

exposed to the training context 48 h after the training session all groups tested showed significantly increased fear-expression compared to habituation (Bonferroni *post hoc* test comparing % freezing during habituation and 48h after training, $p<0.001$ for all groups). Two-way ANOVA comparing all groups for fear-expression in either ambiguous environment (expression I, EI) or in the training

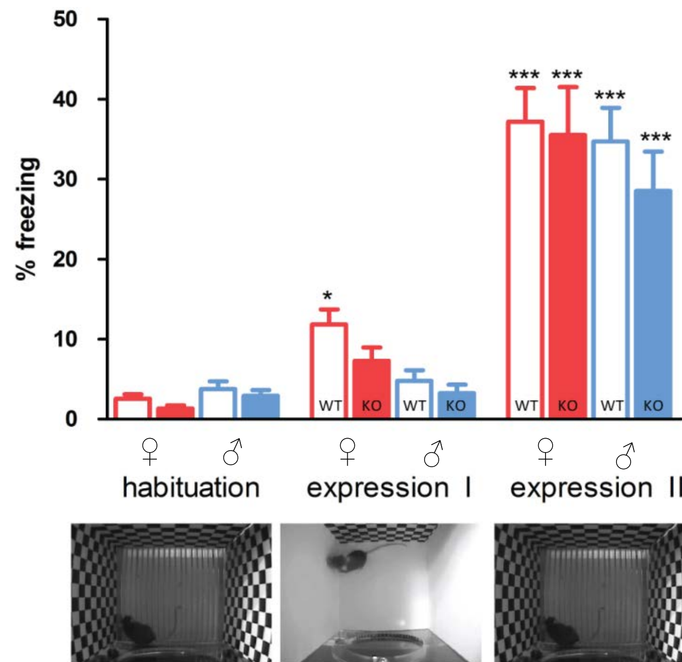

Figure 28 Fear expression evoked by ambiguous and training environment.
% of freezing in the ambiguous cue environment was significantly increased only in female WT mice when compared to % freezing during habituation 24h. % freezing behavior of all mice tested was significantly increased in comparison to baseline levels 48 h after training, when re-exposed to the training environment. The pictures below the graph show the training and the ambiguous cue environment.* significantly different from % freezing during habituation

environment (expression II, EII) revealed a gender difference for EI (main effect of gender trials $F_{(1,47)}=12.47$ $p<0.001$; Bonferroni *post hoc* $p<0.01$ for male vs female WT) and no difference between groups for EII (two-way ANOVA main effect of gender $F_{(1,47)}=0.64$ $p=0.43$, main effect of genotype $F_{(1,47)}=10.92$ $p=0.34$). Although not significantly different, it should be noticed that male 5-HT_{1a} receptor KO mice show tendency for lower amount of freezing than all the other groups tested.

In total, females are more prone to respond to the ambiguous environment than are males. However, the amount of % freezing evoked by the ambiguous environment is rather low, which might be due to the fact that the environment has been altered significantly and only one wall paper can remind the animal of the former training environment. This might also be the reason why 5-HT_{1a} receptor KO mice do not show the expected increase in % freezing to ambiguous cues as described by Klemenhagen *et al* (2006). As all groups show increased % freezing when re-exposed to the training environment, we can

conclude that the procedure itself was applied correctly and an association between the training environment and the foot shock has been formed.

Cue discrimination in fear conditioning

Fear conditioning was used to identify differences in fear memory and the ability to distinguish between partial and complete predictive cues. One day before training, mice were exposed to the fear conditioning boxes and the amount of % freezing was scored to ensure that the boxes themselves did not induce freezing behavior. Figure 29 A shows that none of the groups exhibited more than 1.5 % of freezing behavior, confirming that the context, itself did not induce abnormal levels of freezing behavior. During training, mice were confronted with five tone exposures that were only three times followed by light-shock pairing. This means that the tone only partially predicted the shock, whereas the light was always paired with the shock and, hence, was a complete cue.

24 h after training, mice were re-exposed to the training context (Figure 29, C). The overall amount of % freezing for the 15 min of exposure trial showed that male 5-HT_{1a} receptor KO mice exhibit significantly less amount of % freezing than all the other groups (two-way ANOVA main effect of gender $F_{(1,72)}$=3.73 p=0.0.057; main effect of genotype $F_{(1,72)}$= 25.53 p<0.0001; gender genotype interaction $F_{(1,72)}$= 4.12 p=0.0462, Figure 29, B). The exposure trial was divided into three different parts: 3 min of habituation (hab), tone and light. The first 3 min of re-exposure were used to measure baseline % freezing before cue presentation to confirm the absence of context-dependent conditioning to the fear conditioning box (Figure 29, D). Animals exhibited low levels of freezing during this period, but increased levels in comparison to the habituation trial indicating that at least partially re-exposure to the fear conditioning box evoked context dependent memories. Again, male 5-HT_{1a} receptor KO mice show lowest levels to the context dependent freezing, similar to the trends seen in the fear conditioning paradigm described above. Furthermore, females showed higher levels of % freezing than males in all three parts of the trial. However, no significant differences between groups were observed (two-way repeated measures ANOVA main effect of gender $F_{(1.72,\ 123.66)}$=0.09 p=0.12; main effect of genotype $F_{(1.72,\ 123.66)}$= 0.08 p=0.053). Next, we compared % freezing during the three parts of the test within groups, to examine the outcome of the exposure to partial and complete cue. A significant effect could be observed (two-way repeated measures ANOVA main effect of cue exposure $F_{(1.72,\ 123.66)}$=4.08, p<0.0001). Bonferroni *post hoc* tests revealed a significant difference between the amount of % freezing during the habituation phase and the tone as well as light exposure (p<0.01 for all groups comparing % freezing during habituation and tone exposure; p<0.001 for all groups comparing % freezing during habituation with freezing during light exposure). Bonferroni *post hoc* tests also revealed that male WT mice are the only mice that are able to distinguish between the partial (Figure 29, D tone) and the complete (Figure 29, D light) cue as shown by the significant difference between the amount of %

freezing during tone and light exposure ($p<0.001$) (Figure 29, D). Taken together male 5-HT_{1a} receptor KO mice respond different than the other mice tested to re-exposure of the context, partial and complete stimulus exhibiting lower % freezing levels, indicating that they have altered fear memory as indicated by weaker association of cue and the aversive stimulus.

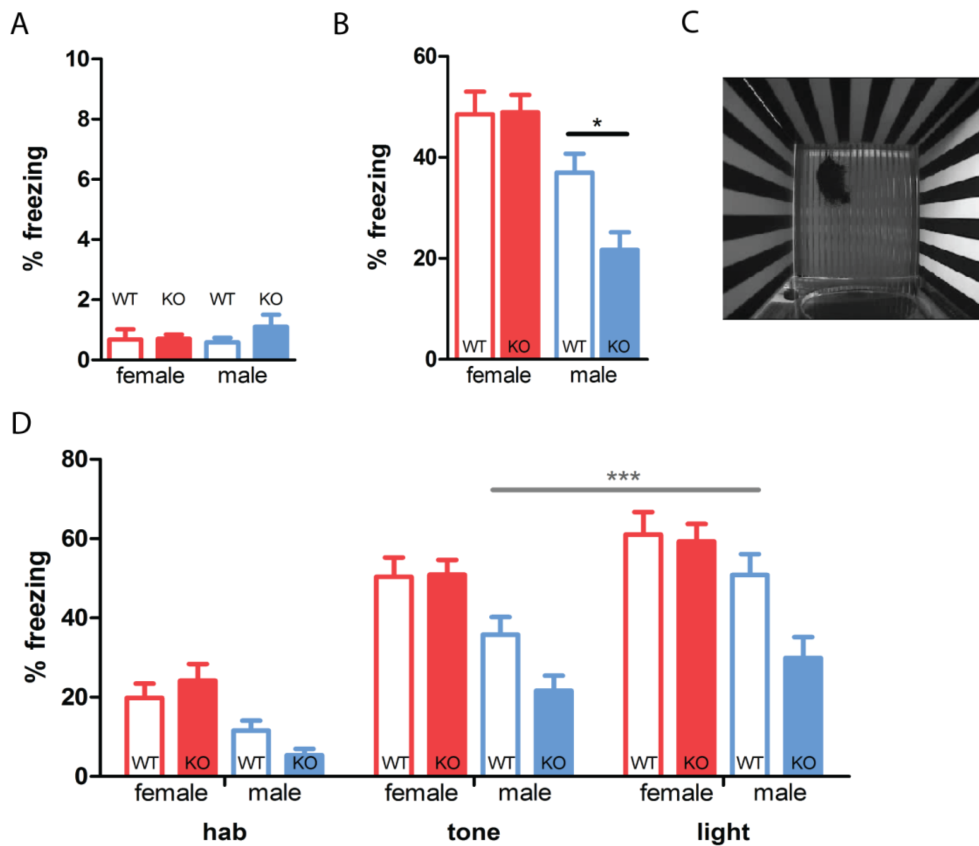

Figure 29 Cue discrimination is affected in 5-HT_{1a} receptor KO male.
No freezing behavior was evoked by the context of the fear conditioning boxes during habituation, % freezing < 2 % (A). Setup of the fear conditioning box is depicted in C. Total amount of % freezing was increased during the 15 min of expression trial, with male 5-HT_{1a} receptor KO mice showing lower levels of % freezing that all other groups (B). % freezing, however, was different during the different phases of the exposure trial (D).Low levels of freezing behavior were seen in the first 3 min of fear expression (D, hab). Freezing expression was significantly increased when mice were exposed to the partial cue (tone) and stayed elevated when switching from partial to complete cue (light) exposure. Only male WT mice were able to distinguish between partial and complete cue as they exhibit a significant increase in % freezing when exposed to light as compared to tone exposure.

Social dominance

Anxiety disorders and depression in humans have been shown to be influenced by the perception of social status of the patient (Weisman *et al*, 2011; Zink *et al*, 2008). To investigate to which extent the anxious phenotype of the 5-HT_{1a} receptor KO male mice is linked to social dominance, we exposed them to different WT opponents in the tube test. The tube test is a non-violent behavioral test in which two opponents start at one end of a Plexiglas tube, meet in the middle and are required to push the opponent backwards in order to exit the tube at the other (Wang *et al*, 2011). The animal pushed out of the tube in this case is considered subordinate. Testing included 36 pairs of male 5-HT_{1a} receptor KO and WT mice, whereby each mouse was exposed to 3 different opponents. Results indicated no differences in social dominance between the two genotypes (two-tailed t-test p=0.74) (Figure 30).

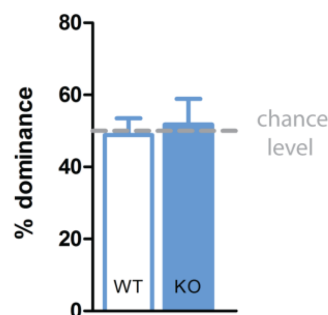

Figure 30 Social dominance of WT and 5-HT_{1a} receptor KO male mice.
No difference was observed comparing dominance-related behavior in WT and 5-HT_{1a} receptor KO male mice. % dominance for both groups is around chance level of 50 %.

Depressive-like behavior

The 5-HT_{1a} receptor has been shown to be strongly involved in depression in humans (Strobel *et al*, 2003).(Lemonde *et al*, 2004a) Constitutive 5-HT_{1a} receptor KO mice, however, show antidepressant-like behavior in the forced swim test and the tail suspension test (Heisler *et al*, 1998; Parks *et al*, 1998; Ramboz *et al*, 1998). We decided to investigate aspects of depressive-like behavior that have not been previously tested in 5-HT_{1a} receptor KO mice.

First, we monitored food intake and sucrose preference using the Phenotypers (Noldus, The Netherlands). A lack of sucrose preference in rodents is commonly used as a measure of anhedonia – the loss of interest or pleasure in otherwise rewarding activities. Second, we estimated mice's self-care and motivation by conducting the splash test, which measures the propensity to self-groom after being squirted with 10 % sucrose solution (Ducottet *et al*, 2004). Finally, we assessed olfaction in the buried

food test and bedding preference, as olfactory function is strongly decreased in patients with major depression (Negoias *et al*, 2015).

Sucrose preference

To identify differences for measures of anhedonia, a sucrose preference test was run during the time the animals spent in the Phenotypers. Two bottles - one filled with water and one containing 2 % sucrose solution - were available to the animals for the entire time of testing. The amount of liquid intake was determined by weighing the bottles at the end of testing and the amount of licks for either of the bottles was compared between gender and genotype. No difference for sucrose preference (two-way ANOVA main effect of gender $F_{(1,37)}$=0.11 p=0.11; main effect of genotype $F_{(1,37)}$= 0.00, p=0.95) was observed

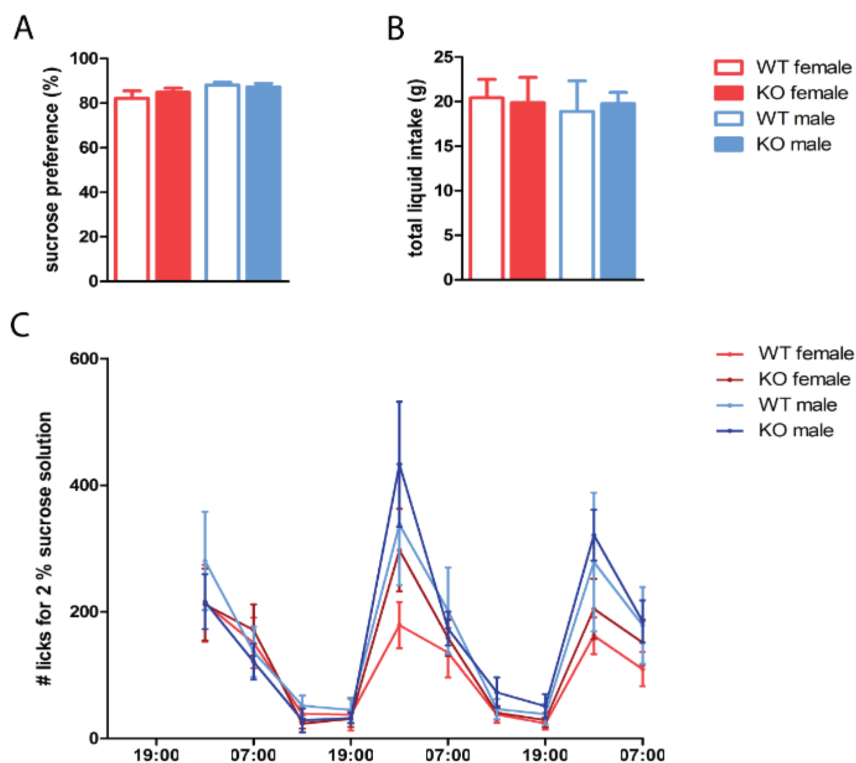

Figure 31 Sucrose preference.
No difference was observed for % sucrose preference between male and female WT and 5-HT_{1a} receptor mice (A). Data was not confounded by difference in total amount of liquid intake, as similar amounts of liquid were drank over the total test period (B). C displays the amount of licks in 12 h time bins.

for either of the groups tested (Figure 31, A). Total liquid intake (Figure 31, B) did not differ either (two-way ANOVA main effect of gender $F_{(1,37)}$=3.70 p=0.06; main effect of genotype $F_{(1,37)}$= 0.19 p=0.67). In addition, no difference could be seen comparing total licks for sucrose solution over time (Figure 31, C) for all groups (two-way repeated measures ANOVA main effect of gender $F_{(2.04, 59.22)}$=0.25 p=0.13; main effect of genotype $F_{(2.04, 59.22)}$=0.17 p=0.45; gender by genotype interaction $F_{(2.04, 59.22)}$=0.068 p=0.67).

SPLASH TEST

The splash test measures the amount and frequency of self-grooming over a 5 min time period after spraying the subjects with a 10 % sucrose solution. The amount and frequency give indications about the depressive-like state of the subject, as low amount of grooming is associated with depressive-like behavior. Figure 32 shows the time and the frequency of self-grooming of male and female 5-HT_{1a} receptor KO and WT mice. Two-way ANOVA for the duration of self-grooming and the frequency showed a significant impact of gender on both parameters, but Bonferroni *post hoc* tests did not highlight differences between groups (two-way ANOVA for time spent grooming: main effect of gender $F_{(1,30)}=7.80$ $p=0.001$; main effect of genotype $F_{(1,30)}=1.13$ $p=0.30$; gender by genotype interaction $F_{(1,30)}=0.00$ $p=0.98$; two-way ANOVA for frequency: main effect of gender $F_{(1,30)}=6.74$ $p=0.015$; main effect of genotype $F_{(1,30)}=1.53$ $p=0.23$; gender by genotype interaction $F_{(1,30)}=0.00$ $p=0.99$).

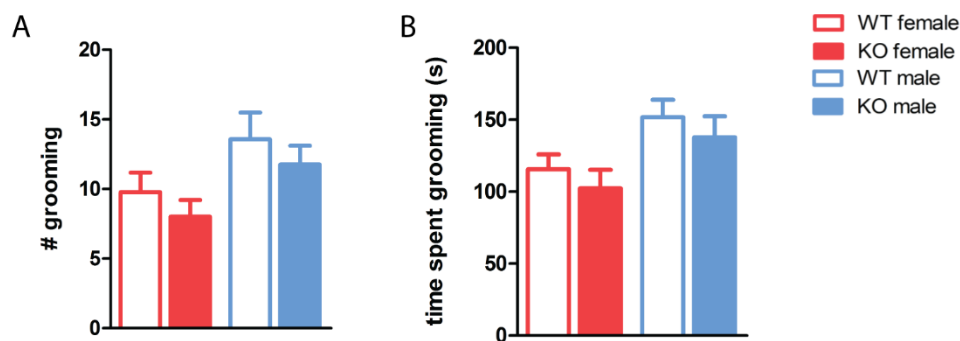

Figure 32 Splash test.
No significant difference could be observed comparing male and female 5-HT_{1a} receptor KO and WT mice for the amount of grooming periods (A) and the time spent grooming (B) after being sprayed with 10 % sucrose solution.

OLFACTION

BURIED FOOD TEST

For this test, an Oreo cookie was covered with clean bedding, the latency to find the cookie within a 10 min period was measured and compared between groups. This procedure was repeated at three consecutive days, to examine possible improvements in the latency. Although previous data suggested an impairment of male 5-HT_{1a} receptor KO mice in this task, two-way ANOVA revealed no significant impact of gender ($F_{(1.64,50.80)}=0.27$ $p=0.07$) or genotype ($F_{(1.64,50.80)}=0.06$ $p=0.48$), nor any effect of time ($F_{(1.64,50.80)}=0.10$ $p=0.17$) (Figure 33, A), indicating no impairment of any of the groups tested for olfactory functioning in this task.

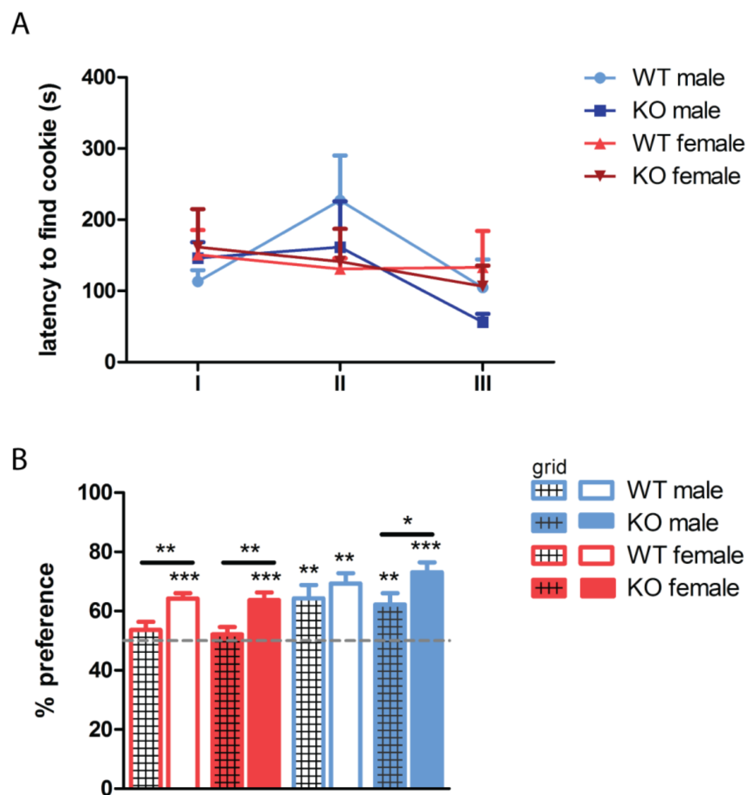

Figure 33 Olfactory functioning tested in the buried food and bedding preference test.
(A) No impact of gender or genotype was seen for the latency to find the buried cookie on either of the three test days. (B) A gender difference could be seen in the bedding preference, with female mice showing significantly lower preference for the dirty bedding than males. When the bowl containing dirty bedding was covered by a grid, female mice did not show a preference for dirty bedding at all (* indicated significant difference from 50 % chance level).

BEDDING PREFERENCE

The bedding preference was measured in two sessions using two bowls containing either clean or dirty bedding. In the first session, the bedding was covered by a grid, whereas in the second session bedding was accessible for the mice. Two-way ANOVA testing bedding preference when bedding was covered with a grid revealed an overall effect of gender ($F_{(1,47)}=9.25$, $p=0.004$), but no effect of genotype ($F_{(1,47)}=0.28$ $p=0.60$), with female mice showing lower levels of % preferences than males (Figure 33, B). Bonferroni *post hoc* test, however, did not reveal significant difference between groups. Using a two-tailed, one sample t-test we investigated whether % preference was significantly different from 50 % chance level. Male (5-HT_{1a} receptor KO $p=0.009$, WT $p= 0.008$), but not female mice showed a significant % preference. Two-way ANOVA of testing bedding preference when mice had access to the bedding showed similar results, with an overall influence of gender ($F_{(1,47)}=6.65$ $p=0.013$) on bedding preference, but no effect of genotype ($F_{(1,47)}=0.35$ $p=0.56$). Two-tailed, one sample t-test to test for significance of % bedding preference above 50 % chance level revealed that all groups showed significant preference (male 5-HT_{1a} receptor KO $p<0.0001$, WT $p=0.0002$; female 5-HT_{1a} receptor KO

p<0.0001, WT p=0.0002). Interestingly, the % preference increased significantly for all groups but WT males, when mice were allowed to access the bedding and to search for volatile cues (two-way repeated measures ANOVA main effect of time ($F_{(1,47)}$=18.58 p<0.0001; *post hoc* t-test female 5-HT_{1a} receptor KO p=0.004, male 5-HT_{1a} receptor KO p=0.04, female WT p=0.004, male WT p>0.05) (Figure 33, B). This data indicates that there is a significant impact of accessibility to the bedding on % bedding preference, which is more pronounced in female than male mice.

III.3 Downstream targets of 5-HT_{1A} receptor

Previous electrophysiological data of our lab comparing excitatory postsynaptic field potentials (fEPSPs) in the CA1 region of male 5-HT_{1a} receptor KO and WT mice revealed significantly increased amounts of NR2B-containing NMDA receptors at the CA3-CA1 synapse of 5-HT_{1a} receptor KO males at P25. During early postnatal development, the subunit composition of NMDA receptors typically changes from predominantly NR2B containing to NR2A containing NMDA receptors, and notably increased NR2A/NR2B ratio is also considered as a marker for the maturation of the synapse (Paoletti *et al*, 2013). Elevated levels of NR2B-containing NMDA receptors as seen in male 5-HT_{1a} receptor KO mice are hence likely to have pronounced effects on neuronal development, synaptic plasticity and sensitivity to excitotoxicity. To investigate the cause of increased NR2B expression at the synapse of 5-HT_{1a} receptor KO mice, we analyzed potential differences of total hippocampal NR2A and NR2B expression during early postnatal development. Furthermore, synaptoneurosomal fractions of total hippocampal lysate and mRNA expression of NR2B were used to specify differences in localization.

Subunit Switch of NMDA receptors

To investigate possible differences between male 5-HT_{1a} receptor KO and WT mice for the NMDA receptor subunit switch during the critical developmental period, we studied the expression of NR2A and NR2B subunits in hippocampal lysate of mice aged 10 (WT n=5, KO n=5), 15 (WT n=8, KO n=5), 20 (WT n=3, KO n=5), 30 (WT n=6, KO n=5) and 60 days (WT n=5, KO n=4). Total hippocampal lysate was used, as dissection of CA1, CA3 and DG as described by Lein *et al* (2004) was not feasible in P10 mice. Figure 34 shows the expression of NR2A and NR2B subunit relative to P10 WT after normalization to total protein expression measured by Ponceau staining. No significant influence of genotype could be seen for the expression of either subunit over time (NR2A, two-way ANOVA main effect of genotype $F_{(4,41)}$=0.03 p=0.85; main effect of time $F_{(4,41)}$=38.52 p<0.0001; time by genotype interaction $F_{(4,41)}$=

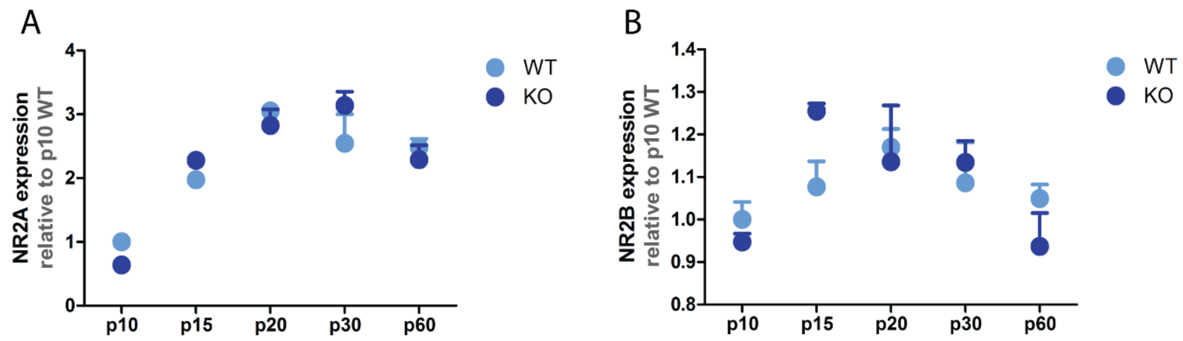

Figure 34 Timeline of NR2A and NR2B subunit expression.
Expression of NR2A (A) and NR2B (B) subunit in hippocampal lysate relative to P10 WT. Increased expression of NR2B subunit in 5-HT_{1a} receptor KO mice can be seen at P15.

2.41 p=0.09; NR2B, main effect of genotype $F_{(4,41)}=0.01$ p=0.92; main effect of time $F_{(4,41)}=2.64$ p=0.048; time by genotype interaction $F_{(4,41)}=1.02$ p=0.41). NR2B levels at P15, however, seemed to be upregulated in 5-HT_{1a} receptor KO mice (t-test at P15 $p<0.05$). Measuring mRNA levels of NR2B subunit via qRT-PCR at P15 confirmed the differences observed in western blot data. 5-HT_{1a} receptor KO mice (n=5) had significantly increased NR2B mRNA levels at P15, when compared to WT (n=8) mice (Figure 35).

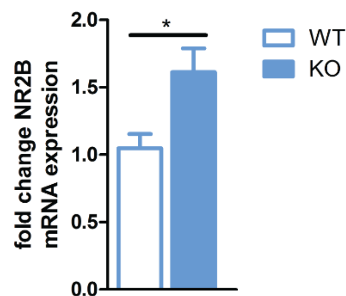

Figure 35 mRNA levels of NR2B of at P15.
At P15, 5-HT_{1a} receptor KO male mice express significantly increased amounts of NR2B mRNA in comparison to WT mice.

EXPRESSION OF NR2B AT THE SYNAPSE

NR2B subunit expression, as measured by western blot analysis of total hippocampal lysate, revealed a difference in NR2B concentrations at P15, but not at later stages. In contrast, electrophysiological studies in our lab showed increased contribution of NR2B-containing NMDA receptors to fEPSPs measured in the CA1 region after Schaffer collateral stimulation in 5-HT_{1a} receptor KO mice at later stages. We therefore wondered whether we could pick up this difference at the protein level by

analyzing protein content of synaptoneruosomes. The synaptoneurosomal fraction of total hippocampal lysate of 5-HT_{1a} receptor KO and WT mice was compared at P30 and P60 (Figure 36). 5-HT_{1a} receptor KO and WT mice expressed similar levels of NR2B subunit at the synapses when comparing synaptoneurosomal fractions of total hippocampal lysate at both time points. The discrepancy between electrophysiological and biochemical data are likely to be due to the lack of region-specificity of total hippocampal lysate used for the preparation of synaptoneurosomes, which, unlike the fEPSPs recordings, is not restricted to CA3-CA1 synapses.

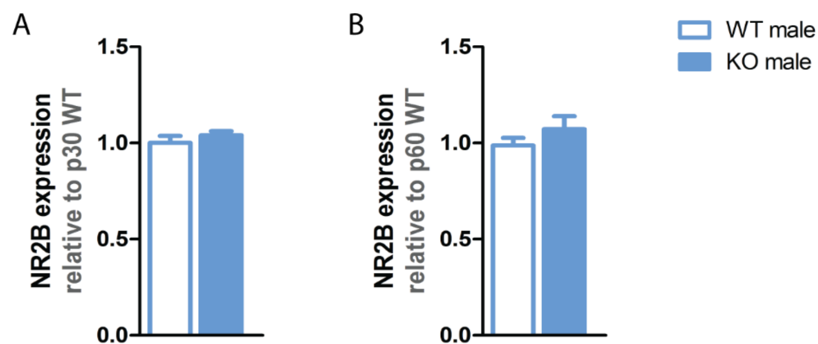

Figure 36 Synapotoneurosomal fraction of hippocampal lysate of 5-HT_{1a} receptor KO and WT mice.
No difference was seen between the amount of NR2B expressed at the synapse of 5-HT_{1a} receptor KO and WT mice at P30 (A) or P60 (B).

REGION SPECIFICITY FOR NR2B EXPRESSION

As biochemical analysis from total hippocampal lysate precludes detection of regional differences, we sought to examine topological expression of NR2B-containing NMDA receptors by DIG *in situ* hybridization of NR2B mRNA, which was performed on hippocampal slices of 15 day-old WT mice.

The primer pair used to produce DIG-labeled RNA probes for NR2B mRNA was the same as the one used to quantify NR2B mRNA expression in qRT-PCR. Figure 37 shows labeling of NR2B mRNA in the hippocampus of P15 WT mice. We can see higher levels of NR2B subunit mRNA in the CA1 region as compared to the CA3 and DG, which is in line with data obtained in earlier studies (Monyer *et al*, 1994). The primers used are therefore sufficient to generate probes that can detect NR2B mRNA in the brain and can be used for follow-up studies to compare NR2B mRNA expression of 5-HT_{1a} receptor KO and WT mice at different time points.

Figure 38 depicts labeling of 5-HT_{1a} receptor mRNA in 15 days old WT mice. A difference between the levels of expression can be noticed in the subregions of the hippocampus. The CA1 region shows higher expression of 5-HT_{1a} receptor mRNA than the DG. Differences between CA3 and CA1 as described by

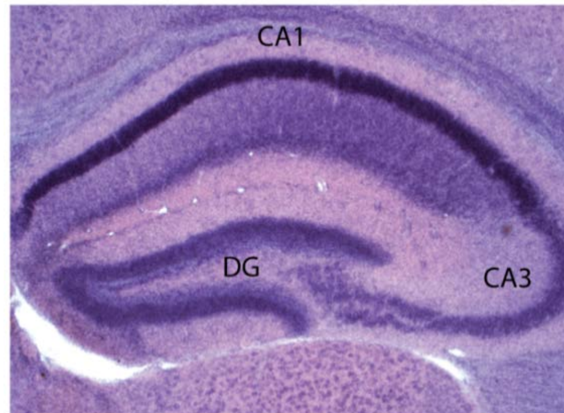

Figure 37 *In situ* hybridization of NR2B mRNA in the hippocampus of P15 WT mice.
Increased detection of NR2B mRNA can be seen in the CA1 region in comparison to the CA3 and DG region of the hippocampus of a 15 day-old WT male mouse.

Tanaka *et al* (2012) are not as obvious, which in this case might be due to long incubation times. A stronger difference between mRNA expression in the CA1 and CA3 region was observed after 3 hours of incubation. To better demonstrate the region specific differences in 5-HT$_{1a}$ receptor mRNA expression, this experiment will need to be repeated and incubation needs to be stopped already after 3 hours. Intriguingly, expression levels of NR2B and 5-HT$_{1a}$ receptor mRNA seem to overlap, emphasizing the possibility of 5-HT$_{1a}$ receptor to impact NR2B subunit expression.

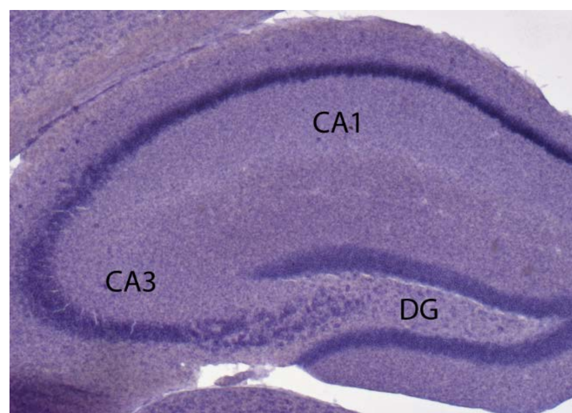

Figure 38 *In situ* hybridization of 5-HT$_{1a}$ receptor in the hippocampus of P15 WT mice.
Differences of mRNA labeling can be seen between the CA1 region and DG, with CA1 region showing stronger labeling. Expression patterns of 5-HT$_{1a}$ receptor mRNA do not differ as strongly between CA3 and CA1 region.

REST

In 2012, Rodenas-Ruano and colleagues showed that increased levels of REST mRNA are responsible for the subunit change of NMDA receptors (Rodenas-Ruano *et al*, 2012). REST mRNA in rats was shown to be upregulated between P13 and pP16, while NR2B mRNA levels were starting to decrease from P13 onwards. When blocking the increase of REST mRNA with shRNA during the critical developmental timeframe, electrophysiological data revealed increased contribution of NR2B-containing NMDA receptors to postsynaptic currents evoked in the DG. The resemblance between these findings and our observations at CA3-CA1 synapses in 5-HT_{1a} receptor KO male mice at P25 prompted us to investigate whether REST mRNA is affected by 5-HT_{1a} receptor deletion during the critical period.

qRT-PCR at 4 different time points (P10, P15, P20 and P30) revealed no significant impact of 5-HT_{1a} receptor deletion REST mRNA levels ($F_{(1,35)}$=0.84, p=0.36). Furthermore, we did not find a significant increase of REST mRNA in the time period between P10 and P30 ($F_{(3,35)}$=2.11 p=0.12), in contrast to the previous report. Interestingly, mRNA levels of NR2B seem to be decreasing in WT, but not 5-HT_{1a} receptor KO mice. However, two-way repeated measures ANOVA did not reveal significant differences between genotypes overtime (main effect of genotype $F_{(1,34)}$=0.93, p=0.34; main effect of time $F_{(3,34)}$=1.26, p=0.30).

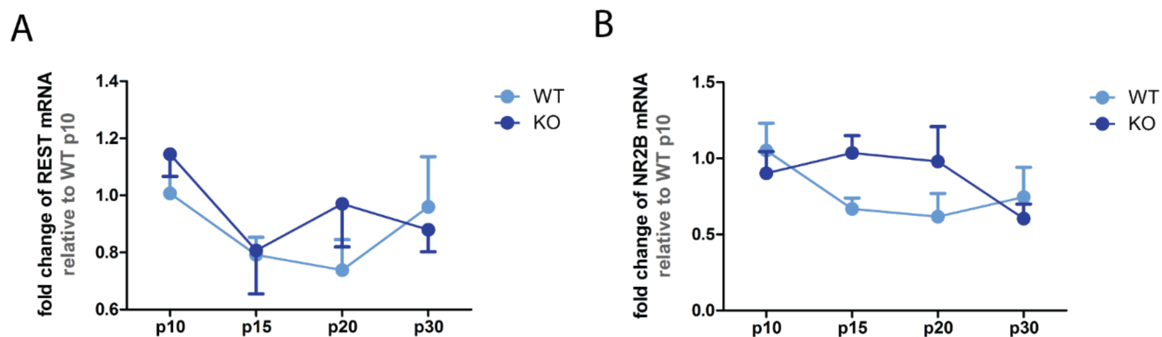

Figure 39 Fold change of REST and NR2B mRNA levels of male 5-HT_{1a} receptor KO and WT mice.
(A) No difference in mRNA expression can be seen comparing 5-HT_{1a} receptor KO and WT males during the critical developmental period. (B) Although a significant difference in mRNA levels can be seen when focusing on P15 samples, no overall impact of genotype on NR2B mRNA levels can be seen between P10 and P30.

DENDRITIC COMPLEXITY IN ORGANOTYPIC CULTURES

NR2B-containing NMDA receptors in the CA1 pyramidal neurons of the hippocampus influence the amount of arborization in the *stratum radiatum*. Increased NR2B-containing NMDA receptor expression at the synapse can result from overall increased expression of NR2B-containing NMDA receptors, or occur exclusively at synaptic sites, due to increased transportation to the synapse, decreased removal

from the synapse or a mixture of the mentioned alterations. As we could only see trends for increase expression of overall NR2B-containing NMDA receptors at P15 in western blots, but observed increased levels at the synapse at P25 in electrophysiological recordings, we further investigated the effects of transportation to and the removal from the synapse in organotypic cultures. This method allowed us to examine the effect of chronic pharmacological treatment on cell morphology. 5-HT_{1a} receptor KO cultures were used to study the effect of U0126, an Erk1/2 inhibitor which is expected to downregulate the transport of NR2B-containing NMDA receptors to the synapse (Yuen *et al*, 2005). The casein kinase 2 inhibitor TBB was applied to block the removal of NR2B containing NMDA receptors from the synapse (Sanz-Clemente *et al*, 2010). The two compounds were applied in the medium at a concentration of 10 µM and 1 µM, respectively for 15 days. In addition we tested the effect of the NR2B specific blocker ifenprodil (IFEN, 3 µM, 15 days) on dendritic branching (Figure 40).

Kruskal-Wallis test revealed significant difference between the amount of primary branches per cell depending on drug treatment ($H=25.87$, $p<0.0001$). Dunn's Multiple Comparison Test comparing drug treated groups with the control group showed a significantly decreased number of branches per cell for ifenprodil (IFEN, $p<0.001$) and U0126 ($p<0.01$) treatment . Application of TBB did not decrease the number of branches in comparison to control levels, but also did not result in significantly elevated amount of branches per cell when compared to IFEN and U0126 treated slices. Thus, the excessive dendritic arborization in 5-HT_{1a} receptor KO mice is likely to derive from upregulated insertion of NR2B-containing NMDA receptors.

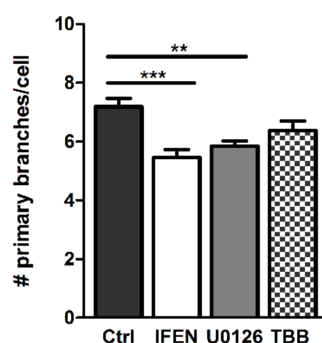

Figure 40 Amount of primary branches per cell in slices of 5-HT_{1a} receptor KO mice.
Blockage of NR2B subunit containing NMDA receptor activity (IFEN) as well as blockage of its transportation to the synapse (U0126) in 5-HT_{1a} receptor KO hippocampal slice cultures decreases the amount of primary branches.

CELL DEATH DURING EARLY POSTNATAL DEVELOPMENT

Excessive amounts of NR2B subunit expression sensitize the cell for excitotoxicity (Zhou *et al*, 2013). NR2B-containing NMDA receptors have slower deactivation kinetics and show reduced CA^{2+}-dependent desensitization in comparison to NR2A containing NMDA receptors. Excessive glutamate release and hence NMDA receptor activation is therefore more likely to result in excitotoxicity (Zhou *et al*, 2013). We observed increased amounts of NR2B during a critical developmental period, which resulted in increased amounts of branching of pyramidal neurons. 5-HT_{1a} receptor is expressed to a higher extent in CA1 than in CA3 region of the hippocampus, suggesting that the CA1 region is more affected by 5-HT_{1a} receptor deletion than CA3. We hypothesized that increased NR2B would lead to increased amount of cell death in CA1 via excitotoxicity. In turn, this could indirectly increase the amount of CA3-CA1 contacts as a result of rewiring of the circuit, as an unaltered amount of inputs coming from the CA3 region would now be distributed on less postsynaptic partners. We measured the amount of cell death by comparing the intensity of propidium iodide (PI) staining in the CA1 region of 5-HT_{1a} receptor KO slice cultures before and exposing the slices to 1x PBS overnight, which induces cell death (Figure 41). This was tested under control conditions (no drug application) and in concomitance with pharmacological treatment (3 IFEN µM, 1 µM TBB, 10 µM U0126 for 9 days). Kruskal-Wallis test revealed significant difference between % cell death depending on drug treatment ($H=13.86$, $p<0.0031$). Dunn's multiple comparison test comparing drug treated groups with control showed significantly decreased amount of % cell death for ifenprodil (IFEN, $p<0.05$) and U0126 ($p<0.05$) treatment. 5-HT treatment, as expected, had no effect on % cell death in 5-HT_{1a} receptor KO cultures. This data indicates that indeed increased NR2B-containing NMDA receptor expression at the synapse can regulate the amount of cell death during the critical developmental period in slices cultures.

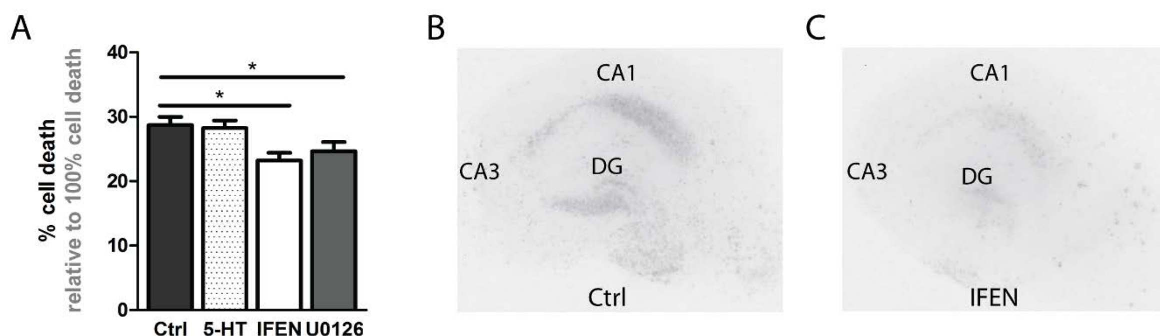

Figure 41 % of cell death in 5-HT_{1a} receptor KO organotypic cultures after nine days in culture.
Graph A shows that untreated (Ctrl) and 5-HT treated organotypic slice cultures show high levels of % cell death in the CA1 region after 9 days in culture (B). Treatment with IFEN (C) that blocks the NR2B subunit of the NMDA receptor as well as U0126 that downregulates transportation of NR2B to the synapse, results in reduced amounts of cell death in the CA1 region. B and C depict a black and white image of acute PI staining of untreated (B) and IFEN treated cultures (C). Intensity of staining is higher in untreated cultures in the CA1 and DG in comparison to treated cultures.

IMPACT OF 5-HT$_{1A}$ RECEPTOR DELETION ON ADULT NEUROGENESIS

As 5-HT$_{1a}$ receptor deletion impacts survival of hippocampal cells during the early postnatal period, we wanted to know whether receptor deletion would also affect the development of adult born neurons. Therefore, we examined the expression of specific markers used to identify the different stages in the cycle of newly generated cells in the dentate gyrus. By analyzing the amount of Ki67 positively stained of male 5-HT$_{1a}$ receptor KO (n=5) and WT (n=5) mice, we observed no difference in cell proliferation (two-tailed, one sample t-test, p=0.44). 5-HT$_{1a}$ receptor deletion, however, had a huge effect on the survival and maturation of cells. The amount positively stained cells for DCX (two-tailed, one sample t-test, p=0.0001) and BrdU (two-tailed, one sample t-test, p=0.021) was significantly decreased in 5-HT$_{1a}$ receptor KO males in comparison to WT male mice (Figure 42). This data is in line with previous publications showing that survival but not proliferation is altered in 5-HT$_{1a}$ receptor KO males (Santarelli *et al*, 2003). To investigate whether this effect is gender dependent another batch of mice was used.

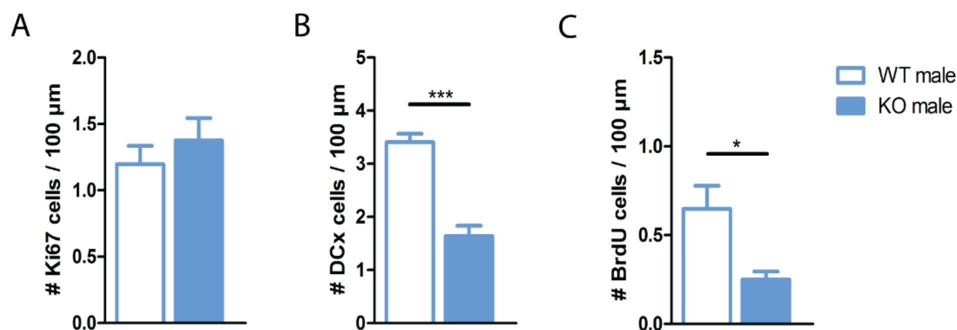

Figure 42 Effects of 5-HT$_{1a}$ receptor deletion on adult neurogenesis in male mice.
(A) No differences in cell proliferation could be seen in the hippocampus of adult male 5-HT$_{1a}$ receptor KO (n=6) and WT mice (n=6), but 5-HT$_{1a}$ receptor deletion significantly affected maturation (B) and cell survival (C). All values are presented as the amount of cells per 100 µm of DG length.

Sections were cut horizontally to ensure that subventricular zone and DG could be analyzed in the same mouse. Figure 43 shows the results of the impact of 5-HT$_{1a}$ receptor deletion in male and female mice. Although no difference was seen for the proliferation rate as measured by the amount of Ki67 positive cells (two-way ANOVA main effect of gender $F_{(1,12)}$=0.86, p=0.37; main effect of genotype $F_{(1,12)}$=0.58, p=0.46) a significant effect was seen for cell survival (two-way ANOVA main effect of gender $F_{(1,12)}$=9.05, p=0.011; main effect of genotype $F_{(1,12)}$=13.74, p=0.003; gender x genotype interaction $F_{(1,12)}$=8.70, p=0.012). Bonferroni *post hoc* test revealed significant differences between male 5-HT$_{1a}$ receptor KO (n=4) and WT (n=4) mice (p<0.01) as well as significantly lower levels of cell survival in female (n=4) mice (p<0.01). Data for the subventricular zone has not been acquired yet. This data strongly suggests a significant difference in cell survival between male 5-HT$_{1a}$ receptor KO and WT mice, which can be

observed in both horizontal and coronal sections and a clear gender-dependent effect on cell survival with female showing lower levels of BrdU positive cells than WT males.

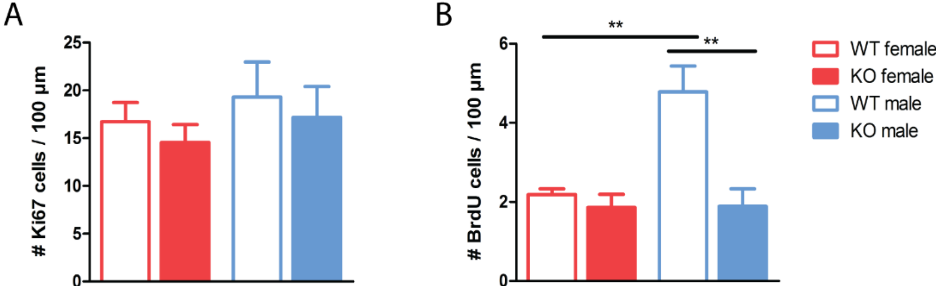

Figure 43 Gender-dependent differences for survival of adult born neurons in the DG.
Whereas there is no difference in the amount of cell proliferation between groups (A), impairments of adult born neuron survival can be seen in female mice and 5-HT_{1a} receptor KO males.

IV Discussion

The aim of this work was to study whether gender-dependent differences as seen on morphological level could also be observed on behavioral level. In our lab we showed, that constitutive 5-HT_{1a} receptor deletion leads to exuberant dendritic branching in the *stratum radiatum* of hippocampal pyramidal neurons. This difference can only be observed in adult male, but not female 5-HT_{1a} receptor KO mice when compared to WT animals in adulthood. Therefore, we compared male and female 5-HT_{1a} receptor KO mice to WT animals in tests for anxiety-related and depressive-like behavior as well as learning and memory. Furthermore, we investigated the downstream mechanisms of 5-HT_{1a} receptor that influence arborization and therefore behavior of 5-HT_{1a} receptor KO mice to identify targets that enable a gender-dependent rescue mechanism.

IV.1 GENDER-DEPENDENCY FOR THE EFFECT OF 5-HT_{1A} RECEPTOR DEPLETION

Results gathered in the open field and the light/dark box for measures of anxiety-related behavior show that male 5-HT_{1a} receptor KO mice are more severely affected by 5-HT_{1a} receptor deletion than females, when compared to WT controls. More specifically, male 5-HT_{1a} receptor KO mice spent less time in the center of the open field and increased time at the wall of the maze, while showing decreased amount of entries into the center and decreased locomotor activity. No effect on anxiety-related measures was seen for female 5-HT_{1a} receptor KO mice. In the light/dark box, both male and female 5-HT_{1a} receptor KO mice exhibit decreased visits to the lit compartment, and male but not female 5-HT_{1a} receptor KO mice showed decreased locomotor activity. This data is in line with the initial description of 5-HT_{1a} receptor KO mice in 1998 (Heisler *et al*, 1998; Parks *et al*, 1998; Ramboz *et al*, 1998), in which male mice are described as more severely affected by the knock out than females. Considering that our animals have been bred in our hands for over ten years and have been crossed with mice of different genetic background strains, this data highlights a robust gender-dependent effects of 5-HT_{1a} receptor deletion. Moreover, these results suggest that gender-dependent differences seen in other behavioral tasks of this study can be generalized and are comparable to other results gathered on 5-HT_{1a} receptor KO mice.

Gender-dependent differences could also be seen in the probe trial of the Barnes maze, indicating that learning and memory abilities of male 5-HT_{1a} receptor KO mice are at least partially impaired. In the probe trial of the Barnes maze, male 5-HT_{1a} receptor KO mice did not spent significantly more time in the target quadrant than in the quadrant opposite to the target hole. This indicates that male 5-HT_{1a} receptor KO mice associated the target quadrant less strongly with the escape box than did all the other mice tests. Deficits of 5-HT_{1a} receptor KO males are in line with previous studies showing impaired

abilities to associate the target quadrant with the platform location in the water maze (Sarnyai *et al*, 2000). Nonetheless, no differences in the total time needed to reach the target hole during the probe trial was seen, suggesting that spatial memory itself was not impaired. The latter is supported by the lack of differences between groups during the training phase in the Barnes and the Morris water maze. This data indicates that the animal's orientation in space was intact, but suggests that either the ability to strongly associate a certain region of the maze with escape was impaired or that the behavior induced by the lack of the escape box is in general different between 5-HT_{1a} receptor KO and WT mice.

In contrast to 5-HT_{1a} receptor KO males, female 5-HT_{1a} receptor KO mice at first sight seem to be impaired in learning, as the latency for them to reach the target hole does not decrease. However, data for the errors made, distance moved and mean velocity until reaching the target hole reveals that the difference seen for female 5-HT_{1a} receptor mice is attributable their locomotor reduced activity rather than to an impairment of learning.

Data gathered in the fear conditioning paradigm also revealed gender-dependent differences. We first perturbed fear expression of mice by exposing them to an ambiguous context, which had modified walls and floor as compared to the original training environment. When re-exposed to the training context, male 5-HT_{1a} receptor KO mice showed a trend for reduced % freezing as compared to WT mice. This was a first indication for a gender-specific disruption of associative learning, which prompted us to further investigate fear conditioning with a more elaborated test. In the cue discrimination task, associative learning was challenged by exposing mice to a partial cue and a complete cue, with only the latter being predictive of the foot shock. When tested for fear expression, male 5-HT_{1a} receptor KO mice showed low amounts of total % freezing in comparison to WT males during re-exposure to partial and complete cue, whereas female 5-HT_{1a} receptor KO mice showed similar amounts of % freezing as WT females. This finding confirmed that 5-HT_{1a} receptor deletion affects fear learning in a gender-dependent manner. Furthermore, when comparing the freezing response to the partial and complete cue, only male WT mice showed a significant increase of % freezing for the complete cue when compared with the partial cue. In contrast, male 5-HT_{1a} receptor KO mice could not distinguish between partially predictive and completely predictive cue. Notably, both female WT and 5-HT_{1a} receptor KO mice exhibited the same response to partial and complete cue, suggesting that the performance of females in this task is independent of 5-HT_{1a} receptor deletion. Importantly, all the results gathered with fear conditioning tests were not biased by altered sensitivity of male and female subjects to the foot shock, as this caused the very similar behavioral response in all group tested (flinching, movement or jumping).

Altogether, gender-dependent differences were observed in anxiety-related behavior and learning and memory paradigms. As the hippocampus is involved in anxiety-related behavior as well as spatial

learning and memory, it is likely that the behavioral alterations in male 5-HT_{1a} receptor KO mice derive from gender-specific alterations of the hippocampal circuitry. The differences in behavior are indeed consistent with the changes seen in arborization of hippocampal pyramidal neurons in the *stratum radiatum*, which exhibited abnormal branching in male but not female 5-HT_{1a} receptor KO mice. Whether the extent of dendritic arborization of hippocampal pyramidal neurons tightly correlates with the degree of behavioral phenotype, still needs to be further investigated in a quantitative manner using *ex vivo* morphological analyses.

The differences seen in the gender-dependent effect of 5-HT_{1a} deletion suggest a rescue mechanism occurring in female 5-HT_{1a} receptor KO mice. Consistently, the alterations seen in arborization of hippocampal pyramidal neurons were restored to WT level in female 5-HT_{1a} receptor KO mice upon development, strengthening the link between cellular and behavioral findings Differences observed in organotypic cultures and horizontal sections focusing on the impact of 5-HT_{1a} receptor deletion in the ventral part of the hippocampus, highlight that alterations in branching are similar in the dorsal an ventral part of male 5-HT_{1a} receptor KO mice. As the ventral hippocampus is known to be more strongly involved in emotionality than the dorsal hippocampus, it is likely that the gender-dependent differences in anxiety-related behavior are regulated via mechanisms in the ventral hippocampus.

In contrast, the dorsal hippocampus is well known for its involvement in spatial learning and memory tasks, such as contextual fear conditioning. Recent publications have emphasized the importance of the hippocampal-entorhinal network in context learning in response to an aversive cue and its retrieval (Lovett-Barron *et al*, 2014; Tanaka *et al*, 2014). The dorsal CA1 pyramidal neurons play a crucial role during memory processing. These neurons receive their major inputs from two excitatory synaptic pathways: Schaffer collaterals provide input from the CA3 pyramidal cells which imping on apical dendrites in the *stratum radiatum* and basal dendrites in the *stratum oriens*; layer III neurons of the entorhinal cortex contact the apical dendritic tuft of CA1 neurons in the *stratum lacunosum moleculare* (perforant path synapses). These two distinct pathways provide different kind of information. Whereas input from the Schaffer collaterals encodes information of the context (Kesner, 2007), input from the entorhinal cortex provides information regarding discrete sensory attributes of the context (Lovett-Barron *et al*, 2014) (Maren and Fanselow, 1997; Sparta *et al*, 2014). The interaction of inputs provided from the perforant path and CA3-CA1 synapses can therefore lead to a variety of different cellular or network activation patterns (Dudman *et al*, 2007; Takahashi and Magee, 2009). For example, Jarsky *et al* (2005) demonstrated that input from the perforant path, which by itself elicits dendritic spikes that are insufficient to propagate to the soma, can be amplified by concomitant input from the Schaffer collaterals at the proximal dendrites, resulting in forward propagation of distal dendritic spikes and subsequent generation of action potentials. Interestingly, Lovett-Barron *et al* (2014) provided evidence

that controlled excitatory input from the perforant path plays a crucial role during fear conditioning, in particular for proper encoding of the memory of the aversive stimulus. Aversive stimuli were shown to induce cholinergic activation of s*tratum oriens* interneurons that inhibit the distal dendrites of CA1 pyramidal neurons, hence dampening the excitatory input from the entorhinal cortex. Blocking inhibitory functions of *stratum oriens* interneurons, thus amplifying perforant path excitation, prevented fear learning. The impaired fear memory in male 5-HT_{1a} receptor KO mice might be explained by a similar unbalance of sensory input from the entorhinal cortex. Electrophysiological analyses conducted in our lab have demonstrated that male 5-HT_{1a} receptor KO mice have >1.5-fold increased synaptic strength and respond more efficiently to weak input from the Schaffer collaterals in comparison to WT mice, consistent with the increased arborization in the *stratum radiatum* (Figure 44). In contrast to this excessive excitation at proximal apical dendrites, the input coming from the entorhinal cortex at the distal tuft of pyramidal neurons is processed normally in 5-HT_{1a} receptor KO mice (Fan, 2010)which is in line with normal amounts of branching of pyramidal neurons in this area However, a rather weak input at the distal dendritic tuft of the pyramidal neuron of 5-HT_{1a} receptor KO mice is more likely to propagate to the soma due to strong amplification of coinciding input from the Schaffer collaterals. As a consequence, input from the entorhinal cortex that is normally blocked by inhibitory neurons might be able to reach the soma and elicit action potentials, thereby disrupting proper hippocampal signaling in 5-HT_{1a} receptor KO mice in response to aversive stimuli. Involvement of Schaffer collateral input in fear memory is further supported by experiments done on 5-HT_{1a} receptor KO mice in cue discrimination. Inhibiting granule cells in the DG, thereby downregulating the input of Schaffer collaterals to CA1 pyramidal neurons via the trisynaptic pathway (DG-CA3-CA1), rescued abnormal behavior of male 5-HT_{1a} receptor KO mice in cue discrimination (Tsetsenis *et al*, 2007). Taken together, our data and the findings in the literature provide a mechanistic explanation for the involvement of 5-HT_{1a} receptor mediated changes in the *stratum radiatum* in acquisition of fear memory. It should be mentioned, however, that the report by Tsetsenis *et al* (2007) presents behavioral data that seem to be in conflict with our findings: 5-HT_{1a} receptor KO mice showed increased fear memory for the aversive stimuli, which, as mentioned above, could be rescued by inhibiting Schaffer collateral input. Our male 5-HT_{1a} receptor KO mice showed instead decreased amount of freezing. This discrepancy is unlikely to be attributable to a difference in the background strains. However, no gender distinction is presented in Tsetsenis *et al* (2007), which is likely to impact the outcome of this study, as female 5-HT_{1a} receptor KO mice show higher amount of freezing than males.

In our study, we noticed that male WT mice were the only group that exhibited decreased freezing to the partial cue in comparison to the complete cue. Why female mice, both WT and 5-HT_{1a} receptor KO, are unable to make this distinction might be due to reasons independent of the serotonergic system.

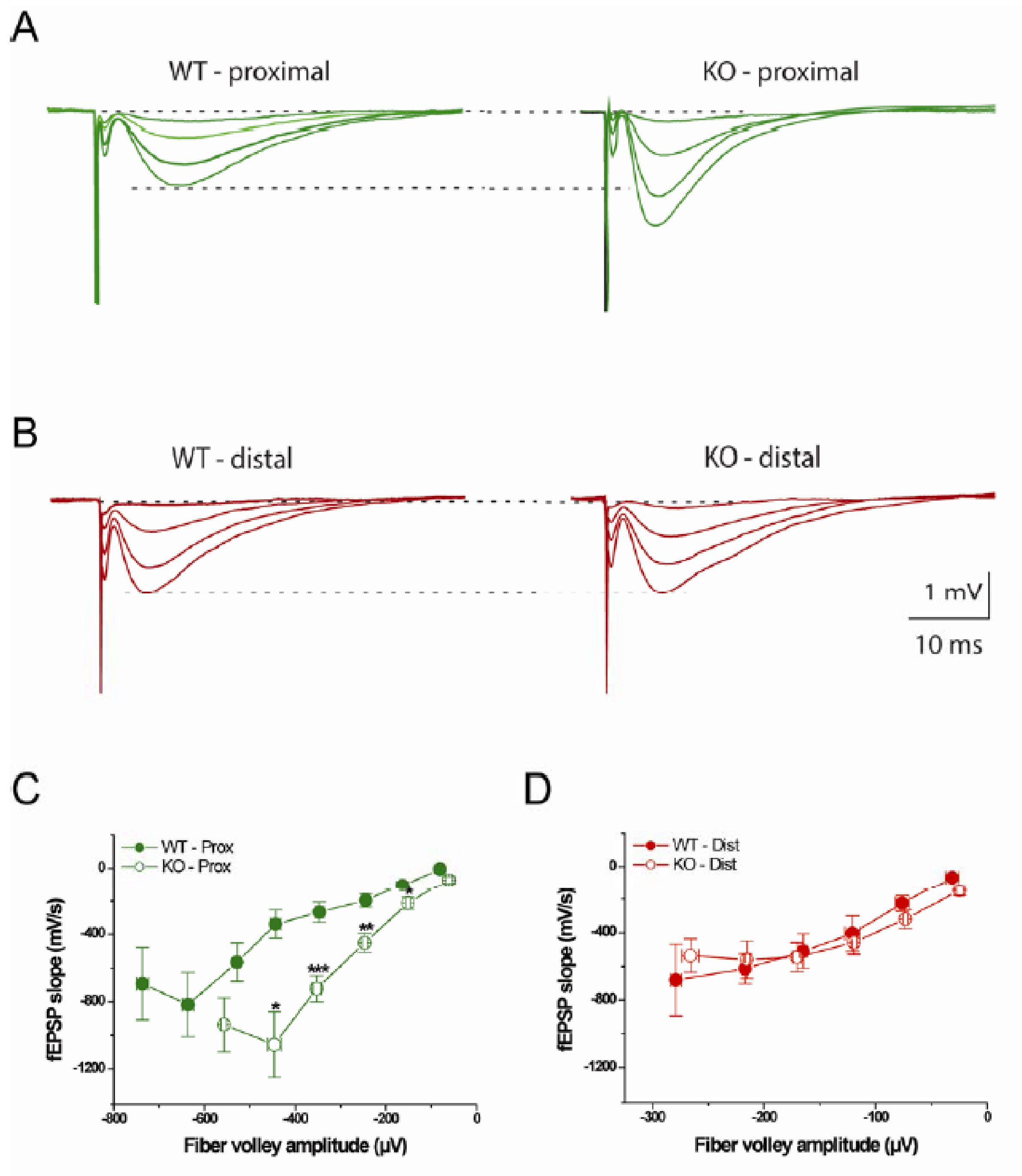

Figure 44 Increased synaptic strength of 5-HT$_{1a}$ receptor KO mice in the *stratum radiatum.*

(A) Representative fEPSP recordings in the proximal afferent pathways of CA1 *stratum radiatum* in WT and KO slices. Stimulation intensities were chosen to remain sub-threshold for population spike generation. Dotted lines denote baseline and peak response of WT-proximal traces. (B) Representative fEPSP recordings in the distal afferent pathway of CA1 *stratum lacunosum moleculare* for WT (WT-distal) and KO (KO-distal). (C) Averaged fEPSP slopes plotted against fiber volley amplitude recordings from WT and KO slices. The fEPSP slopes were averaged for 0.1 mV bins of the fiber volley amplitudes. The right shift in the fiber volley amplitude indicates higher response to stimuli in 5-HT$_{1a}$ receptor KO than WT mice in the *stratum radiatum*. No difference could be seen in the distal part of the pyramidal neurons, which receive inputs form layer II of the entorhinal cortex. Figure from Fan (2010)

Different factors, such as the estrous cycle or the survival rate of adult born neurons, might have an influence on % freezing of females and their ability to distinguish properly between cues. Interestingly, female 5-HT_{1a} receptor KO and WT mice show levels in the survival rate of adult born neurons that are similar to the levels in male 5-HT_{1a} receptor KO mice, and decreased compared to WT males. As adult born neurons are implicated in spatial learning and memory processes involving pattern separation (Denny *et al*, 2014; Gu *et al*, 2012; Seo *et al*, 2015), it is possible that decreased amounts of survival and hence decreased integration of adult born neurons might have affected the animals' ability to distinguish between the partial and complete cue. As gender effects have not been systematically tested in this fear conditioning paradigm before, our findings may provide first indication for a gender-specific impact of adult neurogenesis, which will require further investigation.

In conclusion, our study highlights the importance of gender-dependent differences of 5-HT_{1a} receptor KO mice and suggests that there are mechanisms in female 5-HT_{1a} receptor KO mice which can at least partially rescue the behavioral phenotype and which might extend to different brain areas involved in anxiety and fear learning. This information is highly valuable, as gender-dependent differences for the likelihood to develop mental disorders have been known for some time. For example, depression in women is more than twice as prevalent as in men between the ages of 14-25 years (Albert, 2015). Furthermore, depression-related illnesses are different between women and men. Women especially suffer from hormone-dependent illnesses such as premenstrual dysphoric disorder, postpartum depression and postmenopausal depression and anxiety (Albert, 2015), indicating that hormones play a crucial role in the ontogeny of mental disorders. In our laboratory, we demonstrated that CA1 dendritic arborization in female 5-HT_{1a} receptor KO mice is rescued via activation of G-protein coupled estrogen receptor 1 (GPER-1) by estrogen. Taken together, we suggest that alterations in 5-HT_{1a} receptor signaling during the early postnatal developmental period can be buffered by hormones. However, if hormonal levels are disturbed dramatically, alterations in 5-HT_{1a} receptor signaling cannot be reversed anymore and are likely to lead to behavioral alterations in adulthood. Whether behavioral abnormalities as well as dendritic arborization can be normalized by application of hormones in adulthood though remains to be investigated.

IV.2 IMPACT OF BREEDING SCHEME AND STRAIN ON BEHAVIORAL OUTPUT

Although the gender-dependent increase of anxiety-related behavior in male 5-HT_{1a} receptor KO mice was reproduced in the open field and the light/dark box, no difference for either gender was seen between 5-HT_{1a} receptor KO and WT mice in the elevated plus maze. Reproducibility of data on anxiety-

related behavior of 5-HT_{1a} receptor KO mice across laboratories seems to have been a recurrent issue within the last decade. For example, Groenink *et al* (2003) studying 5-HT_{1a} receptor KO mice on the 129SV background (Ramboz *et al*, 1998) described a lack of differences for anxiety-related behavior in the open field, light/dark box and elevated plus maze. Furthermore, Lo Iacono *et al* (2008) observed effects of 5-HT_{1a} receptor blockage from P14-35 on anxiety related behavior in the open field and novelty suppressed feeding task, but stated that there is no difference between injected and control animals for anxiety-related behavior in the elevated plus maze. Similarly, Richardson-Jones 2011 reported no differences in the elevated plus maze between WT and mice with permanent 5-HT_{1a} autoreceptor depletion. On the other hand, Donaldson *et al* (2014), using the same mice as described by Richardson-Jones 2011, reported increased anxiety-related behavior of adult mice in the elevated plus maze when decreasing 5-HT_{1a} autoreceptor expression between P14 and P30. Different reasons might be at the base of these discrepancies. Gleason *et al* (2010), for example, showed that heterozygous breeding can impact anxiety-related behavior of 5-HT_{1a} receptor KO and WT mice in the open field and the elevated plus maze differently. Anxiety-related behavior is strongly influenced by breeding scheme in the elevated plus maze resulting in highly increase anxiety related behavior of WT mice of HET mothers, no effect of breeding scheme is seen on behavior in the open field. As most of the mice mentioned above were obtained from heterozygous breeding pairs, part of the missing effects on the elevated plus maze might have been induced by heterozygous breeding over multiple generations. Furthermore, the background strain can influence behavior dramatically. 129SV mice are thought to be more anxious than C57BL/6 mice (Kulesskaya and Voikar, 2014) and show decreased amounts of locomotor behavior (Loos *et al*, 2014; Molenhuis *et al*, 2014), which can further impact measures of anxiety-related behavior in the above mentioned tests. The impact of the strain on the performance in specific anxiety-related behavioral tests has been demonstrated by systemically comparing different strains in the open field, light/dark box and the elevated plus maze: whereas mice of the AKR strain were considered the most anxious in the elevated plus maze, they scored as the least anxious in the open field (O'Leary *et al*, 2013). 5-HT_{1a} receptor KO mice used in our study and in all reports mentioned above were originally generated on mixed genetic backgrounds. Depending on the parents, the genetic background can be shifted favoring one or the other background, which in turn can lead to alterations in behavior. By backcrossing our mice further on the C57BL/6 background with a heterozygous breeding scheme, we might have therefore favored aspects of anxiety that appear more prominently in the open field and the light/dark box rather than in the elevated plus maze. Similar effects might also have impacted learning and memory in the water maze and Barnes maze, as we did not observe impaired performance in male 5-HT_{1a} receptor KO mice, in contrast to some previous studies (Sarnyai *et al*, 2000; Wolff *et al*, 2004).

Taken together, our data and the results from recent literature implicate that the anxiety-related behavioral phenotype of constitutive 5-HT_{1a} receptor KO mice is multifaceted and more complex than it was suggested by observations from the earlier studies (Heisler *et al*, 1998; Parks *et al*, 1998; Ramboz *et al*, 1998). In addition to the gender-difference, several factors, such as breeding scheme and background strain, can drastically influence the behavior of 5-HT_{1a} receptor KO mice. Nevertheless, it is general agreement that 5-HT_{1a} receptor deficiency - as comparable to low receptor binding in humans - acts as a predisposition for increase anxiety-related behavior in mice.

IV.3 Depressive-like behavior of 5-HT_{1A} receptor KO mice

Depression is marked by extensive periods of sadness in combination with anhedonia and behavioral despair. In mice, these behaviors can be measured by various tests, however, most of them capture only partial aspects of the complex depressed phenotype in humans. The forced swim test and the tails suspension task, for example, evaluate behavioral despair, whereas the sucrose preference task and the novelty suppressed feeding task provide a read-out for anhedonia. As depressed patients are likely to show impaired learning and memory abilities and abnormal sleep-wake rhythm, mice models of depression are typically analyzed in various learning and memory tasks, such as the Morris water maze or fear conditioning, and their home cage activity is monitored during the light-dark cycle.

In the early description of 5-HT_{1a} receptor KO mice, depressive-like behavior was not observed in the forced swim test or the tail suspension task. All constitutive 5-HT_{1a} receptor KO lines showed antidepressant-like rather than depressive-like behavior (Heisler *et al*, 1998; Parks *et al*, 1998; Ramboz *et al*, 1998). A later study reported depressive-like behavior in the novelty-suppressed feeding task and, depending on which receptor population was deleted, also in the forced swim test (Table 1). However, an extensive depressive-like phenotype of constitutive 5-HT_{1a} receptor KO mice so far has not been observed. In our study, we tested our mice for anhedonia and olfactory functioning to further investigate the depressive-like phenotype of 5-HT_{1a} receptor KO mice. No difference between 5-HT_{1a} receptor KO mice and WT mice was observed in the sucrose preference test or the splash test, confirming the early reports that indicated lack of depressive-like behavior in constitutive 5-HT_{1a} receptor KO mice. Despite preliminary data gathered in trial experiments for olfactory functioning showing genotype-dependent impairments (data not shown), tests for olfaction in this batch of animals showed no marked differences between genotype in the buried food test or the bedding preference test, indicating lack of anhedonia in KO mice.

Taken together, our experiments confirm the absence of strong depressive-like behavior of constitutive male and female 5-HT_{1a} receptor KO mice. Data on olfactory functioning however still needs to be supplemented to be fully conclusive. The lack of a strong depressive-like phenotype is likely to be caused by simultaneous deletion of 5-HT_{1a} hetero- and autoreceptors, which have been shown to differently impact depressive like symptoms (Garcia-Garcia *et al*, 2015; Richardson-Jones *et al*, 2011). Selective 5-HT_{1a} receptor deletion of either of the populations might therefore be a more suitable model to study developmental and acute effects of abnormal 5-HT_{1a} receptor signaling on depressive-like behavior. Breeding of floxed 5-HT_{1a} receptor KO mice are ongoing to study the effects of 5-HT_{1a} receptor deletion on olfaction functioning on the behavioral level as well as on molecular level by measuring early growth response 1 (Erg-1) and c-fos activation in the olfactory bulb after exposure to dirty bedding. The latter experiments might help to reproduce the differences seen in the trial experiments in which animals from homozygous and heterozygous breeding were used.

IV.4 Molecular mechanism regulating dendritic complexity

5-HT_{1a} receptor KO mice show increased dendritic branching in the *stratum radiatum* of pyramidal neurons during early postnatal development. In female 5-HT_{1a} receptor KO mice, this increase normalizes with the onset of puberty, whereas male 5-HT_{1a} receptor KO mice express even further increased amounts of dendritic branching throughout adulthood. A gender-dependent rescue mechanism seems to attenuate the effect of 5-HT_{1a} receptor deletion in female 5-HT_{1a} receptor KO mice. The importance of this mechanism is highlighted by behavioral difference of male and female 5-HT_{1a} receptor KO mice in anxiety-related behavior and fear memory indicating that these morphological alterations can have drastic effects on the animals' behavior. It was thus our aim to understand the downstream mechanism of 5-HT_{1a} receptor signaling regulating arborization.

Expression levels of NR2B during development

Electrophysiological analyses conducted in our lab point at increased contribution of NR2B-containing NMDA receptors to CA3-CA1 glutamatergic transmission in male 5-HT_{1a} receptors KO mice during the early postnatal developmental period. It remained to be investigated whether this was due to differences in global expression and/or synaptic localization of the receptor as compared to WT mice. Using western blot and qRT-PCR, we identified increased NR2B mRNA and protein levels at P15 in male 5-HT_{1a} receptor KO mice. P15 lies within the critical developmental period that is crucial for dendritic arborization. In culture, various studies have shown that NR2B-containing NMDA receptors are involved

in dendritogenesis during the first three weeks of postnatal development (Bustos *et al*, 2014; Espinosa *et al*, 2009; Henle *et al*, 2012; Sepulveda *et al*, 2010). Overexpression of NR2B-containing NMDA receptors therefore, will have a strong impact on branching. However, significant differences in NR2B subunit mRNA and protein expression were only observed at P15, and not in the adjacent time points analyzed at P10 and P20. The restricted difference is unlikely to be sufficient to permanently alter the amount of branching, as molecular processes to stabilize the neurons' morphology outlast this short time window. Furthermore, electrophysiological data also suggested increased synaptic expression of NR2B-containing NMDA receptors of 5-HT_{1a} receptor KO mice around P25. This difference was not detected in the synaptoneurosomal fraction of our samples, which is most likely due to the utilization of total hippocampal lysate. Hence, experiments applying *in situ* hybridization at different developmental time points to detect region-specific differences in hippocampal NR2B mRNA expression will be needed to further examine the expression of NR2B-containing NMDA.

To understand the cause of NR2B subunit overexpression at P15 we analyzed REST mRNA levels during the critical developmental period starting from P10 onwards. REST has been described to regulate NR2B subunit expression in the hippocampus of rats during the critical period of NMDA receptor subunit switch between P13-16 (Rodenas-Ruano *et al*, 2012). The temporal match between the reported peak activity of REST and the developmental profile of NR2B expression in male 5-HT_{1a} receptors KO mice prompted us to investigate a possible link between REST and 5-HT signaling. Interestingly, REST has been shown to at least partially repress 5-HT_{1a} receptor expression (Lemonde *et al*, 2004b), which in turn could impact NR2B-containg NMDA receptors. We found no difference in REST mRNA expression between male 5-HT_{1a} receptor KO and WT mice, suggesting that the absence of 5-HT_{1a} receptor does not alter NR2B subunit expression via REST mediated pathways. However, our experimental setting might need to be refined before drawing a definitive conclusion. In contrast to the reported increase in protein levels of REST between P13 and P16, we have not found changes in REST mRNA levels in the developmental age range we have examined (P10-P30). Additional data at better time resolution in an extended range might provide a more detailed picture of REST activity in 5-HT_{1a} receptor KO mice and unravel a link that went undetected on the first analyses.

NR2B-containing NMDA receptors and branching

Overexpression of NR2B-containing NMDA receptors, as mentioned above, is in line with data on the impact of NR2B subunit on dendritogenesis of neurons. For example, increased expression of NR2B-containing NMDA receptors in developing hippocampal neurons due to downregulation of NR2A subunit expression resulted in significant increase of branches (Sepulveda *et al*, 2010). NMDA

application in culture induced generation of filopodia and secondary branches of hippocampal neurons, which could be blocked by application of the NR2B-selective antagonist ifenprodil (Henle *et al*, 2012). Consistent with this data, 5-HT_{1a} receptors have been shown to regulate dendritic growth cone dynamics in hippocampal dissociated cultures and dendritic arborization Neuronal growth cones contain high amounts of filopodia which are rich in F-actin (Mattila and Lappalainen, 2008), a molecule that is enriched in 5-HT_{1a} receptor KO mice (Ferreira *et al*, 2010). The amount of F-actin, growth cone dynamics and dendritic arborization can be normalized in WT hippocampal dissociated cultures following application of 5-HT (30 nM) (Ferreira *et al*, 2010). This data strongly supports the link between 5-HT_{1a} receptor and dendritic branching and gives reason to believe that 5-HT_{1a} receptor impacts dendritic branching by indirectly acting through NR2B-containing NMDA receptors. This hypothesis is supported by data showing that regulation of NR2B-containing NMDA receptor on branching is controlled by Erk1/2 activation via RAS protein-specific guanine nucleotide-releasing factor 1 (RASGRF1) (Sepulveda *et al*, 2010). NR2B-containing NMDA receptor activation leads to interaction with RASGRF1 via the NR2B subunit and subsequent activation of RAS and Erk1/2 (Ivanov *et al*, 2006; Krapivinsky *et al*, 2003), which in turn leads to increased branching in hippocampal neurons (Sepulveda *et al*, 2010). An increase in NR2B-containing NMDA receptors, as seen in our male 5-HT_{1a} receptor KO mice, is therefore likely to promote activation of this downstream pathway resulting in higher amount of arborization (Figure 45). Data from organotypic cultures supports the involvement of Erk1/2-dependent pathways in the downstream mechanisms of NR2B-containing NMDA receptors. As we could show, application of the Erk1/2 inhibitor, U0126, normalizes the amount of branching in 5-HT_{1a} receptor KO slices.

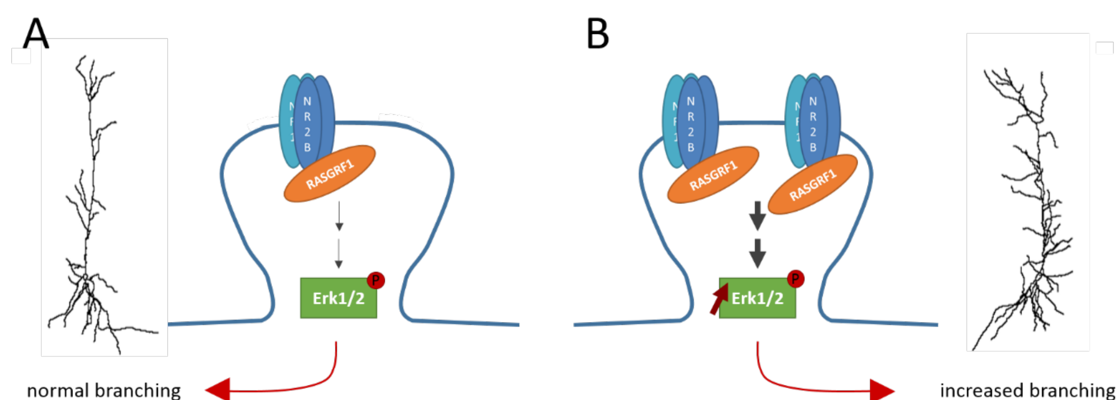

Figure 45 NR2B-containing NMDA receptor activate Erk1/2 via interaction with RASGRF1.
(A) Binding of RASGRF1 to the NR2B subunit of the NMDA receptor is sufficient to result in Erk1/2 phosphorylation regulating the amount of branching under normal conditions. B) In 5-HT1a receptor KO mice, however, increased NR2B-containing NMDA receptor expression is likely to lead to increased activation of Erl1/2 which in turn results in high levels of dendritic branching.

5-HT_{1A} RECEPTOR DOWNSTREAM SIGNALING

Although the above mentioned mechanism sheds light on the impact of NR2B-containing NMDA receptors and its downstream pathway on increased arborization, information on the exact downstream pathways of 5-HT_{1a} receptor involved in increased NR2B-containing NMDA receptor expression still need to be determined. Unraveling the intracellular link between these two receptors is particularly challenging, as 5-HT_{1a} receptor signaling involves postsynaptic downstream effectors that have been shown to vary in a cell-dependent and age-dependent manner (signal transduction p.15). Nevertheless, the main candidates can be recognized in CaMKII and Erk1/2, that are well known to regulate NR2B-containing NMDA receptors.

The link between 5-HT, Erk1/2 and NR2B-containing NMDA receptors is complex. On the one hand, NR2B-containing NMDA receptors can phosphorylate Erk1/2, which limits dendritic branching, as described above. On the other hand, 5-HT_{1a} receptors have been shown to activate Erk1/2 in HN2-5 cells and in hippocampal slices (Adayev *et al*, 1999; Adayev *et al*, 2003; Mehta *et al*, 2007). Therefore, it can be expected that 5-HT_{1a} receptor depletion would result in decreased activation of Erk1/2. To directly test this, the ability of the 5-HT_{1a} receptor to activate the Erk1/2 should be selectively blocked, while leaving the CaMKII pathway unaffected. Mutations of the second intracellular loop of the 5-HT_{1a} receptor (T149A) have been shown inhibit interaction of Gβγ upon 5-HT_{1a} receptor activation receptor while leaving coupling with the Gα subunit unaffected (Kushwaha and Albert, 2005). Electroporation of cultured hippocampal 5-HT_{1a} receptor KO cells with plasmids containing the mutated 5-HT_{1a} receptor and investigation of arborization thereafter would allow dissociating between 5-HT_{1a} receptor dependent direct effect on Erk1/2 and indirect effect through NR2B-containing NMDA receptors.

NR2B-containing NMDA receptors are highly regulated via CamKII, which is likely to be modulated by serotonergic signaling. CamKII is implicated in pathways that regulate NR2B-containing NMDA receptor transportation via KIF17 by altering the stabilization of microtubule (Yuen *et al*, 2005) and its involvement in cargo release at the synapse (Guillaud *et al*, 2008). Furthermore, CaMKII is phosphorylated by NMDA receptor dependent Ca^{2+} influx, which leads to subsequent binding of CaMKII to NR2B (Raveendran *et al*, 2009; Sessoms-Sikes *et al*, 2005; Strack *et al*, 2000). Binding of CaMKII to the NR2B subunit can lead to phosphorylation of serine 1303 residue, which would cause dissociation of the kinase from the receptor. Moreover, CaMKII binding can induce CK2-dependent mechanisms resulting in the removal of NR2B-containing NMDA receptors from the synapse and subsequent endocytosis (Sanz-Clemente *et al*, 2013; Sanz-Clemente *et al*, 2010). 5-HT_{1a} receptor KO mice and mice treated with the 5-HT_{1a} receptor inhibitor WAY-100,635 express increased amounts of phosphorylated CaMKII after exposure to an unfamiliar environment, suggesting strong upregulation of activated CaMKII in 5-HT_{1a} receptor KO mice. Upregulated CaMKII activation could result in increased phosphorylation of NR2B

subunit at serine 1303, which in turn could lead to increased NR2B-containing NMDA receptor conductance (Paoletti *et al*, 2013) or removal of the receptor from the synapse (Sanz-Clemente *et al*, 2013). Sampling NR2B phosphorylation at serine 1303 would therefore give valuable information about the impact of 5-HT_{1a} receptor deletion on the regulation of NR2B-containing NMDA receptors. Furthermore, as removal of NR2B-containing NMDA receptors from the synapse is dependent on association of activated CaMKII, NR2B subunit and CK2, resulting in phosphorylation of the latter, measuring CK2 phosphorylation will indicate whether removal from the synapse in 5-HT_{1a} receptor KO mice is downregulated. Another way to study efficient removal of NR2B-containing NMDA receptors from the synapse is by blocking CK2 activity. Applying TBB, a CK2 inhibitor, to organotypic cultures we could not observe further increase of branching due to increased amount of NR2B-containing NMDA receptors at the synapse. This suggests that NR2B-containing NMDA receptor expression has reached a ceiling effect in 5-HT_{1a} receptor KO slices as increasing NR2B-containg NMDA receptor expression by blocking its removal leads to cell death rather than further increased in branching.

In conclusion, in addition to clarify the involvement of Erk1/2 signaling, we suggest that further investigations should test the role of CaMKII in regulating trafficking of NR2B-containing NMDA receptorsdownstream of 5-HT_{1a} receptor activation.

INCREASED NR2B SUBUNIT EXPRESSION INFLUENCES CELL DEATH

As mentioned above, applying TBB in cultures lead to increased amount of cell death which prompted us to further investigate the effects of 5-HT_{1a} receptor deletion on NR2B-containing NMDA receptor mediated cell death. Comparing 5-HT_{1a} receptor KO slices treated with or without U0126 or ifenprodil revealed a significant impact of treatment on the amount of cell death in organotypic hippocampal cultures after 9 days of treatment. Propidium iodide staining revealed increased levels of cell death in the CA1 region of the hippocampus in control and 5-HT treated 5-HT_{1a} receptor KO slices. In contrast, blocking NR2B-containing NMDA receptors during the early developmental period in cultures decreased the amount of propidium iodide staining in the CA1 region. Similar results were obtained when applying U0126, which also decreased the amount of cell death in 5-HT_{1a} receptor KO cultures. This data suggests the involvement of NR2B-containing NMDA receptors in downstream pathways leading to cell death of pyramidal neurons in hippocampal slices.

By cutting Schaffer collaterals in organotypic hippocampal cultures of 5-HT_{1a} receptor KO mice we could show that input of CA3-CA1 synapse and, thus, the activation of NR2B-containing NMDA receptors via glutamatergic synaptic drive is needed to regulate branching. Hyperactivatioin of increased amounts of NR2B-containing NMDA receptors at the postsynaptic membrane, therefore, seems to trigger

downstream pathways that favor cell death. In turn, the subsequent decreased amounts of cells in the CA1 region of the hippocampus would lead to a rewiring in the stratum radiatum as the input coming from the Schaffer collaterals now needs to be distributed on lesser postsynaptic partners. This would sustain the preservation of increased branching of CA1 pyramidal neurons in the *stratum radiatum*.

Similar mechanisms could explain the decreased survival rate of adult born neurons in the DG of 5-HT_{1a} receptor KO mice. We showed that in comparison to male WT mice, male 5-HT_{1a} receptor mice showed decreased amounts of DCX positive cells in the DG of the hippocampus. DCX is a marker for immature granule cells start migration and dendritic outgrowth. These cells exhibit synaptic NMDA-currents with slow kinetics at the synapse that can be blocked by ifenprodil, indicating high amounts of NR2B containing NMDA receptors at the synapse (Spampanato *et al*, 2012). Adult born granule cells exhibit a similar critical period of NMDA receptor subunit exchange as hippocampal neurons during the first few postnatal weeks (Ge *et al*, 2007). Accumulation of NR2B-containing NMDA receptors could be responsible for increased cell death of adult born neurons in male 5-HT_{1a} receptor KO mice.

The exact mechanisms regulating cell death of adult born neurons and the involvement of NR2B-containing NMDA receptors and Erk1/2 - both known to be involved in excitotoxicity - remains to be investigated.

An interesting perspective is provided by gender-related differences in survival of adult born granule cells, as female mice show a survival rate comparable to that of 5-HT_{1a} receptor KO mice. It seems that in general the survival rate of adult born neurons in females is decreased in comparison to male mice (Martini *et al*, 2014; Naninck *et al*, 2015). Different hormonal-dependent mechanisms might be responsible, and our findings suggest the additional implication of 5-HT_{1a} receptors.

IV.5 Conclusions and perspectives

We used constitutive 5-HT_{1a} receptor KO mice to analyze the effects of serotonergic depletion in CA1 pyramidal neurons of the hippocampus, the limbic forebrain structure with the highest receptor density. In our laboratory, we have identified morphological and physiological alterations in CA1 pyramidal neurons of 5-HT_{1a} receptor KO mice, which are more strongly expressed in male than in female mice. The present study provides evidence for a gender-specific impact of 5-HT_{1a} receptor deletion on a wide range of behaviors, including anxiety-related behavior and cognitive function. Our analysis in male and female 5-HT_{1a} receptor KO mice revealed differences which could be associated with the morphological and physiological changes induced by 5-HT_{1a} receptor deletion. Moreover, we have identified NR2B-containing NMDA receptors as a downstream effector of 5-HT_{1a} receptors during development

regulating hippocampal circuit formation. The higher expression of NR2B-containing NMDA receptors not only alters electrophysiological parameters, including the amplitude of postsynaptic potentials and the propensity to undergo synaptic plasticity in the hippocampal circuit, but also exerts a detrimental effect on adult neurogenesis, possibly excitotoxicity, and eventually could be associated with altered olfactory function.

Altogether, our findings highlight the association between structural and functional changes in the hippocampus and behavior, and indicate possible hormone-regulated rescue mechanisms in female mice implicating downstream signaling of NR2B-containing NMDA receptors. The identification of this link opens avenues for further investigations on the serotonergic system in hippocampal-related behavior. Hormone-regulated rescue experiments will be a straightforward approach to directly assess a gender-specific link between 5-HT_{1a} receptors, NR2B-containing NMDA receptors and the cellular and behavioral outcome. Gonadectomy in P25 female knock-out mice should prevent the reduction in dendritic branches and cause hippocampal overexcitability, thus removing the gender-dependent behavioral differences of 5-HT_{1a} receptor KO mice. The opposite is to be expected in gonadectomized male 5-HT_{1a} receptor KO mice treated with GPER1 agonist from P25 onwards.

Furthermore, focusing on intra hippocampal circuits, hyperexcitability of CA1 neurons by Schaffer collaterals could be transiently lowered by optogenetically hyperpolarizing CA3 pyramidal neurons expressing exogenous Kv2.1. Hyperpolarization of CA3 pyramidal neurons during the training phase fear conditioning, or during the critical developmental period between P25 and P40, should rescue the behavioral, physiological or morphological phenotypes. A further line of investigation involves the role of auto- and hetereceptors in mediating the physiological and pathological impact of serotonin. Different receptor populations could be addressed by using conditional deletion of 5-HT_{1a} receptors in restricted brain areas. Breeding of floxed 5-HT_{1a} receptor mice with region-specific and cell-specific Cre-lines would allow the identification of the time window and the receptor population responsible for the behavioral phenotype observed in our 5-HT_{1a} receptor KO mice. The mechanisms reported in the hippocampus following 5-HT_{1a} deletion are likely to be found in a number of other areas of the forebrain in which the same group of receptors (5-HT_{1a}, NR2B-containing NMDA, GPER-1) are located. Due to its involvement in serotonergic release from the raphe nuclei via negative feedback mechanisms, the prefrontal cortex would be a main target region for further investigations (Altieri *et al*, 2013).

The gender-dependent differences observed in our 5-HT_{1a} receptor KO mice open a large area of investigation for pathologies reported in humans, as the occurrence of anxiety and mood disorders are also influenced by gender (Bangasser and Valentino, 2014; Catuzzi and Beck, 2014; Mueller *et al*, 2014). Given the fact that NR2B-containing NMDA receptors act downstream of 5-HT_{1a} receptors, further targeting downstream effectors of the NMDA receptor might unravel novel therapeutic targets.

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