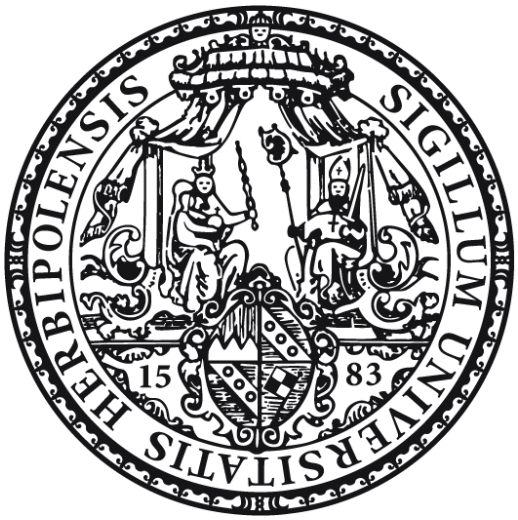

Electrophysiological investigation of two animal models for emotional disorders - serotonin transporter knockout mice and tryptophan hydroxylase 2 knockout mice

Elektrophysiologische Untersuchung bei zwei Tiermodellen für emotionale Störungen - Serotonin Transporter knockout Mäuse und Tryptophan Hydroxylase 2 knockout Mäuse

Dissertation zur Erlangung des naturwissenschaftlichen Doktorgrades der Graduate School of Life Sciences,
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vorgelegt von

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Abstract

Serotonin (5-HT) has been implicated in the regulation of emotions as well as in its pathological states, such as anxiety disorders and depression. Mice with targeted deletion of genes encoding various mediators of central serotonergic neurotransmission therefore provides a powerful tool in understanding contributions of such mediators to homeostatic mechanisms as well as to the development of human emotional disorders. Within this thesis a battery of electrophysiological recordings were conducted in the dorsal raphe nucleus (DRN) and the hippocampus of two murine knockout lines with deficient serotonergic systems. Serotonin transporter knockout mice (5-Htt KO), which lack protein responsible for reuptake of 5-HT from the extracellular space and tryptophan hydroxylase 2 knockout (Tph2 KO) mice, which lack the gene encoding the neuronal 5-HT-synthesising enzyme. First, 5-HT_{1A} receptor-mediated autoinhibition of serotonergic neuron firing in the DRN was assessed using the loose-seal cell-attached configuration. Stimulation of 5-HT_{1A} receptors by a selective agonist, R-8-hydroxy-2-(di-n-propylamino)tetralin (R-8-OH-DPAT), showed a mild sensitisation and a marked desensitisation of these receptors in Tph2 KO and 5-Htt KO mice, respectively. While application of tryptophan, a precursor of 5-HT and a substrate of Tph2, did not cause autoinhibition in Tph2 KO mice due to the lack of endogenously produced 5-HT, data from 5-Htt KO mice as well as heterozygous mice of both KO mice lines demonstrated the presence of autoinhibitory mechanisms as normal as seen in wildtype (WT) controls. When the Tph2-dependent step in the 5-HT synthesis pathway was bypassed by application of 5-hydroxytryptophan (5-HTP), serotonergic neurons of both Tph2 KO and 5-Htt KO mice showed decrease in firing rates at lower concentrations of 5-HTP than in WT controls. Elevated responsiveness of serotonergic neurons from Tph2 KO mice correspond to mild sensitisation of 5-HT_{1A} receptors, while responses from 5-Htt KO mice suggest that excess levels of extracellular 5-HT, created by the lack of 5-Htt, stimulates 5-HT_{1A} receptors strong enough to overcome desensitisation of these receptors. Second, the whole-cell patch clamp recording data from serotonergic neurons in the DRN showed no differences in basic electrophysiological properties between Tph2 KO and WT mice, except lower membrane resistances of neurons from KO mice. Moreover, the whole-cell patch clamp recording from CA1 pyramidal neurons in the hippocampus of 5-Htt KO mice showed increased conductance both at a steady state and at action potential generation. Lastly, magnitude of long-term potentiation (LTP) induced by the Schaffer collateral/commissural pathway stimulation in the ventral hippocampus showed no differences among Tph2 KO, 5-Htt KO, and WT

counterparts. Taken together, lack and excess of extracellular 5-HT caused sensitisation and desensitisation of autoinhibitory 5-HT_{1A} receptors, respectively. However, this may not directly translate to the level of autoinhibitory regulation of serotonergic neuron firing when these receptors are stimulated by endogenously synthesised 5-HT. In general, KO mice studied here showed an astonishing level of resilience to genetic manipulations of the central serotonergic system, maintaining overall electrophysiological properties and normal LTP inducibility. This may further suggest existence of as-yet-unknown compensatory mechanisms buffering potential alterations induced by genetic manipulations.

Zusammenfassung

Serotonin (5-HT) ist an der Regulation von der Emotionen, sowie ihrer pathologischen Zustände, wie Angststörungen und Depressionen beteiligt. Mäuse denen, mittels einer zielgerichteteten Deletion von Genen, die verschiedenste Proteine involviert in der zentralen serotonergen Nerotransmission fehlen, dienen daher als ein nützliches Tiermodell, um die Rolle dieser Mediatoren bei Homöostasemechanismen und der Entwicklung emotionaler Störungen beim Menschen zu verstehen. Im Rahmen dieser Thesis wurde eine Batterie von elektrophysiologischen Ableitungen im Hippocampus sowie in der dorsalen Raphe Nucleus (DRN) zweier Knockout-Mauslinien mit einem defizienten serotonergen Systems durchgeführt. Serotonintransporter Knockout-Mäuse (5-Htt KO), denen das Protein zur Wiederaufnahme von 5-HT aus dem extrazellulären Raum fehlt und Tryptophanhydroxylase 2 Knockout-Mäuse (Tph2 KO), denen das Gen für das 5-HT-synthetisierende Enzym im Gehirn fehlt. Zunächst wurde mittels der “loose-seal cell-attached” Aufnahmemethode die Eigenhemmung der serotonergen Neuronen untersucht, die durch 5-HT_{1A} Rezeptoren in der DRN vermittelt wird. Stimulierung der 5-HT_{1A} Rezeptoren durch einen selektiven Agonist, R-8-hydroxy-2-(di-n-propylamino)tetralin (R-8-OH-DPAT), zeigte eine milde Sensibilisierung und eine deutliche Desensibilisierung dieser Rezeptoren in Tph2 KO bzw. in 5-Htt KO Mäusen. Während die Anwendung von Tryptophan, eine Vorstufe von 5-HT und ein Substrat

der Tph2, keine Eigenhemmung, aufgrund des Mangels an endogen produziertem 5-HT, in Tph2 KO Mäusen verursachte, wiesen Daten von 5-Htt KO Mäusen sowie von heterozygoten Mäusen beider KO Mauslinien die Existenz der Eigenhemmungsmechanismen wie in den Wildtypen (WT) nach. Wurde der Tph2-abhängige Schritt im 5-HT Syntheseweg durch Anwendung von 5-Hydroxytryptophan (5-HTP) umgangen, zeigten sowohl Tph2 KO als auch 5-Htt KO Mäuse eine Verminderung der serotonergen neuronalen Feuerungsrate bei niedrigeren Konzentrationen von 5-HTP im Vergleich zu den WT. Die erhöhte Reaktionsfähigkeit der serotonergen Neuronen von Tph2 KO Mäusen entsprechen der milden Sensibilisierung der 5-HT_{1A} Rezeptoren. Stattdessen deuten die Reaktionen der serotonergen Neuronen von 5-Htt KO Mäusen darauf hin, dass das überschüssige Niveau von extrazellularem 5-HT, welches durch den Mangel an 5-Htt verursacht wird, 5-HT_{1A} Rezeptoren stark genug stimuliert, um eine Desensibilisierung dieser Rezeptoren zu überwinden.

Zweitens zeigten die Daten der whole-cell Patch Clamp Ableitung von serotonergen Neuronen im DRN keine Unterschiede in grundlegenden elektrophysiologischen Eigenschaften zwischen Tph2 KO und WT, außer niedrigen Membranwiderständen in KO Mäusen. Darüber hinaus zeigte die whole-cell Patch Clamp Ableitungen von CA1 Pyramidenzellen im Hippocampus der 5-Htt KO Mäuse eine erhöhte Leitfähigkeit sowohl bei Ruheständen als auch bei Aktionspotentialerzeugungen. Schließlich zeigte die Stärke der Langzeitpotenzierung (long-term potentiation: LTP) durch die Stimulation der Schaffer-Kollateralen/kommissuralen Fasern im ventralen Hippocampus keine Unterschiede zwischen Tph2 KO, 5-Htt KO, und jeweiligen WT. Zusammengefasst verursachten der Mangel und der Überschuss von extrazellularen 5-HT eine Sensibilisierung bzw. Desensibilisierung der autoinhibitorischen 5-HT_{1A} Rezeptoren. Dies kann jedoch nicht direkt in die Regulierung von serotonergen Neuronen Feuerung umgesetzt werden, wenn die 5-HT_{1A} Rezeptoren durch endogen synthetisiertes 5-HT stimuliert werden. Im Allgemeinen zeigten die hier untersuchten KO Mäuse, ein erstaunliches Maß an Widerstandskraft, die die allgemeinen elektrophysiologischen Eigenschaften und die normale LTP Induzierbarkeit trotz genetischer Manipulationen des zentralen serotonergen Systems aufrechterhielt. Weiterhin deutet dies auf die Existenz noch unbekannter Kompensationsmechanismen hin, die diese potentiellen Veränderungen abzudämpfen scheinen.

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1 . Introduction

1.1. Serotonin and affective disorders

1.1.1. Depression and monoamine neurotransmitters

Major depressive disorder (MDD) is one of the most common and yet debilitating psychiatric disorders, affecting about 121 million people worldwide (WHO, 2012) and being one of the major causes of productivity loss worldwide. In 2004, it was ranked as the third most leading cause of burden of disease and is estimated to reach the first place in 2030 (WHO, 2008). Its epidemiology is socio-economic dependent, yielding the average lifetime prevalence of 14.6% in high-income countries, whereas 11.1% in low- to middle-income counties (Bromet et al., 2011). Symptoms of depression include: persistent sad feelings, feelings of guilt, low self-esteem, irritability and restlessness, loss of appetite or overeating, loss of interest, anhedonia, sleep disturbance, fatigue and low energy, poor concentration, difficulty in decision-making, and feeling of hopelessness (NIMH, 2012). In its worst case, depression can lead to suicide, taking lives of about 850,000 people every year (WHO, 2012). The etiopathogenetic mechanisms of depression are still unknown but it is believed that genetic factors which determine certain personality of individuals as well as stressful life events trigger the onset of major depression. Neurobiologically, several lines of pharmacological evidence suggest involvement of monoamine neurotransmitters, especially serotonin (5-HT) and norepinephrine (NE) but also dopamine (DA) (Jacobsen et al., 2012a; Haenisch and Bonisch, 2011; Robinson, 2007). For instance, tricyclic antidepressants (TCAs) exert its therapeutic effects through primarily blocking reuptake of 5-HT and NE. Moreover, monoamine oxidase inhibitors (MAOIs), which inhibit enzymes metabolising monoamine neurotransmitters and thus enhances neurotransmission of 5-HT, NE, and DA, are known to alleviate some depressive symptoms. At present, selective serotonin reuptake inhibitors (SSRIs), which inhibit 5-HT reuptake and thus elevate synaptic 5-HT levels, are considered to be the first-line antidepressants, while therapeutic efficacy of serotonin norepinephrine reuptake inhibitors (SNRIs), e.g. venlafaxine and duloxetine, as well as norepinephrine dopamine reuptake inhibitors (NDRIs), e.g. nomifensine and bupropion, are also acknowledged. The involvement of 5-HT in depression is further evidenced by the observation that dietary supplementation of 5-HT precursor, 5-hydroxy-L-tryptophan (5-HTP), works positively to alleviate depressive symptoms (reviewed in Turner et al., 2006), forming together with other findings the so-called “5-HT deficiency theory of depression”.

1.1.2. Depression and anxiety disorders

In humans, depression and anxiety disorders fall in different categories of psychiatric disorders under the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) as well as under the 10th revision of International Classification of Diseases (ICD10) definition. Depression and anxiety, however, share dimensionally overlapping symptoms such as sleep disturbance, appetite disturbance, irritability, fatigue, impaired concentration, oversensitivity to criticism, self-consciousness, dysphoria, feelings of rejection, as well as subjective experiences of nervousness, restlessness, and worry (Gorman, 1996; Ressler and Nemeroff, 2000). The difference between anxiety and depression is rather subjectively defined, whether the patient primarily has a depressed or anxious mood (Ressler and Nemeroff, 2000). Moreover, it has been suggested that depression may occur secondary to a generalized anxiety disorder (GAD) or panic disorder (Mongeau et al., 1997 and references therein). Indeed, SSRIs have been shown to exert their therapeutic effects on these two anxiety disorders, too (Hirschfeld, 2001), indicating that dysfunctional serotonergic system is involved in the pathogenesis of anxiety disorders (Gorman, 1996 and references therein). In this context, depression may be understood as a maladaptive defence mechanism of individuals when they experience unresolvable anxiety or aversive events, developing a false concept that they cannot control recurrent stress or obtain gratification (Mongeau et al., 1997). This model well explains high comorbidity of depression and anxiety. Based on a number of studies, it is generally established that approximately two thirds of depressive patients have a comorbid anxiety disorder whereas about one third or more of patients with GAD or panic disorder have comorbid depression (Gorman, 1996 and references therein). In addition to the serotonergic system, a series of experimental data suggest existence of other common biological substrates between anxiety and depression, such as the gamma-aminobutyric acid (GABA) neurotransmitter system (Paul, 1988; Kalueff and Nutt, 2007), the hypothalamic-pituitary-adrenal (HPA) axis and secretion of associated hormones (Gorman, 1996; Kalueff and Nutt, 2007 and references therein), the noradrenergic system (Weiss et al., 1994), brain metabolism associated with mitochondrial functions and brain derived neurotrophic factor (BDNF) (Burroughs and French, 2007). Familial studies also indicate common genetic predispositions between depression and anxiety; a higher incidence for developing anxiety disorders was reported among relatives of patients with major depression alone (Paul, 1988). Although there are still on-going debates whether depression and anxiety exist as separate, comorbid, or mixed symptoms (Gorman, 1996) and whether depression sequentially develops after anxiety disorders or *vice versa* (Moffitt et al., 2007), a multitude of pharmacological and

biological findings indicate that depression and anxiety disorders are closely related and that several neurotransmitters including 5-HT link these two psychiatric disorders.

1.1.3. Animal models of depression and anxiety disorders

When animals are exposed to uncontrollable aversive events, they exhibit behavioural deficits called "learned helplessness" which have certain resemblance to human depressive behaviours. Depression, however, is highly human-specific, implicating complex socio-economic constellations along with biological factors. Modelling depression in animals, thus, has a certain limitation. Anxiety-related traits, on the other hand, are considered to be more biologically ancient and can be observed in non-human primates as well as in rodents. Furthermore, while anxiety-like behaviours of animals are indicative of defensive adaptation to potential distant threats, such as potential attack from predators, animals develop fear responses against immediate inescapable threats such as electric shocks in experimental contexts. Similarly, human anxiety is defined as a diffuse, unpleasant, vague sense of apprehension and thus often objectless (Sadock and Sadock, 2000). Whereas fear is an emotional response to a known and immediate threat and has a clear object. Since fear and anxiety are closely related and depression can be seen as an exacerbated extension of anxiety disorders, investigation of fear- and anxiety-related behaviours of animals are believed to contribute to the understanding of human depression. Since the advent of transgenic techniques, animals with targeted deletion of genes encoding various mediators of neurotransmitters implicated in emotional regulation further enhanced our understanding of the contribution of such mediators to the development of human disorders of emotion regulation. Given that monoamine transporters, such as the serotonin transporter (5-HTT), the norepinephrine transporter (NET), and the dopamine transporter (DAT), are the major targets of antidepressant drugs (see section 1.1.1.), knockout mice lacking genes encoding these transporters were created as model organisms for investigating neural mechanisms of anxiety disorders and depression (Haenisch and Bonisch, 2011; Perona et al., 2008). Moreover, mice with disrupted genes of the central serotonin-synthesising enzyme, tryptophan hydroxylase-2 knockout (Tph2 KO) mice, have been generated as novel models for investigating human emotional disorders (Gutknecht et al., 2008; Savelieva et al., 2008; Alenina et al., 2009). The validity of such animal models is generally assessed in three aspects (Haenisch and Bonisch, 2011): face validity, i.e. animal models represent similar disease symptoms as in humans; predictive validity, i.e. pharmacological correlation between humans and animal models can be detected; construct validity, i.e. symptoms in animal models should be based on the same neurobiological mechanisms as in humans. Since the precise neurobiological mechanisms of

depression are yet to be elucidated, construct validity of animal models of depression is only of theoretical value. Despite this limitation, reductionistic approaches embodied by animal models of depression are expected to represent symptom characteristics, causative relationships, and treatment reactions of depression as seen in humans.

1.2. Serotonin and serotonergic neurons

1.2.1. Synthesis and metabolism

5-HT plays multifaceted physiological roles in both the peripheral and the central nervous system (CNS), including eating, gastrointestinal activity, reproductive activity, sensory processing, pain perception, motor activity, sleep, aggression, cognition, and mood. The diversity of serotonergic functions has been bolstered by the presence of more than 14 different 5-HT receptor subtypes (reviewed in Barnes and Sharp, 1999). 5-HT is stored in three main cell types (Sigma-RBI, 2006): serotonergic neurons in the CNS and in the intestinal myenteric plexus; enterochromaffin cells in the mucosa of the gastrointestinal tract; blood platelets. Among them, only serotonergic neurons and enterochromaffin cells can produce 5-HT themselves, while platelets uptake and store externally synthesised 5-HT. Biosynthesis of 5-HT is summarised in **Fig. 1.1.** First, an essential amino acid L-tryptophan (Trp), which can be found in such foods as banana, milk, eggs, cheese, and oats, is converted into 5-HTP by tryptophan hydroxylase (TPH), requiring O_2 and tetrahydrobiopterin (BH4) as cofactors. Synthesised 5-HTP is then converted to 5-HT by 5-hydroxy-tryptophan decarboxylase (and aromatic L-amino acid decarboxylase; AADC) (Carlsson et al., 1972) with pyridoxal phosphate as a cofactor. Of these, the catalysis mediated by TPH is the rate-limiting step. Two isoforms of TPH are known to exist: TPH1 in the periphery and the pineal gland; TPH2 in the CNS (see section 1.6.1.). 5-HT synthesised in the pineal gland serves as a precursor for the subsequent enzymatic pathway leading to the formation of the pineal hormone, melatonin. Produced 5-HT is then converted to 5-hydroxyindole acetaldehyde by monoamine oxidase (MAO), which can be found in two molecular subtypes, namely MAO-A and MAO-B. MAO-A has higher affinity for 5-HT than MAO-B and metabolise 5-HT with a much lower K_m value than MAO-B. Contradictorily, however, immunohistochemical studies have shown that serotonin-containing neurons themselves may contain only MAO-B. 5-hydroxyindole acetaldehyde is in turn metabolised mainly by an isoform of aldehyde dehydrogenase (ALDH2) located in mitochondria to 5-hydroxyindoleacetic acid (5-HIAA), which then is excreted into the urine (Sigma-RBI, 2006).

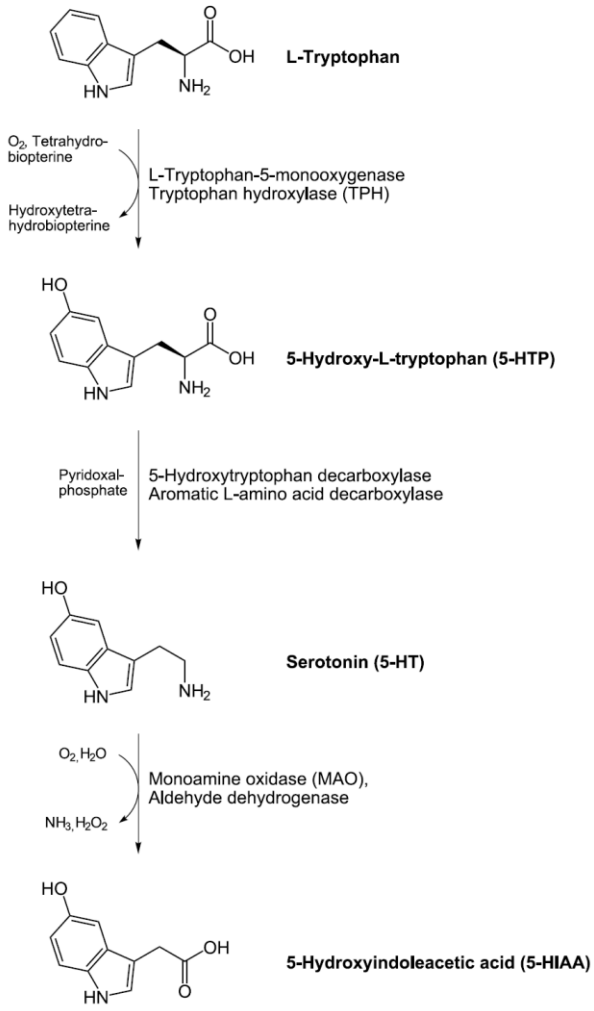

Fig. 1.1. Biosynthesis of serotonin. An amino acid L-tryptophan is converted into 5-hydroxy-L-tryptophan (5-HTP) by the rate limiting enzyme tryptophan hydroxylase (TPH). TPH requires tetrahydrobiopterin (BH4) as a cofactor. Produced 5-HTP is then converted to serotonin (5-HT) by 5-hydroxytryptophan decarboxylase and aromatic L-amino acid decarboxylase. 5-HT is metabolised to 5-hydroxyindoleacetic acid (5-HIAA) by monoamine oxidase (MAO). (Figure source: http://en.wikipedia.org/wiki/File:Serotonin_biosynthesis.svg)

1.2.2. Anatomy of brain serotonin system

In mammals, most serotonergic neurons originate from the raphe nuclei of the brainstem. **Fig. 1.2** illustrates the organisation of serotonergic neurons in the rat brain, where serotonergic neurons in the raphe nuclei are grouped into nine nuclei (B1-B9). B1-3 comprise the caudal group, consisting of the raphe pallidus nucleus (B1), the raphe obscurus nucleus (B2), and the raphe magnus nucleus (B3), and send descending projections to the medulla, cerebellum, and spinal cord to control motor activity, pain perception, and the autonomic nervous system. B7-9, on the other hand, form the rostral group, including the dorsal raphe nucleus (DRN; B7) and the medial raphe nucleus (MRN; B8). The rostral group of serotonergic neurons send ascending projections to the forebrain, including the cortex, amygdala, basal ganglia, hippocampus, thalamus and hypothalamus to control cognition, emotion, perception, as well as food intake, circadian rhythms, and reproduction (reviewed in Cools et al., 2008; Lesch et

al., 2012; Waider et al., 2011). B4-6 are an intermediate group and may send projections both into the ascending and descending groups.

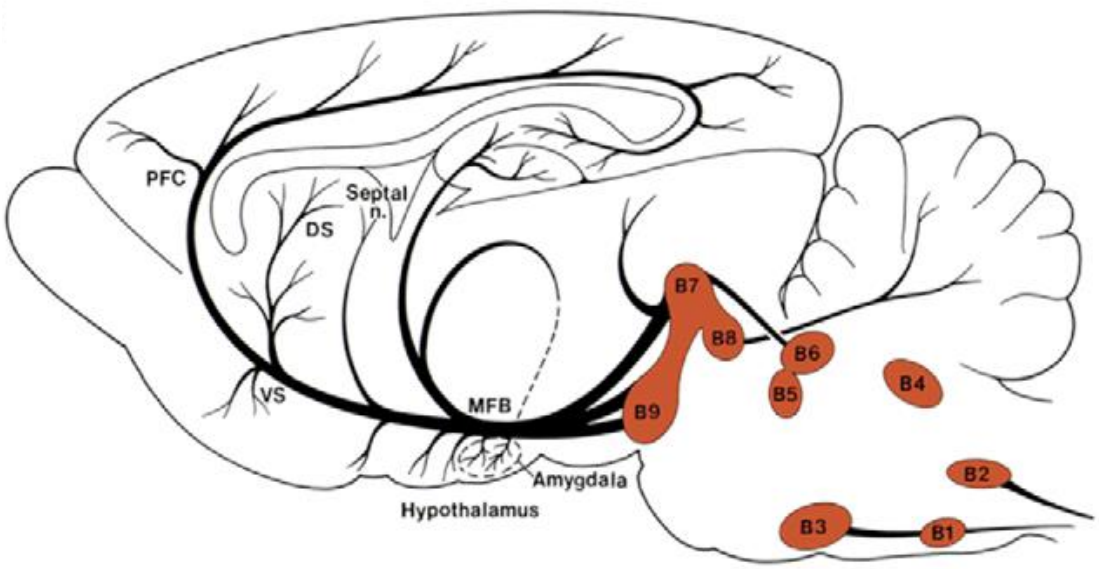

Fig. 1.2. Anatomy of serotonergic system in the rat brain. Bold branching lines show neuroanatomical projections. B7-B9: the rostral group of neurons in the upper midbrain which send ascending projections into the forebrain. B1-B3: the caudal group of neurons in the lower brainstem which send descending projections into the medulla and spinal cord. B4-B6: an intermediate group, which may have both ascending and descending projections. B7: the dorsal raphe nucleus (DRN). B8: the medial raphe nucleus (MRN). MFB: medial forebrain bundle. Septal n.: septal nucleus. DS: dorsal striatum. VS: ventral striatum. PFC: prefrontal cortex. (Figure source: Cools et al., 2008. Serotoninergic regulation of emotional and behavioural control processes. Trends in cognitive sciences, 12(1): 31)

The raphe nuclei, on the other hand, receives afferent inputs from the forebrain structures such as the prefrontal cortex and superior vestibular nuclei, as well as nucleus propositus of hypoglossy, nucleus of solitary tract, hypothalamus, amygdala, locus coeruleus (LC), lateral habenula, ventral tegmental area (VTA), and bed nucleus of stria terminalis (BNST) (reviewed in Gobbi, 2005). Moreover, intercellular connections within the DRN as well as reciprocal connections between the DRN and MRN also exist (reviewed in Hensler, 2006). Local connections between serotonergic neurons in the DRN bear particular importance in that firing of serotonergic neurons is controlled by somatodendritic 5-HT_{1A} autoreceptors, which are activated by released 5-HT. It should also be noted that not all the cells in the DRN are serotonergic, although they are the major type of cells in this nucleus. Neurons releasing glutamate, DA, GABA, nitric oxide and various neuropeptides (e.g., galanin, neuropeptide Y, thyrotropin-releasing hormone, somatostatin) are also known to be present along with

serotonergic neurons (Lesch and Waider, 2012). For further description of the anatomy of brain serotonergic system, see Araragi and Lesch (submitted).

1.2.3. Serotonergic neurons

i). Electrophysiological signature

One of the most prominent electrophysiological characteristics of serotonergic neurons in the dorsal raphe nucleus (DRN) is its slow (0.5-2.5 Hz) and regular firing rate with a long action potential width (0.8-1.2 ms) (Aghajanian et al., 1968 as in Gobbi, 2005). Furthermore, it has been reported that serotonergic neurons have high input resistance (150-400MΩ; Burlhis and Aghajanian, 1987) and large afterhyperpolarisation (AHP) (amplitude: 10-20 mV, duration: 200-800 ms; Vandermaelen and Aghajanian, 1983). It should be noted, however, that recent study revealed that these parameters are not statistically different between serotonergic and non-serotonergic neurons in the DRN (Kirby et al., 2003). In the study of Kirby and colleagues, input resistances of non-serotonergic neurons were somewhat higher than those of serotonergic neurons, and both fell within the range of input resistances of serotonergic neurons previously reported. Moreover, AHP amplitude and duration of non-serotonergic neurons also fell within the range of serotonergic neurons previously reported. It was proposed that the time constant of the initial phase of AHP and the membrane time constant are more reliable parameters to distinguish serotonergic and non-serotonergic neurons. On the other hand, Beck et al. (2004) reported that serotonergic neurons and non-serotonergic neurons are very different from each other in the MRN (see the previous section). Moreover, serotonergic neurons in the DRN and MRN are different in that serotonergic neurons in the MRN had a shorter membrane time constant and larger AHP amplitude than serotonergic neurons in the DRN. While, in the DRN, both serotonergic and non-serotonergic neurons responded to 5-HT_{1A} receptor agonists, non-serotonergic neurons in the MRN did not respond to 5-HT_{1A} receptor stimulation. The response of serotonergic neurons in the DRN to 5-HT_{1A} receptor stimulation was larger in its efficacy compared to serotonergic neurons in the MRN, implying that autoinhibitory mechanisms (see section 1.2.3.iii).) of serotonergic neuron firing is more robust in the DRN (Beck et al., 2004)

ii). Spontaneous firing

The slow rhythmic pacemaker activity of serotonergic neurons is generated by interaction between a calcium-activated potassium current ($I_{K(Ca^{2+})}$), low-threshold Ca^{2+} conductance, and

a voltage-dependent early transient outward K^+ current (I_A). (Burlhis and Aghajanian, 1987). Both low-threshold Ca^{2+} conductance and I_A are inactive at rest and can be relieved from inactivation (i.e. de-inactivated) with membrane hyperpolarisation. During the action potential, Ca^{2+} enters neurons through high-threshold Ca^{2+} channels and activates outward $I_{K(Ca^{2+})}$ current, generating a large AHP, which is characteristic of serotonergic neurons (Aghajanian, 1985; Vandermaelen and Aghajanian, 1983). AHP then de-inactivates both low-threshold Ca^{2+} current and I_A. Due to the sequestration and extrusion of intracellular Ca^{2+}, AHP diminishes gradually, bringing the membrane potential back to the appropriate voltage rage (-60 to -58 mV) to activate both currents, which compete with each other, i.e. I_A tends to slow the rate of depolarisation, the low-threshold Ca^{2+} conductance tends to facilitate it. Under normal physiological conditions, the low-threshold Ca^{2+} conductance is more dominant in this competition, generating a slow depolarising voltage ramp until it reaches the threshold of the next action potential initiation (Burlhis and Aghajanian, 1987 and references therein).

It is known that, in living animals, the spontaneous activity of serotonergic neurons depends on tonic noradrenergic input (Baraban and Aghajanian, 1980a; Baraban and Aghajanian, 1980b as in Burlhis and Aghajanian, 1987). However, the spontaneous rhythmic activity itself is intrinsic since such an activity can be observed *in vitro* as well, although the pacemaker activity can be greatly enhanced by application of α_1-adrenoceptor agonist, such as phenylephrine (Burlhis and Aghajanian, 1987 and references therein). Such a facilitating effect of noradrenergic inputs is mediated by the suppression of I_A current and reduction of a resting K^+ conductance (Aghajanian, 1985 as in Burlhis and Aghajanian, 1987), which bring the membrane potential to the action potential threshold. Evidence suggests that most of the noradrenergic inputs to DRN derive not from the LC but from nuclei outside the LC (Levine and Jacobs, 1992 and references therein).

Spontaneous firing activity of serotonergic neurons in known to be regulated under the sleep-wake cycle. Namely, extracellular electrophysiological recordings in freely moving cats have demonstrated that serotonergic neurons in the DRN fire tonically during wakefulness, decrease its firing rate during slow-wave sleep (SWS) and nearly stops its activity during the rapid-eye-movement (REM) sleep (Trulson and Jacobs, 1979). It is assumed that GABA exerts its inhibitory effects on serotonergic neuron firing during sleep although its precise mechanism is still under investigation. Studies in the awake cat by Levine and Jacobs (1992) reported that microiontophoretic application of the $GABA_A$ antagonist bicuculline into the DRN reversed suppression of firing during SWS while it did not generally affect suppression

of firing during the REM sleep, neither had any effect on the spontaneous firing during wakefulness. Moreover, the same study revealed that further application of norepinephrine or phenylephrine during wakefulness does not alter the spontaneous firing, indicating that the noradrenergic input in living animals already exerts its maximum facilitative effects during wakefulness. On the contrary, in studies by Gervasoni et al. (2000) in the awake rat, bicuculline levelled suppression of serotonergic firing during SWS as well as the REM sleep and increased firing rates during quiet waking, too. Findings from a more recent study by Brown et al. (2008) suggest that GABAergic neurons in the subcoeruleus and a subset of GABAergic neurons in the pontine nucleus oralis are excited by cholinergic inputs, which then suppress activity of serotonergic neuorns in the DRN through inhibitory projections during the REM sleep.

Measuring spontaneous firing activity of dorsal raphe serotonergic neurons bears importance from the following reasons. First, firing activity corresponds to the amount of 5-HT release and thus affects activity of target cells (Jacobs and Azmitia, 1992 and references therein). Second, several classes of antidepressant drugs (e.g. SSRIs, TCAs, MAOIs, and bupropion) and drugs of abuse as well as neurotoxins (e.g. 3,4-methylenedioxymethamphetamine [MDMA], lysergic acid diethylamide [LSD], amphetamine, and cocaine) modulates firing rate (for review; Gobbi, 2005). Third, several genetically modified animal models of emotional disorders, such as 5-Htt KO mice, show an altered baseline firing rate (Gobbi et al., 2001; Lira et al., 2003). Importantly, a series of studies done by Gobbi and colleagues demonstrated a strong correlation between serotonergic neuron firing activity and the effect of acute and chronic antidepressant administration (Gobbi, 2005 and references therein).

iii). Autoinhibition of serotonergic neuron firing

Activity of serotonergic neurons is limited by a homeostatic control, which is mediated by negative feedback exerted by extracellular 5-HT via somatodendritic inhibitory 5-HT_{1A} autoreceptors of serotonergic neurons (Audero et al., 2008 and references therein). The role of 5-HT_{1A} receptors in suppression/regulation of serotonergic neuron firing activity is considered to be important in the pathophysiology of emotional disorders (Pineyro and Blier, 1999; Sharp et al., 2007). The importance of 5-HT_{1A} receptor function is further bolstered by the proposed mechanism of antidepressant drugs such as SSRI and MAOIs (Pineyro and Blier, 1999; Blier and de Montigny, 1998; Blier et al., 1998). After acute administration of such antidepressant drugs, extracellular 5-HT contents transiently increase. Therapeutic doses of SSRIs are known

to decrease 5-HTT function as much as 60-80% (Stein et al., 2006). Extracellular excess of 5-HT, in turn, activates somatodendritic 5-HT_{1A} receptors on serotonergic neurons, leading to a decrease in serotonergic neuron firing activity. After chronic administration of antidepressant drugs, these 5-HT_{1A} receptors are desensitised, resulting in the net increase of 5-HT levels. This mechanism explains why antidepressant drugs start to exert their therapeutic effects with a certain time lag. Further evidence supporting the notion that increased 5-HT neurotransmission mediates therapeutic effects of antidepressants comes from the fact that administration of para-chlorophenylalanine (PCPA), a 5-HT synthesis inhibitor, caused relapse of depression in patients who had responded to antidepressants (Mongeau et al., 1997 and references therein).

In this context, dysfunction of autoinhibitory 5-HT_{1A} receptors has been proposed as a potential factor contributing to the pathogenesis of emotional disorders. However, reports on the expression level of 5-HT_{1A} receptors in depressed patients measured by positron emission tomography (PET) or in postmortem brains of depressed patients have not reached consensus: Some reported decreased expression (Arango et al., 2001; Drevets et al., 1999; Meltzer et al., 2004; Bhagwagar et al., 2004), while some others reported enhanced expression (Stockmeier et al., 1998) or no difference compared to controls (Stockmeier et al., 1997; Lowther et al., 1997; Parsey et al., 2006). Moreover, PET imaging data revealed reduced 5-HT_{1A} binding in several brain regions including the raphe nucleus in panic disorder patients either with or without comorbid depression (Neumeister et al., 2004). To date, most studies concentrated on association between expression levels of 5-HT_{1A} receptors with depressive disorders and there has been no direct evidence demonstrating how altered 5-HT_{1A} receptor levels translate to magnitude of autoinhibition. The discrepancies among literature describing relationship between 5-HT_{1A} receptor expression levels and depression imply the need for understanding precise mechanisms linking 5-HT_{1A} receptor levels with autoinhibition.

iv). Serotonin release

As reviewed by Zoli et al. (1998), it has been suggested that interneuronal communication can be categorised into two different types; wiring transmission and volume transmission (VT; also termed paracrine transmission). Wiring transmission occurs in a spatially constrained cellular chain (wire), i.e. across the synaptic clefts. On the other hand, VT is three-dimensional diffusion of neurotransmitters in the extracellular fluid beyond the realm of synaptic contacts (Zoli et al., 1998). Using carbon fibre microelectrodes, Bunin and

Wightman (1998) demonstrated that 5-HT release in the substantia nigra reticulate and the DRN is primarily of the VT type. This is also in agreement with anatomical findings that 5-HT uptake sites are not synaptically localised but rather are widely distributed across the serotonergic neurons so that they can optimally control the extracellular 5-HT levels (Bunin and Wightman, 1998 and references therein). Further in accordance with the VT concept, the DRN is known to display little synaptic specialisation (Bunin and Wightman, 1998). Thus, VT allows 5-HT to interact with many extrasynaptic elements.

The VT type of 5-HT release has important consequences in the mechanism of autoinhibition. Namely, if the autoinhibition is regulated by pulsatile release of vesicular 5-HT, as is seen in the wiring transmission mode of neurotransmission, serotonergic neuronal firing becomes erratic, while in fact the firing of serotonergic neurons is very regular. The paracrine type of 5-HT release, on the other hand, creates homogeneous concentration of extracellular 5-HT, enabling tonic autoinhibition, leading to the typical regular firing of serotonergic neurons.

v). Mechanism of 5-HT_{1A} receptor action

When activated by their ligands, 5-HT_{1A} receptors stimulate the opening of Kir.3 K^+ channels, also known as voltage-dependent G-protein-coupled inwardly rectifying K^+ (GIRK) channels, via pertussis toxin-sensitive G-proteins ($G_{i/o}$) both in the DRN (Innis and Aghajanian, 1987; Colmers and Williams, 1988; Innis et al., 1988; Williams et al., 1988; as in Mannoury la Cour et al., 2004) and in CA1 pyramidal cells of the hippocampus (Andrade et al., 1986). Subsequent outflow of K^+ ions from the intracellular to the extracellular space hyperpolarises the neuron. This inhibitory effects on neuronal excitability is complemented by inhibition of N- and P/Q-type Ca^{2+} channels (Millan et al., 2008). Although 5-HT_{1A} receptors are known to be negatively coupled to adenylyl cyclase via α-subunit of $G_{i/o,}$ the level of cAMP apparently does not affect opening of GIRK channels. This is because neither intracellular injection of cAMP nor bath application of 8-Br-cAMP, the membrane-soluble analogue of cAMP, reduces the hyperpolarising effect of 5-HT_{1A} receptor activation (Aghajanian, 2000). On the contrary, evidence suggests that the opening of GIRK channels by 5-HT_{1A} receptor activation is mediated through the interaction between G-protein βγ-subunits of $G_{i/o}$ (Doupnik et al., 1996, 1997 as in Raymond et al., 2001).

1.2.4. The DRN and emotion regulation

The DRN is seen as a structure playing a critical role in the modulation of emotion. This is indicated by its unique anatomical connections with limbic and prefrontal cortical structures. Graeff et al. (1996) proposed an integrative theory accounting for two different roles of 5-HT in the regulation of emotion, namely anxiety and unconditioned fear such as fight/flight escape response. In their theory, serotonergic innervation from DRN to the amygdala facilitates anxiety to potential threat via postsynaptic 5-$HT_{2A/2C}$ and 5-HT_3 receptors, whereas afferents from DRN to the periaqueductal grey (PAG) suppress panic-like fight/flight behaviours in response to immediate threat via postsynaptic 5-$HT_{2A/2C}$ and 5-HT_{1A} receptors. On the contrary, afferents from the MRN to the hippocampus is hypothesised to help organisms to cope with chronic stress by disconnecting impacts of aversive events from psychobiological processes underlying appetitive and social behaviours by activating postsynaptic 5-HT_{1A} receptors.

Serotonergic innervation to the amygdala attracts particular attention because the amygdala has been repeatedly implicated in literature describing neural mechanisms of anxiety and fear responses. Anatomically, the amygdala comprises three major nuclei, the lateral nucleus (LA), the basolateral nucleus (BLA), and the central nucleus (CeA). It is understood that LA serves as an input station for sensory stimuli, BLA relays the information, and the CeA sends projections to effector structures mediating fear responses, such as PAG for panic response or freezing (Kim and Jung, 2006). Serotonergic regulation of amygdaloid activity is supported by a number of experimental observations. In anatomical terms, both pyramidal neurons and interneurons in the BLA receive intense serotonergic innervation. Immunohistochemical studies also demonstrated expression of 5-HT_{1A} receptors on pyramidal cells and of 5-HT_{2A} and 5-HT_{3A} receptors on interneurons of the basolateral complex (Muller et al., 2007; Mascagni and McDonald, 2007; McDonald and Mascagni, 2007). This is in correspondence with a previous electrophysiological study by Stutzmann and LeDoux (1999) showing that application of 5-HT activates GABAergic interneurons in the LA through 5-HT_2 and 5-HT_3 receptors, which then inhibits pyramidal neurons activated by glutamatergic innervation from the sensory afferents. In a clinical setting, it was demonstrated in an imaging study, that the amygdala of patients with anxiety or mood disorders was overactive, whereas the chronic SSRI treatment, which is supposed to enhance serotonergic neurotransmission, normalised the amygdala activity along with amelioration of clinical symptoms (Muller et al., 2007).

Not only sending projections to other brain areas, the DRN is known to receive inputs from other brain areas. Particularly, the DRN receives virtually all of its cortical inputs from the infralimbic (IL) and the prelimbic (PL) areas of the ventral medial prefrontal cortex (mPFCv). Amat et al. (2005) reported that this mPFCv imput sends information to the DRN whether a stressor to the organism is controllable or not. When a stressor is controllable, mPFCv imput suppresses stress-induced activation of the DRN and subsequent behavioural consequences of uncontrollable stress.

Finally, a retrograde staining study revealed a unique afferent input into the DRN originating from the BNST, a structure implicated in the mediation of anxiety-related behaviours (Lowry et al., 2005). Evidence suggests that BNST exerts its influence on DRN through corticotrophin-releasing hormone (CRH) (Hammack et al., 2003; Davis et al., 2010). Conversely, serotonergic neurons in the DRN also send projections to the BNST and modulate anxiety (Robinson et al., 2012). It has been proposed that the BNST receives excitatory CRH release from the amygdala in response to aversive stimuli and express anxious behaviours. By activating 5-HT_{1A} receptors, serotonergic inputs from the DRN suppress this excitatory effect of CRH on BNST (Robinson et al., 2012; Hammack et al., 2009). Importantly, this DRN control on BNST is, in turn, controlled by top-down regulation from medial prefrontal cortex (mPFC) on the DRN, as mentioned earlier.

Taken together, multiple lines of evidence indicate elaborated interaction between the DRN and other limbic and cortical structures in mediating anxiety and fear. This interaction is mediated by several different types of excitatory and inhibitory 5-HT receptors. The DRN and other structures are often connected in a hierarchical manner, i.e. the DRN receives commands from some other structures such as mPFC and then send afferents to other subordinate structures which are more directly related to the expression of fear and anxiety.

1.3. Serotonin transporter (5-HTT) and its association with emotional disorders

The 5-HTT is a plasma membrane protein, which reuptakes 5-HT from the extracellular space into the serotonergic neurons by coupling the transport of 5-HT to the electrochemical gradients for Na^+ and Cl^-. The 5-HTT has been implicated in emotional regulation based on three lines of evidence: i) Several classes of antidepressants inhibit the 5-HTT, such as SSRI, and TCA; ii) it is also a target of drugs of abuse such as MDMA and cocaine; iii) genetic polymorphisms which lead to variability in 5-HTT expression and function has been

associated with anxiety, depression, suicidal behaviour, substance abuse, irritable bowel syndrome, autism, obsessive-compulsive disorder, and schizophrenia (Quick, 2003; Canli and Lesch, 2007; Murphy and Lesch, 2008). With regard to the last point, the human 5-HTT protein is known to be encoded by a single gene, SLC6A4, whose transcriptional activity is modulated by several gene variants including a repetitive sequence termed 5-HTT linked polymorphic region (5-HTTLPR; reviewed in Canli and Lesch, 2007). The short variant (s-allele) of the 5-HTTLPR is known to lead to less expression of 5-HTT compared to the long variant (l-allele), resulting in an increased level of 5-HT in the synaptic cleft. At the phenotypic level, s-allele has been associated with depression (Collier et al., 1996) and anxiety-related personality traits (Lesch et al., 1996). Moreover, it was found that s-carriers show higher rates of a particular type of anxiety disorders, namely post-traumatic stress disorder (PTSD) (Lee et al., 2005a). Functional magnetic resonance imaging (fMRI) study by Hariri and colleagues provided direct evidence that s-carriers have higher amygdala activity in response to fearful facial stimuli in comparison to non-carriers (Hariri et al., 2002). In addition, structural MRI revealed reduced grey matter volume in s-allele carriers in the perigenual anterior cingulate cortex (pACC) and the amygdala, limbic regions involved in the processing of negative emotion (Pezawas et al., 2005). The same group, using fMRI, further demonstrated functional coupling of pACC and the amygdala during perceptual processing of fearful stimuli. Importantly, s-allele carriers were shown to have weaker coupling of these structures, which may lead to dysregulation of the amygdala in response to fearful stimuli (Pezawas et al., 2005; reviewed in Canli and Lesch, 2007). Subsequently, Canli and associates (Canli et al., 2005) provided evidence that the increased amygdala activation in s-carriers in response to negative stimuli, as reported earlier, were in fact due to the decreased activation to neutral stimuli which were used as a control in earlier studies. Overall, influence of 5-HTTLPR polymorphism on brain structure and function, especially emotion regulation and cognition, seems even more complicated than initially perceived.

S-allele of 5-HTTLP has been shown to influence learning abilities as well (Borg et al., 2009 and references therein). For example, carriers of the s-allele were shown to have enhanced acquisition in fear-conditioning tasks (Garpenstrand et al., 2001). Interestingly s-carriers were shown to perform better than l-carriers in some cognitive tasks such as sustained attention task (Strobel et al., 2007), risky decision-making and a visual-planning task (Roiser et al., 2006), and episodic memory and attention tests (Roiser et al., 2007).

It should be noted that genetic variability in 5-HTTLPR does not directly determine the onset of a particular affective disorder. Rather, it represents an individual's predisposition which

influences its reactivity to certain aversive environmental factors. Studies done in primates demonstrated an influence of rh5-HTTLPR (rhesus monkey 5-HTTLPR; orthologous to the human 5-HTTLPR) and early life stress (e.g. maternal separation) on variation in serotonergic function, which led to emotion dysregulation, increased stress reactivity, social incompetence, aggression, increased alcohol consumption observed in their later life (reviewed in Canli and Lesch, 2007). Similarly, in human cohorts, the number of stressful life events (childhood maltreatment, socioeconomic factors, personal relationship, health status, employment stressors) was more strongly correlated with major depression episodes and suicide ideation/attempts in s-carriers than l-carriers of 5-HTTLPR (Caspi et al., 2003).

1.4. Serotonin transporter knockout (5-Htt KO) mice as a model for depression-related emotionality

1.4.1. Characteristics of 5-Htt KO mice

Given the importance and complexity of 5-HTTLPR functions in humans mentioned above, mice with reduced or completely absent 5-Htt protein were generated by targeted disruption of murine *5-htt* gene (Slc6a4) (Bengel et al., 1998) to better understand functions of 5-HTT. Mice with complete lack of 5-Htt protein (5-Htt KO mice) have 60-80 % reduction in brain serotonin content (Bengel et al., 1998; Kim et al., 2005), although 5-HT synthesis itself is 30-60 % increased in different brain regions, reflecting an inadequate compensatory mechanism (Kim et al., 2005). In contrast, the extracellular 5-HT concentrations at baseline are ~3-fold and ~6-fold elevated in 5-Htt heterozygous (Het) and KO mice, respectively (Fabre et al., 2000; Mathews et al., 2004; Shen et al., 2004, as in Fox et al., 2010). 5-HT clearance rate was shown to be decreased in Het mice and more markedly so in KO mice, as expected from the decreased expression and complete absence of 5-Htt, respectively (Montanez et al., 2003). Moreover, the total number of serotonergic neurons in the DRN appears to be reduced (Lira et al., 2003; Rumajogee et al., 2004).

5-Htt KO mice exhibit more than 50 different phenotypic alterations (reviewed in Murphy and Lesch, 2008) including gastrointestinal dysfunction, skeletal fragility, change in somatosensory cortex (whisker barrels), hypothermia (Li et al., 1999, 2000; Bouali et al., 2003), sleep disturbance and late-onset obesity driven by metabolic syndrome (Erritzoe et al., 2010; Uceyler et al., 2010; as in Gutknecht et al., 2012), and physiological responses to stressors (Lanfumey et al., 2000; Li et al., 1999; Tjurmina et al., 2002;Tjurmina et al., 2004).

At the behavioural level, 5-Htt KO mice are known to exhibit increased anxiety-like behaviour and diminished exploratory locomotion, manifested in behavioural paradigms such as elevated plus maze (EPM), light-dark exploration/emergence, and open field (OF) test (Holmes et al., 2003). In contrast, transgenic mice overexpressing 5-Htt exhibited less anxiety behaviours in EPM and hyponeophagia tests (Jennings et al., 2006). The low anxiety observed in the EPM test was reversed by acute administration of the SSRI paroxetine. The finding further corroborates the link between the low expressing variant of the 5-HTTLPR with anxious phenotypes. Additionally, 5-Htt KO mice are known to burry fewer marbles than their wildtype (WT) counterparts, a behavioural test often used as an indication of obsessive-compulsive traits (Zhao et al., 2006).

Moreover, depression-like behaviours of 5-Htt KO mice have been reported, reflected as increased immobility time in paradigms such as forced swim test (FST) (Wellman et al., 2007; Renoir et al., 2008) and tail suspension (TS) test (Zhao et al., 2006; Lira et al., 2003). Since immobility represents behavioural despair in these experimental paradigms, it is interpreted that KO mice have higher tendency toward the "depression-like" behavioural trait. However, the opposite result, i.e. reduction in immobility, was also reported in TS test (Renoir et al., 2008), possibly due to the difference in genetic background (i.e. 129 or C57BL/6Jx129S6 mixed background vs. C57BL/6J. See also discussion in Savelieva et al., 2008).

5-Htt KO mice are also known to feature alterations in their learning abilities. For example, Wellman et al. (2007) showed that KO mice had impaired extinction recall in fear extinction learning although they had normal fear conditioning and extinction capabilities compared to WT counterparts. Since the deficit in acquiring or retaining extinction memory is a defining feature of anxiety disorders in humans, the result from this behavioural test suggests that genetic loss of 5-HTT function may be a risk factor for this disorder. In fact, it was found that individuals carrying the low-expressing variant 5-HTTLPR show higher rates of a particular type of anxiety disorders which has close relevance to extinguishing negative memories, namely PTSD (Lee et al., 2005a; Wellman et al., 2007).

On the other hand, 5-Htt KO mice exhibit improvements in certain domains of learning which require cognitive flexibility such as reversal learning, where mice have to switch from responding to a previously rewarded stimulus to a previously unrewarded stimulus in the same stimulus dimension. Brigman et al. (2010) showed that both constitutive 5-Htt null mutants and mice treated with chronic fluoxetine made fewer errors in reversal learning than

control animals. On the other hand, constitutive 5-HT depletion by Pet1 KO or acute 5-HT depletion by PCPA did not change reversal learning efficiency compared to control mice. A probabilistic reversal learning test in 5-Htt KO mice also revealed that 5-HT modulates processes mediating reward-stay and punishment-shift behaviour (Ineichen et al., 2012). The findings suggests involvement of the serotonergic system in cognitive flexibility and bears importance since growing evidence indicates that deficient cognitive flexibility prevails among several neuropsychiatric disorders including depression (Cools et al., 2008).

Importantly, evidence suggests that various behavioural changes in 5-Htt KO mice as compared to WT mice could be, to some extent, traced down to cellular and molecular levels. Indeed, increased depressive-like behaviours and impaired fear extinction recall in 5-Htt KO mice were accompanied by morphological changes in key limbic structures such as the IL and the BLA (Wellman et al., 2007). Specifically, they found that the apical dendritic branches of Golgi-stained pyramidal neurons in the IL of 5-Htt KO mice were significantly increased in length. Moreover, pyramidal neurons in the BLA had significantly greater spine density in 5-Htt KO mice, although their dendritic morphology was not different from that of WT counterparts. Nietzer et al. (2011) also reported a spine density increase in several dendritic compartments of pyramidal neurons in the BLA and LA. In addition, they found that, in general, the dendritic morphology of the cingulate cortex (CG), CA1 pyramidal neurons, and of amygdaloid interneurons does not differ between genotypes. In contrast to the findings of Wellman et al. (2007), however, they reported that pyramidal neurons in the IL tended to have shorter and less spinous distal apical dendrites in KO mice. The authors speculated that this discrepancy could be due to the behavioural paradigms used prior to sacrificing of animals and the time period elapsed after the cessation of such behavioural paradigms.

It is beyond the scope of this section to cover all the behavioural changes of 5-Htt KO mice, which include reduced aggression (Holmes et al., 2002), reduced locomotion and resultant late-onset obesity (Uceyler et al., 2010), serotonin syndrome-like behaviours (Kalueff et al., 2007b), and behavioural responses to various stress paradigms, e.g. mild predator stress (Adamec et al., 2006); mild early life stress (Carroll et al., 2007); social defeat (Jansen et al., 2010); low maternal care (Carola et al., 2008). For further description of behavioural changes in 5-Htt KO mice, refer to more comprehensive reviews (Murphy and Lesch, 2008; Haenisch and Bonisch, 2011). Pharmacological studies done on 5-Htt KO mice, especially effects of psychoactive drugs working on the serotonergic system, are described in Araragi and Lesch (submitted).

1.4.2. Electrophysiology

i). *In vitro* electrophysiology

Using extracellular recording, Mannoury la Cour et al. (2001) demonstrated that the potency of 5-HT_{1A} receptor partial agonists ipsapirone and 5-carboxamidotryptamine (5-CT) in inhibiting the discharge of serotonergic neurons in the DRN was markedly decreased in 5-Htt KO compared to WT mice. Similarly, their intracellular recording data showed that the potency of 5-CT to hyperpolarise serotonergic neurons in the DRN was significantly lower in 5-Htt KO than in 5-Htt WT animals. On the contrary, 5-CT was equipotent to hyperpolarise CA1 pyramidal neurons of the hippocampus in both mutant and wildtype mice. In line with this, there were no significant differences between WT and KO in the basic membrane properties of CA1 pyramidal neurons, such as the resting membrane potential and the input resistance. Based on these results, the authors concluded that 5-Htt knockout induced a marked desensitisation of 5-HT_{1A} autoreceptors in the DRN without altering postsynaptic 5-HT_{1A} receptor function in the hippocampus. Loucif et al. (2006) showed enhanced 5-HT_{1A} receptor desensitisation in the DRN of female in comparison to male mice in their patch clamp experiments. In detail, they demonstrated that the 5-Htt KO mutation reduced the GIRK current density induced by Gpp(NH)p (a nonhydrolysable GTP analogue) in females but not in males. Since $5HT_{1A}$ receptors are coupled to GIRK channels via G-proteins, the data suggest that the decreased response of 5-HT_{1A} receptors to their agonists in KO mice is likely to be mediated by alteration in this G-protein coupling in females but not in males.

Immunohistochemical investigations also support this brain region specific desensitisation of $5HT_{1A}$ receptors. Namely, the number of somatodendritic $5HT_{1A}$ autoreceptors in the brainstem raphe is reduced (>60%) in 5-Htt KO mice with slightly greater reduction in females compared to males and intermediate reduction in Het mice. On the other hand, postsynaptic 5-HT_{1A} receptors are partially reduced only in females in the hypothalamus, septum, and amygdala. By contrast, no changes were observed in the frontal cortex or hippocampus (Fabre et al., 2000; Mannoury la Cour et al., 2001; Li et al., 1999, 2000; Murphy and Lesch, 2008 and references therein).

Since both 5-HT_{1A} receptors and $GABA_B$ receptors activate the GIRK current, it has been postulated that both receptor types share a common pool of G-proteins, and that alterations in the homeostasis of the serotonergic system may therefore influence $GABA_B$ receptor functions. *In vitro* brain slice recording showed that the $GABA_B$ receptor agonist baclofen

induced a lower hyperpolarisation and inhibition of neuronal firing of DRN serotonergic cells in 5-Htt KO mice compared to their WT counterparts. On the other hand, $GABA_B$ receptors in the hippocampus are functionally identical both in KO and WT mice (Mannoury la Cour et al., 2004). This brain-region specific desensitisation of $GABA_B$ receptors in 5-Htt KO mice exhibits close resemblance to that of $5HT_{1A}$ receptors (Mannoury la Cour et al., 2001), further supporting the existence of common downstream pathways between these two receptor types.

ii). *In vivo* electrophysiology

Gobbi et al. (2001) reported that the spontaneous firing rate of serotonergic neurons in the DRN was significantly decreased by 66% in 5-Htt KO and 36% in Het mice, compared to WT controls. Lira et al. (2003) also reported about four-fold reduction of spontaneous firing rate which was accompanied by 50% reduction of the serotonergic cell number in the DRN. It should be noted that such a decrease in the baseline firing rate of DRN serotonergic neurons was not observed in *in vitro* conditions (Mannoury la Cour et al., 2001, 2004; Renoir et al., 2008). Moreover, Gobbi et al. (2001) showed desensitisation of 5-HT_{1A} receptor in response to a 5-HT_{1A}/5-HT_7 agonist, 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) both in KO and Het mice in the DRN. In addition, the time required for hippocampal CA3 pyramidal neurons to return to the baseline firing rate after 5-HT iontophoretic application was significantly increased. Most likely, this is due to the deficiency of rapid clearance of 5-HT from extracellular fluid, as has been demonstrated using high-speed chronoamperometry (Montanez et al., 2003). Gobbi et al. (2001) also demonstrated that hippocampal CA3 neurons in KO mice showed an attenuated response to 8-OH-DPAT, but not to 5-HT itself. Their finding that 5-HT_{1A} receptor is desensitised also in the hippocampus is in contrast to the finding of Mannoury la Cour and coworkers (2001). Although there are some differences in the responsiveness of CA1 and CA3 pyramidal neurons to 5-HT_{1A} receptor agonists (Beck et al., 1992 as in Gobbi et al., 2001), no significant differences were found in another *in vivo* electrophysiological approach (Blier et al., 1993 as in Gobbi et al., 2001). For this reason, Gobbi et al. (2001) speculated that the major reason for the discrepancy is that *in vitro* methodology as was adopted by Mannoury la Cour et at. (2001) is generally less sensitive to detect moderate-to-small alteration in receptor responsiveness. For example, micromolar concentrations of 5-HT agonists are usually required to cause membrane hyperpolarisation of several milivolts *in vitro*, whereas *in vivo* microiontophoretic approach applies a few nanoamperes to a mililimorlar solution to eject agonists.

Similar to the *in vitro* work by Loucif et al. (2006), an *in vivo* study by Bouali et al. (2003) demonstrated enhanced desensitisation of $5HT_{1A}$ receptors in response to 8-OH-DPAT in the DRN of 5-Htt female KO mice (CD-1 strain) compared to their male counterparts. By applying gonadectomy, they showed that this gender difference is mediated by sex hormones; namely, castrated 5-Htt KO males showed a downregulation, and ovariectomised females an upregulation of these receptors, which are restored by administering testosterone or estradiol to gonadectomised males or females, respectively.

Regarding the desensitisation of $5\text{-}HT_{1A}$ receptors, detailed analyses of alteration in G-protein coupling in 5-Htt KO mice have been conducted. For further details see Araragi and Lesch (submitted).

Recent *in vivo* recording on behaving 5-Htt KO mice investigated impact of social defeat on fear extinction learning and on theta synchronisation between the IL cortex and the LA (Narayanan et al., 2011). It was found that naïve 5-Htt KO mice as well as socially defeated 5-Htt Het and KO mice had impairment in fear extinction recall. Moreover, socially defeated 5-Htt Het and KO mice showed a delay in fear extinction learning. Interestingly, both impaired extinction learning and extinction recall were accompanied by augmentation in the IL-LA theta synchronisation. The result indicates that 5-HT modulates fear extinction learning and consolidation of fear extinction via influencing the theta coupling between the IL cortex and LA activities. The result further implies that such interaction is influenced by environmental stressors.

1.5. Hippocampus and depression

1.5.1. Memory and depression

A number of experimental findings demonstrated that aversive stimuli, such as foot shocks, which are known to induce fearful memories, alter activity-dependent hippocampal synaptic plasticity in rodents (Sarkisyan and Hedlund, 2009 and references therein). Anxiety disorders and depression then involve consolidation of fearful memories. When organisms experience unresolvable or inescapable stressful events, they develop so-called “learned helplessness”. It is generally accepted that memory functions play a major role in the formation of learned helplessness. The hippocampus, on the other hand, is known to integrate information about space, time and context and thus plays a crucial role for declarative learning and memory (reviewed in Millan et al., 2012). The so-called “Cognitive-Learning Theory of affective

disorders" links the concept of learned helplessness to depression (Mongeau et al., 1997). Several hormones and neurotransmitters are known to modulate memory formation. While stress hormones such as adrenal corticosteroids are proposed to facilitate consolidation of stressful memories, 5-HT counteracts the consolidation (Graeff et al., 1996). For example, application of selective 5-HT_{1A} receptor agonists, both systemic and in the dorsal hippocampus, is known to disrupt spatial learning in a water maze or impairs performance in a passive avoidance task (Graeff et al., 1996 and references therein). Moreover, application of buspirone or lesion of MRN is shown to interfere with the electrical activity of the hippocampus (Graeff et al., 1996). Based on these findings, it has been proposed that the MRN-dorsal hippocampus 5-HT neurotransmission promotes resilience to chronic, unavoidable stress through the activation of hippocampal 5-HT_{1A} receptors, disengaging behavioural memories that could lead to aversive outcomes. In this way, increased 5-HT transmission in the hippocampus leads to tolerance to chronic aversive events, preventing the onset of depressive reactions against them (Graeff et al., 1996). According to this model, depression occurs when this coping mechanism fails.

In rodent studies, it was demonstrated that 5-Htt deficiency in rats leads to impairment in object memory. Since the s-allele of 5-HTTLPR is associated with developmental structural changes in the hippocampus and the object memory is mediated by the hippocampus (Olivier et al., 2009 and references therein), it may be postulated that lower 5-HTT function predisposes individuals both to affective disorders and memory impairments due to common neurodevelopmental alterations in the hippocampus.

Several lines of evidence point to the involvement of hippocampus in emotional regulation by way of forming fear memories. In experimental fear conditioning, several lesion and pharmacological inactivation studies demonstrated the involvement of hippocampus in contextual fear conditioning (Deacon et al., 2002; McEown and Treit, 2009, 2010) in contrast to the amygdala, which mediates cued fear conditioning. Moreover, it has been suggested that the ventral hippocampus (vHPC) mediates contextual fear conditioning, while its dorsal part (dHPC) plays a role for cognitive tasks such as spatial memory. Anatomical evidence also supports the idea that vHPC regulates fear learning (reviewed in Fanselow and Dong, 2010); the ventral CA1 sends direct projections to the CeA and receives substantial inputs from the LA and BLA. Particularly, it has been shown that the vHPC has a direct efferent to the mPFC (Ferino et al., 1987), a neural region playing a critical role for the modulation of anxiety. For example, Adhikari and colleagues (2010) demonstrated synchronisation of the theta-

frequency activity between the mPFC and vHPC of mice in anxiogenic behavioural environments.

Modulation of hippocampal activity by 5-HT has been linked to the control of mood and emotion, in particular anxiety-related phenotypes and depression (Morini et al., 2011 and references therein). For example, increased 5-HT release was reported in the vHPC during the anxious state induced by the EPM (Rex et al., 2005 as in Morini 2011; Bickerdike et al., 1993 as in Graeff et al., 1996) or in the caudal hippocampus (which may contain both the dorsal and ventral hippocampus) in response to gentle experimental handling and tail-pinch (Kalen et al., 1989 as in Graeff et al., 1996). The relationship between increased 5-HT release and anxious states is also in accordance with the fact that acute administration of SSRIs used to treat anxiety disorders provokes anxiogenic-like effects in animals submitted to tests for anxiety (Rex et al., 2005 and references therein).

1.5.2. Hippocampus and stress

It has been shown that exposing adult rodents to adverse events leads to deficits in long-term potentiation (LTP) and working memory (Millan et al., 2012 and references therein). Importantly, such chronic-stress induced cognitive deficits are often accompanied by neuronal structural changes, including dendritic spine atrophy, decreased neurogenesis in the hippocampus and disruption in the prefrontal cortex-hippocampus coupling (Millan et al., 2012 and references therein). In fact, it has previously demonstrated that the rate of neurogenesis in the hippocampus is associated with spatial learning and memory consolidation (Sarkisyan and Hedlund, 2009). Moreover, several lines of evidence link decreased hippocampal volume with affective disorders, such as PTSD, bipolar disorder, and depression (Fanselow and Dong, 2010 and references therein). It has been reported that the volume loss in some depressed patients reaches as much as 20% (Sapolsky, 2001). The drawback of the hypothesis linking adult neurogenesis with hippocampal volume, however, is that the adult hippocampal neurogenesis occurs in a rather limited area, i.e. dentate granule layer, and it is still unknown to what extent such a restricted neurogenesis and alteration of morphology in a single neuron level contributes to the total volume of the hippocampus. Moreover, the hippocampal volume may well be determined not only by adult neurogenesis but the rate of cell death of already existing neurons should also be taken into account.

One notion for a hypothetical mechanism directly linking stress and the hippocampus claims that stress-related hormone, glucocorticoids (in humans it is called cortisol, in mice

corticosterone) inhibits neurogenesis either directly or indirectly, thus leading to the atrophy of the hippocampus (Sapolsky, 2001). This hypothesis seems plausible given that brain glucocorticoid receptors are expressed mainly in the hippocampus (Bickerdike et al., 1993 as in Graeff et al., 1996) and corticosteroid hormones were shown to influence hippocampal morphology (Woolley et al., 1990 as in Graeff et al., 1996). Moreover, past investigation suggests that the negative feedback on the secretion of glucocorticoids released during stress takes place mainly in the hippocampus. Thus, it has been suggested that depression might be linked to an aberrant feedback mechanism at the level of the hippocampus. Indeed, one of the most consistent observations among depressed patients is their hypercortisolism and in animal models of depression-like behaviours, inhibition of fast glucocorticoid receptors and down-regulation of glucocorticoid receptors have been reported selectively in the hippocampus after the animals experienced chronic stress (Mongeau et al., 1997 and references therein).

As outlined here, the precise mechanism linking adult neurogenesis, hippocampal volume loss, and depression is still under investigation. However, the data available to date at least indicate that the hippocampus is one of the most vulnerable regions to stress and stress-related hippocampal dysfunction may convincingly explain some of the well-documented hippocampus-related cognitive deficits that accompany severe depression, including deficits in declarative memory such as episodic and semantic memory.

1.5.3. Pharmacologic interventions on the hippocampus ~Antidepressants, anxiolytics, and drugs of abuse~

Further evidence suggesting the involvement of the hippocampus in depression is based on the fact that long-term treatment with SSRIs, which results in a net increase in 5-HT transmission, induces adult neurogenesis (Schmitt et al., 2007). This action seems to be mediated by postsynaptic 5-HT_{1A} receptors since therapeutic effects of SSRIs were absent in 5-HT_{1A} receptor KO mice. Accordingly, blocking hippocampal neurogenesis appears to abolish some of the therapeutic effects of SSRIs (Santarelli et al., 2003 as in Graeff and Zangrossi, 2010). Moreover, acute or chronic antidepressant treatment induces autophosphorylation and activation of receptors for BDNF in the hippocampus as well as in the prefrontal and anterior cingulate cortex (Saarelainen et al., 2003 as in Graeff and Zangrossi, 2010). It is assumed that the clinical effects of antidepressants thus may be mediated by enhanced neurotrophin signalling and subsequent formation and stabilisation of synaptic connectivity.

Pharmacologically, infusion of benzodiazepines to the hippocampus is known to be anxiolytic (Menard and Treit, 1999 as in Klemenhagen et al., 2006). Moreover, such drugs are shown to impair the theta activity of hippocampus (Gray and McNaughton, 2000 and references therein). In fact, most anxiolytic drug actions are similar to septo-hippocampal lesions in their behavioural effects. Experimentally, it was shown that the behavioural deficits of learned helplessness were rescued by hippocampal lesions (Mongeau et al., 1997 and references therein). Based on these findings, Gray and McNaughton (2000) put much importance on hippocampus in the regulation of anxiety. In detail, they proposed that the hippocampus works as a "comparator" that would compare actual stimuli with expected stimuli, resolving the conflict between different goals of behaviours. In that way, the hippocampus increases the weighting of stimuli which lead to potential harm, shifting behavioural approach-avoidance conflict toward the direction of avoidance both in the present and future events (Gray and McNaughton, 2000). Therefore, the fact that moderate doses of benzodiazepine lead to resolution of conflict between approach and avoidance in favour of approach can be explained as hippocampal dysfunction (Bannerman et al., 2004 and references therein).

Furthermore, the hippocampus is known to be one of the most susceptible brain regions to MDMA-induced serotonergic axotomy. Importantly, long-lasting behavioural impairments observed in frequent MDMA users are characterised not only by deficits of learning and memory but also increased anxiety (Morini et al., 2011 and references therein).

1.5.4. 5-HT receptors in the hippocampus

Several distinct subtypes of 5-HT receptors are known to be present in the hippocampus. 5-HT_{1A} receptors are abundantly expressed on hippocampal pyramidal neurons. On the other hand, GABAergic interneurons possess 5-HT_3 receptors. Moreover, 5-HT_2 receptor family (5-HT_{2A} and 5-HT_{2C}) is present in all areas of the hippocampus. 5-HT_7 receptors are also known to be expressed in the hippocampus and their roles in spatial memory and contextual learning have recently attracted attention (Sarkisyan and Hedlund, 2009; Renner et al., 2012). A role for 5-HT_{1B}, 5-HT_4 and 5-HT_6 receptors in learning and memory has also been proposed (King et al., 2008; Buhot et al., 2000; Perez-Garcia and Meneses, 2008). Although all these receptors are implicated in the context of anxiety and depression, focus will be placed on 5-$HT_{1A,}$ 5-HT_3, and 5-HT_7 receptors for their particular relevance to the present study (for the roles of 5-HT_2 receptors in the hippocampus, see Araragi and Lesch (submitted)).

i). 5-HT_{1A} receptors

The involvement of 5-HT_{1A} receptors in emotional disorders has long been established by the fact that activation of 5-HT_{1A} receptors by partial agonists (e.g. buspirone) or indirectly via SSRIs is effective in treating anxiety disorders (Freeman-Daniels et al., 2011 and references therein). In addition, behavioural deficits of learned helplessness in rats can be rescued by activation of 5-HT_{1A} receptors (Mongeau 1997 and references therein). In postmortem brain of depressed suicides, reduction in the number or mRNA levels of hippocampal 5-HT_{1A} receptors has been reported (Lopez et al., 1998; Cheetham et al., 1990; Graeff et al., 1996; Mongeau et al., 1997). Moreover, high levels of glucocorticoids were shown to decrease the density of 5-HT_{1A} receptors in the rat hippocampus (Lopez et al., 1998; Mendelson and McEwen, 1992).

In animal studies, 5-HT_{1A} receptor KO mice are known to display increased anxiety-like behaviours, specifically in hippocampal-dependent spatial tasks such as contextual fear conditioning or the OF test, but not in paradigms that do not recruit the hippocampus, such as cued fear conditioning or the Vogel test (Klemenhagen et al., 2006). Other behavioural phenotypes include aberrant performances in hippocampus-dependent learning and memory tasks as well as antidepressant-like phenotypes in FST and TS test (Freeman-Daniels et al., 2011 and references therein). Importantly, the regulatory effect of 5-HT_{1A} receptors on emotion seems to be of developmental origin and to act via those receptors expressed postsynaptically, particularly in the hippocampus. For example, using conditional tissue-specific rescue strategies, Gross and co-workers (Gross et al., 2002) demonstrated that the restoration of 5-HT_{1A} receptors in the hippocampus but not in the raphe nuclei recovered normal anxiety-like behaviours of adult mice when the restoration took place during the first three weeks of postnatal development (i.e. until P21) rather than in adulthood.

Several electrophysiological correlates of enhanced anxiety in 5-HT_{1A} receptor KO mice have been found in CA1 pyramidal neurons of the hippocampus such as enhanced neuronal excitability and increased theta oscillations (Gordon et al., 2005 as in Freeman-Daniels et al., 2011), decreased paired-pulse inhibition (Sibille et al., 2000 as in Klemenhagen et al., 2006), and enhanced evoked-EPSC amplitude (Freeman-Daniels et al., 2011). Moreover, impaired hippocampal-dependent learning was shown to be accompanied by impaired paired-pulse facilitation in the dentate gyrus (DG) of the hippocampus (Sarnyai et al., 2000).

As mentioned above (see section 1.2.4.), serotonergic projections to the hippocampus originates from the MRN. Direct interaction between dHPC and MRN has been

experimentally demonstrated. In the study by Dos Santos and coworkers (2008), the elevated T-maze test was used to assess anxiety-related behaviours of rats. In this test, inhibitory avoidance response and escape response are seen as a model for generalised anxiety and panic disorders, respectively. In their study, anxiogenic effects, i.e. inhibitory avoidance, of direct injection of WAY-100635 (WAY), a 5-HT_{1A} receptor antagonist, into the MRN was counteracted by pre-injection of WAY into the dHPC, although WAY injection into dHPC alone had no effect. On the other hand, direct excitation of serotonergic neurons in the MRN by kainic acid facilitated inhibitory avoidance and suppressed escape, both of which were not blocked by pre-injection of WAY into the dHPC. The results suggest 5-HT_{1A} receptor-mediated complex interaction between the dHPC and MRN in the regulation of anxiety and panic related behaviours.

In summary, 5-HT_{1A} receptors are probably one of the most relevant serotonergic receptors for depression. For a more comprehensive review on 5-HT_{1A} receptors in human depression, refer to Drevets et al., 2007 and Savitz et al., 2009.

ii). 5-HT_3 receptors

Hippocampal interneurons express 5-HT_3 receptors. 5-HT_3 is unique in that it is the only ligand-gated ion channel receptor as opposed to other 5-HT receptor subtypes, which are coupled to G-proteins. When activated, 5-HT_3 receptors excite neurons which possess these receptors. Using quantitative autoradiography, Mössner et al. (2004) found upregulation of 5-HT_3 receptors in the stratum oriens of the hippocampal CA3 region (+18%) in 5-Htt KO mice along with increases in the frontal cortex(+46%) and parietal cortex (+42%). No difference was found in the stratum oriens and radiatum of the CA1 region, nor in the stratum radiatum of the CA3 region. However, quantitative *in situ* hybridisation detected significant increases in the density of 5-HT_3 receptor mRNA in the CA1 region of the hippocampus in 5-Htt Het mice (+64.8%) but not in KO mice. Since 5-HT_3 receptors exert excitatory effects on the GABAergic interneurons in the hippocampus, 5-HT may generate inhibitory postsynaptic potentials in the hippocampal pyramidal neurons. Alteration of 5-HT_3 receptor expression in 5-Htt KO mice indicates changes in this inhibitory regulation, which may in turn implicate significant influence on the cognitive processing in the hippocampus. Indeed, 5-HT_3 receptors have been implicated not only in memory formation but also in a number of disorders including depression, anxiety and bipolar disorders, schizophrenia, and the development of substance dependence (Martin and Humphrey, 1994 as in Mössner et al., 2004; Savitz et al., 2009 and references therein). Pharmacologically, 5-HT_3 antagonists such as ondansetron and

tropisetron have been shown to improve learning and memory. As mentioned earlier, 5-HT_3 receptors are located on GABAergic interneurons in the hippocampus and antagonism of 5-HT_3 receptors was shown to decrease firing activity of CA1 interneurons, which consequently increased the firing rate of pyramidal neurons (Reznic and Staubli, 1997). Moreover, it was demonstrated that inactivation of 5-HT_3 receptors increased the frequency of the hippocampal theta rhythm and the magnitude and duration of LTP in the CA1 field in freely moving rats (Staubli and Xu, 1995). Such 5-HT_3 receptor-mediated control of theta rhythm in the hippocampus as well as firing activity and LTP of CA1 pyramidal neurons is believed to underpin the memory-enhancing effects of 5-HT_3 receptor antagonists (Buhot et al., 2000).

iii). 5-HT_7 receptors

The 5-HT_7 receptor is one of the recently characterised 5-HT receptor subtypes. It is a G protein-coupled receptor and facilitates cAMP formation by activating adenylyl cyclase isoforms through G_s proteins. The hippocampus, along with the thalamus and the frontal cortex, is one of the regions where 5-HT_7 receptors are highly expressed (Sarkisyan and Hedlund, 2009). Accumulating evidence suggests that 5-HT_7 receptors play an important role in learning and memory, anxiety, depression, obsessive-compulsive disorder, circadian rhythm, and REM sleep (Hedlund, 2009; Fox et al., 2008). Of clinical relevance, 5-HT_7 receptors have been proposed as a target for a new class of antidepressant drugs (Bonaventure et al., 2007; Sarkisyan et al., 2010; Leopoldo et al., 2011; Mnie-Filali et al., 2011).

As discussed earlier, the hippocampus plays a crucial role for contextual fear learning. Roberts and colleagues (2004) demonstrated that 5-HT_7 receptor knockout mice have specific deficits in contextual fear conditioning and such deficits are accompanied by reduced LTP inducibility in the CA1 region of hippocampal slices. Using the Barnes maze test, it was shown that 5-HT_7 knockout mice have impairments in allocentric spatial memory, one of the hippocampus-dependent spatial memory functions, while striatum-dependent egocentric memory was intact (Sarkisyan and Hedlund, 2009). Emerging evidence suggests that 5-HT_7 receptors form heterodimers with 5-HT_{1A} receptors in certain brain regions, including the hippocampus and the DRN (Renner et al., 2012). Dimerisation of 5-HT_{1A} receptors with 5-HT_7 receptors reduces coupling of 5-HT_{1A} receptors to GIRK channels. Moreover, heterodimerisation facilitates 5-HT-induced internalisation of 5-HT_{1A}-5-HT_7 heteromers from the cell membrane. Importantly, the expression of 5-HT_7 receptors in the hippocampus decreases throughout development, whereas 5-HT_7 receptor expression is kept at high level in the DRN. The resultant dominant form of 5-HT_{1A} homomers in the hippocampus, which is

resistant to receptor agonist-mediated internalisation, may explain why 5-HT_{1A} receptors in 5-Htt KO mice were desensitised only presynaptically in the DRN but not postsynaptically in the hippocampus (see section 1.4.2.) (Renner et al., 2012).

1.5.5. Effects of serotonin on learning and synaptic plasticity

Evidence suggests that depletion of 5-HT enhances learning abilities especially in the domain of negative emotionality, such as fear learning. Burghardt and colleagues (Burghardt et al., 2004; 2007) demonstrated that acute administration of SSRI enhanced the acquisition of fear memories in rats, whereas chronic treatment reduced it. Due to the presence of autoinhibitory 5-HT_{1A} receptors on serotonergic neurons, acute administration of SSRI leads to transient reduction of the net 5-HT transmission, while the chronic SSRI treatment elevates the net 5-HT transmission possibly via adaptive desensitisation of 5-HT_{1A} receptors. In humans, mild 5-HT depletion can be achieved by an experimental procedure called "acute tryptophan depletion (ATD)", where human subjects are given diets which lead to reduced availability of Trp in the brain. Subjects who underwent ATD were shown to exhibit exaggerated aversive processing and deficient response inhibition towards previously rewarded but now punished behaviours (Cools et al., 2008). In detail, subjects after ATD can predict future punishment better than non-ATD subjects. This seems to be achieved by firmly establishing cue-punishment contingency. On the other hand, when the cue-reward/punishment association is swapped, subjects with ATD still responded to cues previously paired with rewards. Interestingly similar results are obtained among subjects given acute SSRI administration, which is also supposed to reduce 5-HT transmission. These results indicate that 5-HT depletion, at least transient depletion, promotes performance of probabilistic learning paradigms, presumably by abnormally enhancing the impact of punishment (Cools et al., 2008).

Compared to other neurotransmitter systems, effects of 5-HT on synaptic transmission and LTP in the neuronal level have much less been explored and remarkably divergent findings were reported in earlier studies. The first study which investigated the effect of 5-HT depletion reported reduction of LTP in the DG *in vivo* (Bliss et al., 1983 as in Andersen et al., 2007). A more recent investigation by Shutoh et al. (2000) demonstrated that 5-HT depletion induced by PCPA led to an increase of the GluR2 subunit of the AMPA receptor, which is known to inhibit Ca^{2+} influx into the neuron. Since Ca^{2+} plays an important role in LTP induction and enhancement of hippocampal LTP in GluR2 null mutant mice had been

reported (Shutoh et al., 2000 and references therein), the authors postulated that reduction of LTP following 5-HT depletion would have been mediated by the increase of GluR2 subunit. The authors also postulated that reduction in LTP was caused by an extensive degree of synaptic loss, which was previously reported in another study using the same 5-HT depletion method (Chen et al., 1994). On the contrary, Stanton and Sarvey (1985) demonstrated that depletion of 5-HT does not affect LTP inducibility either in the DG or CA1 *in vitro*.

Reduction of LTP inducibility following 5-HT depletion may indicate that 5-HT positively modulates and thus facilitates LTP induction. Indeed, activation of certain receptor subtypes such as 5-HT_4 receptors has been shown to enhance LTP. Application of 5-HT_4 receptor agonist SC 53116 was shown to attenuate learning impairment induced by antagonism of muscarinergic acetylcholine receptors in a passive avoidance test (Matsumoto et al., 2001). As a potential mechanism of such 5-HT_4 receptor action, SC 53116 was shown to enhance the population spike amplitude (PSA) in the hippocampal CA1 area evoked by Schaffer collateral stimulation. Moreover, SC 53116 was shown to enhance the tetanus-induced LTP. A following study by Matsumoto et al. (2002) found that SSRI, fluvoxamine, increased PSA in the CA1 and CA3 fields concentration dependently. Using specific antagonists, it was revealed that SSRI fluvoxamine-induced facilitation in the CA1 was mediated by 5-HT_{1A} and 5-HT_4/5-HT_7 receptors in an inhibitory and a stimulatory manner, respectively. On the other hand, in the CA3 field, both 5-HT_{1A} and 5-HT_7 receptors contribute to the facilitatory effects of 5-HT on PSA. A whole-cell patch clamp recording by Mlinar and coworkers (2006) showed that an excitability increase in CA1 pyramidal neurons induced by 5-HT_4 receptor activation is attributable to inhibition of a Ba^{2+}-sensitive inwardly rectifying K^+ current. It should be noted, however, that in the DG of rats, stimulation of the 5-HT_4 receptor was shown to inhibit basal synaptic transmission and LTP (Kulla and Manahan-Vaughan, 2002).

Of particular interest is that stimulation of 5-HT receptors outside of the hippocampus modulates hippocampal LTP. Abe et al. (2009) demonstrated that injection of 5-HT_{2C} receptor agonist MK212 into the BLA facilitated the induction of LTP at the perforant path (pp)-DG granule cell synapses in anesthetised rats, whereas injection of 5-HT_2 antagonist cinanserin into the BLA inhibited the pp-DG LTP. As mentioned earlier (see section 1.5.1.), the hippocampus and the amygdala are anatomically connected. It is likely that this connection enabled the amygdala to modulate hippocampal LTP.

On the other hand, inhibitory effects of 5-HT on LTP have also been reported. Villani and Johnston (1993) demonstrated that 5-HT inhibits induction of LTP at commissural/associational synapses onto CA3 *in vitro*. Similarly, specific antagonists for 5-HT_{2A} receptors (Wang and Arvanov, 1998) and antagonists for 5-HT_3 receptors (Staubli and Xu, 1995; see also section 1.5.4. ii).) have been shown to enhance LTP in the hippocampus. As for the inhibitory effects of 5-HT on LTP, it has been demonstrated that such effects are in part mediated by activation of 5-HT_{1A} receptors in the CA1 *in vitro* (Pugliese et al., 1990; Corradetti et al., 1992; Tachibana et al., 2004) and *in vivo* (Kojima et al., 2003) as well as in the DG *in vitro* (Sakai and Tanaka, 1993). It was shown that such an inhibitory effect of 5-HT on LTP in the CA1 and DG is mediated by hyperpolarisation of pyramidal cells via 5-HT_{1A} receptors (Sakai and Tanaka, 1993; Kojima et al., 2003; Corradetti et al., 1992; Tachibana et al., 2004). Moreover, suppression of LTP in CA1 by increase in spontaneous discharges of GABAergic interneurons induced by activation of 5-HT_3 receptors (Staubli and Xu, 1995; Andersen et al., 2007; Corradetti et al., 1992) has been proposed.

Additional support for the notion that 5-HT prevents LTP derives from the finding that rats with serotonergic deafferentation to the hippocampus induced by 5,7-dihydroxytryptamine (5,7-DHT) showed enhanced spatial discrimination learning (Altman et al., 1990) and LTP in the hippocampus-mPFC pathway *in vivo* (Ohashi et al., 2003). Contribution of 5-HT to LTP becomes even more complicated when different modes of behavioural learning are considered. For example, Sanberg et al. (2006) demonstrated that 5-HT_{1A} receptor antagonist (WAY-100635) blocked pp-DG LTP in freely moving rats when LTP was induced only in novel, but not familiar, environments. Since application of a 5-HT_{1A} agonist to the DG reduced somatic GABAergic inhibition and serotonergic projections from the MRN terminate on inhibitory interneurons in the DG, the authors concluded that 5-HT_{1A} receptors contribute to LTP induction via inhibition of GABAergic interneurons. Contribution of 5-HT_{1A} receptor stimulation to LTP induction was reported in another study demonstrating that stimulation of 5-HT_{1A} receptors specifically impairs associative long-term depression (LTD) of the Schaffer collateral-CA1 synapse and, as an inference, may facilitate LTP (Normann et al., 2000).

The serotonergic system is one of the most relevant targets of psychoactive drugs. Considering the above-mentioned effects that 5-HT exerts on synaptic plasticity, it is reasonable to assume that psychoactive drugs affect synaptic plasticity. MDMA leads to release of monoamine transmitters including 5-HT, NE and DA and transporters such as 5-

HTT and NET mediate the psychoactive effects of MDMA. Moreover, repeated administration of MDMA is known to induce serotonergic deficits, memory impairment, as well as increased anxiety. Recent studies, however, demonstrated that both acute (Rozas et al., 2012) and repeated (Morini et al., 2011) administration of MDMA enhances LTP in the CA1 area of rat brain slices, implying that MDMA-induced memory deficits are not associated with LTP impairment in the hippocampus. Rozas and associates (2012) further demonstrated that such LTP-enhancing effects of MDMA are mediated by presynaptic 5-HT_2 receptors and postsynaptic D1/D5 dopaminergic receptors. Moreover, Mlinar et al. (2008) showed that MDMA induces potentiation of excitatory postsynaptic potential (EPSP)-spikes and its effects are prevented by blockade of both 5-HTT and NET. Simultaneous direct stimulation of both 5-HT_4 and adrenergic β_1 receptors, on the other hand, mimicked MDMA-induced EPSP-spike potentiation, indicating crucial roles for these receptors in MDMA-mediated alterations in neuronal excitability and synaptic plasticity.

Taken together, multiple lines of evidence indicate that 5-HT modulates LTP inducibility and synaptic transmission in the hippocampus. Its effects are, however, exceedingly complicated with both facilitatory or inhibitory mechanisms of action. It is rather likely that the net effects of 5-HT on synaptic plasticity depend on availability of certain receptor subtypes, which are subject to the subregion in the hippocampus, and on behavioural learning paradigms in question. Since hippocampal synaptic plasticity is highly relevant to emotions, serotonergic modulation of synaptic plasticity presented here may indicate that 5-HT controls emotions through changing synaptic plasticity of the hippocampus.

1.6. Tryptophan-hydroxylase 2 (Tph2) and Tph2 KO mice

1.6.1. Discovery of two isoforms of Tph and generation of Tph2 KO mice

The second isoform of Tph, designated as Tph2, has been identified based on the fact that Tph1 KO mice, while having no 5-HT in the periphery, possessed normal levels of 5-HT in brain (Walther et al., 2003; Côté et al., 2003). Tph2 is predominantly expressed in the brainstem and additionally in neurons of the myenteric plexus, whereas Tph1 is expressed in the peripheral tissues (e.g. enterochromaffin cells of the gastrointestinal tract, spleen, thymus) as well as in the pineal gland (Walther and Bader, 2003; Côté et al., 2003). Although subsequent studies have generated substantial controversies regarding the distribution of Tph1 and Tph2 isoforms (reviewed in Waider et al., 2011), generation of Tph2 KO mice has

facilitated understanding of the differential distribution of the two isoforms (Gutknecht et al., 2008; Savelieva et al., 2008; Alenina et al., 2009). In detail, it has been demonstrated that in Tph2 KO mice, Tph2 immunoreactivity and 5-HT are completely absent in the brain implying that Tph2 is the only isoform expressed in the brain. Surprisingly, these mice maintain apparently normal serotonergic-like neurons in their DRN. Their neurons have all the serotonergic neuron-specific markers; 5-Htt, transcription factor Pet1, vesicular monoamine transporter-2 (Vmat2) and 5-HT receptors (Gutknecht et al., 2008, 2009; Kriegebaum et al., 2010). Recordings from brain slices showed that raphe neurons from Tph2 KO mice retain electrophysiological properties typical of normal serotonergic neurons, i.e. slow and regular firing rate, broad action potential width (Gutknecht et al., 2012). Moreover, using 5-HT_{1A} receptor-mediated autoinhibition of serotonergic neuron firing as a functional assay, it was confirmed that serotonergic neurons in Tph2 KO mice are able to produce 5-HT from 5-HTP but not from tryptophan (Gutknecht et al., 2012). Upregulation of 5-HT_{1A} and 5-HT_{1B} receptors as well as reduction in NE concentrations and accompanying reduction in the noradrenergic cell number in the LC were also detected, presumably as an adaptive change in response to the absence of endogenous 5-HT (Gutknecht et al., 2012). Although not serotonergic neuron-specific, levels of MAO-A and neuronal nitric oxidase (nNOS) were also measured (Angoa-Perez et al., 2012). Tph2 KO mice had normal levels of these enzymes except in the frontal cortex where slight but significant reduction was observed in Tph2 KO mice.

1.6.2. Characteristics of Tph2 KO mice

Tph2 KO mice exhibit numerous physiological and behavioural alterations (reviewed in Waider et al., 2011; Lesch et al., 2012). Prominently, they show increased mortality rates and are delayed in the development and during the first two months of postnatal days as reflected by a reduced body weight and size (Alenina et al., 2009; Gutknecht et al., 2012; Savelieva et al., 2008). Correspondingly, Tph2 KO mice were shown to have lower bone mass, significant decrease in appetite, and increase in energy expenditure at early stages of development (Yadav et al., 2009). On the other hand, age-dependent and sex-specific overweight was observed in female Tph2 KO mice (Gutknecht et al., 2012). Moreover, deficiency of Tph2 leads to dysregulation of several physiological and autonomic parameters. While the body temperature of Tph2 KO at the baseline condition was not different from wildtype controls, they showed significant drop in the body temperature when exposed to a cold environment (4°C), suggesting deficits in thermoregulation. In addition, the respiration rate of Tph2 KO

mice was significantly reduced, although the circadian variations were normal. Finally, Tph2 KO mice showed extended daytime sleep and a decrease in blood pressure and heart rate during night time (Alenina et al., 2009).

Behaviourally, Tph2 KO males display more aggression (Angoa-Perez et al., 2012; Mosienko et al., 2012; Gutknecht et al., submitted). In a modified resident-intruder test, where a resident mouse was singly caged only shortly before the introduction of a group-caged intruder, even female KO mice and weanlings (3-4 weeks old) of both sexes showed elevated aggressive behaviours compared to controls (Angoa-Perez et al., 2012). Moreover, female Tph2 null mutant mice show impaired maternal care, which may lead to poor survival of their pups (Alenina et al., 2009). Further, Tph2 KO mice exhibit increased obsessive-compulsive-like traits, reflected as frequent burying of marbles (Savelieva et al., 2008; Angoa-Perez et al., 2012). In the OF test, they did not differ from wildtype counterparts in terms of total locomotor activity or exploratory behaviours but spent less time in the central field, indicating elevated anxiety-like traits (Savelieva et al., 2008). On the other hand, in the light-dark box test, Tph2 KO mice showed less anxiety-like behaviours, while they are no different from controls in the EPM test (Angoa-Perez et al., 2012). Other phenotypes such as acoustic startle response and sensorimotor gating, motor coordination, learning and memory, and tonic inflammatory pain sensitivity did not differ between KO and wildtype as assessed by pre-pulse inhibition, inverted screen, cued fear conditioning, and formalin paws tests, respectively (Savelieva et al., 2008). Recently, it was discovered that Tph2 KO male mice lose sexual preference to females over males, and such a phenotype was restored by application of 5-HTP, an immediate precursor of 5-HT (Liu et al., 2011). Another defining behavioural feature of Tph2 KO mice observed is enhanced fear acquisition and retention in Pavlovian fear conditioning (Gutknecht et al., submitted). After pairing an auditory tone (conditional stimulus, CS) with an electric footshock (unconditional stimulus, US), mice establish association between the tone and the aversive footshock (cued fear conditioning) or between the footshok and the context where the footshock was given (contextual fear conditioning). Both in fear conditioning and fear memory retention tests, Tph2 KO mice exhibited prolonged freezing time in response to both cue and context stimuli and shorter latencies to freezing when placed in the conditioning context. Enhanced fear learning and retention of Tph2 KO mice were even more pronounced when mice underwent chronic mild stress (CMS) prior to fear conditioning (Gutknecht et al., submitted).

Finally, evidence suggests that GABAergic interneurons play an important role for disorders of emotion regulation (Goren et al., 2007; Tunnicliff et al., 1999; Tunnicliff and Malatynska, 2003; Ehrlich et al., 2009). A recent study revealed that GABA concentrations were significantly increased in the hippocampus of Tph2 KO mice and in the amygdala of Tph2 Het mice, while GABA concentrations were significantly decreased in the PFC of Tph2 Het mice (Waider et al., 2012). On the other hand, the number of GABAergic neurons in the anterior BLA was significantly decreased in Tph2 KO mice. The results indicate that neuronal 5-HT is involved in the development and maintenance of the GABAergic system. Given the importance of the GABAergic system in emotional regulation, disruption of serotonergic transmission may have contributed to the altered emotional behaviours of Tph2 KO mice by acting on the GABAergic system.

1.6.3. Association of TPH2 with neuropsychiatric disorders

Analysis of SNPs in the upstream regulatory region of *TPH2* revealed significant genotype frequency differences between the control and the patient group comprising either cluster B (dramatic-emotional, i.e. borderline, antisocial, narcissistic, and histrionic) or cluster C (anxious-fearful, i.e. obsessive-compulsive, avoidant, and dependent) according to the DSM-IV criteria (Gutknecht et al., 2007). In both patient groups, overrepresentation of the T-allele in the SNP (G703T, rs4570625) was observed. Furthermore, in the control group, significant effect of *TPH2* variation on anxiety-related personality traits (defined by Tridimensional Personality Questionnaire (TPQ) Harm Avoidance) was found. Importantly, the SNP was previously shown to be associated with attention-deficit/hyperactivity disorder (ADHD) (Walitza et al., 2005; Canli et al., 2005) and bias responsiveness of the amygdala (Brown et al., 2005; Canli et al., 2005; see below). Taken together, these studies link a *TPH2* functional polymorphism with anxiety-related personal trait as well as neuropsychiatric symptoms, both reflected in the activity of the neuronal system involved.

Functional MRI study provided the first *in vivo* evidence that the functional variant rs4570625 of *TPH2* modulates amygdala activity. T-allele carriers had greater amygdala activity in response to fearful or angry faces (Brown et al., 2005) as well as sad and happy faces (Canli et al., 2005). Taken together, Canli and colleagues (2005) concluded that this genetic variance affects the responsiveness of amygdala to emotionally relevant stimuli, both with positive and negative valence. The additive effects of both *TPH2* and *5-HTT* variation have been demonstrated using event related potentials (ERP) focusing early posterior negativity (EPN).

Herrmann et al. (2007) found that when the subjects carry both the short variant of *5-HTT* and T-variant of *TPH2*, they show the highest neuronal activity to emotional stimuli. Subsequently, the finding was replicated in fMRI study (Canli et al., 2008) revealing the putamen as the most robust site where the gene-by-gene interaction takes place, as well as the amygdala, at a less stringent threshold. The additive effects were more robust for visuospatial stimuli than for verbal, and more robust for negatively than positively valenced stimuli.

ADHD, manifested as deficient response inhibition and failing emotion regulation, has been hypothesised to be linked to reduced PFC function. Baehne and colleagues investigated the influence of *TPH2* functional variants, which have previously been shown to be linked to ADHD (rs4570625 and rs11178997), on the PFC function in the Go-NoGo task (Baehne et al., 2009). It was found that carriers of risk alleles had reduced NoGo-anteriorisation (NGA) in the ERP, indicative of lower prefrontal function, both in the healthy and ADHD cohort. As expected, carriers in the ADHD group had an even smaller mean NGA value than those in the healthy group.

Moreover, an elevated level of TPH2 was detected in the raphe nuclei of the postmortem brains of depressed individuals who committed suicides (Bach-Mizrachi et al., 2006, 2008; Evans et al., 2008). This is interpreted as a regulatory response to counteract deficient 5-HT levels in the brains of depressed individuals. It has also been shown that allelic variation of TPH2 function can be a genetic predictor of depression, suicide risk among depressed patients, and antidepressant treatment response (Evans et al., 2008 and references therein). In sum, multiple lines of evidence implicate *TPH2* variation in the anxious and fearful personality traits as well as in a wide spectrum of disorders characterised by emotion dysregulation ranging from depression, anxiety and obsessive-compulsive disorders, to ADHD.

1.7. Aims of the study

The primary aim of the present study is to understand how deferent levels of brain 5-HT, which is induced by genetic deletion of major mediators of 5-HT neurotransmission, alter neurophysiological properties of the serotonergic neurons and their projection areas. Moreover, since 5-HT has been implicated in emotional regulation and indeed mice used in this study, i.e. 5-Htt KO mice and Tph2 KO mice, have been shown to exhibit anxiety-like and depression-like behaviours, the present study aims to infer functional relevance of

potential neurophysiological changes. In sum, the aim of the present study can be summarised as follows:

- **Investigate homeostatic regulatory mechanisms of serotonergic neuronal activity in the DRN in the continuum of deferent levels of extracellular 5-HT (Study 1)**

  To this end, the loose-seal cell-attached recording configuration was used to monitor firing of serotonergic neurons for a long duration with minimum perturbation to the recorded neuron. Firing of serotonergic neurons reflects release of 5-HT and is under homeostatic regulation, which is influenced by synthesis of 5-HT and sensitivity of autoinhibitory 5-HT_{1A} receptors. For this reason, functionality of autoinhibition was assessed by artificially inducing 5-HT synthesis *in vitro* as well as directly applying a 5-HT_{1A} receptor agonist.

- **Detect potential neurophysiological changes in brain regions involved in emotion regulation (Study 2, Study 3)**

  For this purpose, the whole-cell recording configuration was applied in order to detect potential subtle electrophysiological alterations in a single neuron level. Here detailed analyses of basic membrane properties and action potential shapes were conducted on serotonergic neurons in the DRN of Tph2 KO mice and on CA1 pyramidal neurons in the hippocampus of 5-Htt KO mice.

- **Link electrophysiological alterations with functional consequences (Study 4)**

  For this aim, potential functional alterations which may have derived from changes in 5-HT levels were investigated in one of the postsynaptic target areas of the central serotonergic system, the hippocampus. The hippocampus plays a crucial role in learning and memory and it has been maintained that LTP of synaptic transmission in the hippocampus is the neuronal correlate of learning and memory. Moreover, alteration of synaptic plasticity has been implicated in depression. In particular, it has been proposed that the ventral hippocampus is involved in the context of anxiety and fear learning (see section 1.5.1.). Given this, extracellular field potential recording was conducted to study magnitude of LTP in the CA1 area of the ventral hippocampus in response to the Schaffer collateral/commissural pathway stimulation. It is assumed that LTP inducibility could have been modified by the lack or excess level of extracellular 5-HT in Tph2 KO and 5-Htt KO mice, respectively.

2 . Materials and Methods

2.1. Animal handling and strains

Procedures for animal handling followed the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive 86/609/EEC) and approved by the local committees. The generation of Tph2 KO animals was described previously (Gutknecht et al., 2008). Their genetic background is composed theoretically of 97% C57BL/6N and 3% Sv129/Ola. The 5-Htt KO mice line (Bengel et al., 1998) was backcrossed to a C57BL/6J background for more than ten generations. Animals were housed under a 12 h light and a 12 h dark cycle (lights: from 08:00 to 20:00 h) at ambient temperature of 22±1 °C and the relative humidity of 40–50%

2.2. Genotyping of mice

The following genotyping protocol was used in the laboratory of Prof Corradetti and is a modified version of the original developed in the laboratory of Prof Lesch. Since reagents used in the two laboratories were not the same, it was necessary to modify the original protocol. The protocol described below is the one which worked the best in Prof Corradetti's laboratory.

2.2.1. Tail digestion and DNA purification

A Tip of mouse tail (c.a. 3 mm) was cut and put into an autoclaved 1.5 ml microcentrifuge tube. Tubes were kept in ice until starting digestion. Mixture of lysis buffer (LB buffer, **Table 1**; 500 µl per sample) and proteinase K (lyophilised, Novagen: 70663, 20 mg/ml; 5 µl per sample) were premade in a 50 ml falcon tube for all the samples and dispensed 500 µl each into microcentrifuge tubes. Tubes were then placed in a hot water bath (55 °C) for overnight (c.a. 16-17 hours). Ideally, tubes should be under constant shaking during this procedure to facilitate tissue digestion. On the second day, the tubes were removed from the heat and vortexed shortly (~ 2 seconds). A second set of clean microcentrifuge tubes were labelled and provided with 540 µl of isopropanol (2-propanol) and cooled down in an ice bath. When this was done, the samples were centrifuged at the maximum speed (14,000 – 15,000 rpm) for 5 mins (room temperature is fine). By inclining the tubes containing lysed probes, the supernatant was transferred directly to the tubes with isopropanol. Each tube was mixed gently by inversion till a white glob of DNA precipitate became visible. The probes were

centrifuged again at the maximum speed for 10 mins (4°C). The supernatant was poured off and the liquid was removed as much as possible by patting the tubes onto paper towels several times. At this stage, the DNA pellet should be stuck to the bottom of the tubes.

500 µl of 70 % EtOH (precooled in a fridge at 4°C) was then added to each probe. Subsequently, the probes were centrifuged at the maximum speed for 20 mins. EtOH was discarded and removed as much as possible by patting the tubes onto paper towels several times. EtOH was completely removed by 3-4 hours of air drying and 50 µl of TE buffer (**Table 2**) was added to each probe. DNA was resuspended by constant shaking at 4 °C for at least overnight.

Table 1. LB buffer (final concentration)

- 3.33 ml 1.5M Tris, pH 8.5 (100 mM)
- 0.5 ml 0.5 M EDTA (Na_2EDTA x $2H_2O$, Sigma), pH 8.0 (5 mM)
- 1 ml 10 % SDS (0.2 %)
- 2 ml 5 M NaCl (200 mM)
- measure up to 50 ml with deionised water

Table 2. TE buffer (final concentration)

- 1 ml 1M Tris, pH 8.0 (10 mM)
- 20 µl 0.5M EDTA, pH 8.0 (0.1 mM)
- measure up to 100 ml with distilled water
- Autoclave the whole solution

2.2.2. PCR

i). *5-htt*

Composition of PCR mixture (for 1 sample) for *5-htt* is as follows (µl): 0.76 Primer 1 (5htt-wt-F: 6.67 µM stock); 0.76 Primer 2 (5htt-wt-R: 6.67 µM stock); 0.76 Primer 3 (5htt-mut-F: 6.67 µM stock); 0.76 Primer 4 (5htt-mut-R: 6.67 µM stock); 1.0 dNTP mix (2.5 mM stock); 2.5 Goldstar PCR buffer 10x; 1.5 $MgCl_2$ (25 mM stock); 1.0 Taq DNA polymerase (native without BSA, Fermentas: EP0282, 5u/µl); 10.96 H_2O. 5 µl of DNA suspension was taken from each probe. Primer sequences and the PCR program for *5-htt* genotyping are shown below:

Primer 1: 5htt-wt-F: 5'- AAG ATG GAT TTC CTC CTG TC-3'
Primer 2: 5htt-wt-R: 5'- TTT AGG CTC ACG TCA GCT AC-3'
Primer 3: 5htt-mut-F: 5'- CTT CTA TCG CCT TCT TGA CG-3'

Primer 4: 5htt-mut-R: 5’- GAC TGG AGT ACA GGC TAG C-3’

- Cycle 1: (x1) step 1: 95°C for 5 min
- Cycle 2: (x35) step 1: 95°C for 1 min
  step 2: 55°C for 1 min
  step 3: 72°C for 1 min
- Cycle 3: (x1) step 1: 72°C for 8 min
  step 2: 30°C for 10 min
  end: 10 °C ∞

ii). *Tph2*

Composition of PCR mixture (for 1 sample) for *Tph2* is as follows (µl): 1.0 Primer 1 (3’Del mTph2 F: 10 pmol/µl); 1.0 Primer 2 (5’arm over const. Tph2 F: 10 pmol/µl); 1.0 Primer 3 (3’over constr. Tph2 R: 10 pmol/µl); 1.0 dNTP mix (2.5 mM stock); 2.5 Goldstar PCR buffer 10x; 1.5 $MgCl_2$ (25 mM stock); 1.0 Taq DNA polymerase (5u/µl); 11 H_2O. 5 µl of DNA suspension was taken from each probe. Primer sequences and the PCR program for *Tph2* genotyping are shown below:

Primer 1: 3’Del mTph2 F: 5’- TGG GGC ATC TCA GGA CGT AGT AGT -3’
Primer 2: 5’arm over const. Tph2 F: 5’- CAC CCC ACC TTG CAG AAA TGT TTA -3’
Primer 3: 3’over constr. Tph2 R: 5’- TGG GGC CTG CCG ATA GTA ACA C -3’

- Cycle 1: (x1) step 1: 95°C for 5 min
- Cycle 2: (x38) step 1: 95 °C for 40 sec
  step 2: 64 °C for 40 sec
  step 3: 72 °C for 50 sec
- Cycle 3: (x1) step 1: 72 °C for 5 min
  step 2: 4 °C for 5 min
  end: 10 °C ∞

In order to prevent unspecific amplification of DNA fragments, PCR cycles were commenced as soon as possible after adding DNA to PCR mixtures and PCR probes were set into a PCR cycler only after the cycler was heated up above ~70 °C.

2.2.3. Electrophoresis

5 µl of BBF dye (6x DNA loading dye) was added to each PCR product and about 15 µl of BBF/PCR product mixture was loaded into a well on 2% agarose gel (in TBE buffer, see **Table 3** and **4**) with etidium bromide. As a reference, 10 µl of 90-908 base pair (bp) size marker (pBR322 DNA/AluI Marker, 20, ready-to-use; Fermentas) was also loaded into a well. Electrophoresis was conducted at 110V for 1 hour and visualised under a UV transilluminator. Expected bp sizes for each genotypes are as follows:

5-htt: WT 220 bp, KO 550 bp

Tph2: WT 437 bp, KO 387 bp

When there are two bands, it means that the sample has a Het genotype.

Table 3. TBE 5X (final concentration)

- 54 g Tris Base (MW 121.14; 445 mM)
- 27.g Boric acid (MW 61.83; 445 mM)
- 20 ml 0.5 M EDTA, pH 8.0 (10 mM)
- Add distilled water till 1000 ml
- Filter and autoclave the solution.
- Dilute this stock 1:10 to obtain TBE 0.5X

Table 4. 2 % agarose gel

- 2 g agarose (Sigma, A5093) in 100 ml TBE 0.5 X
- Warm up to 100-150 °C under continuous stirring until the solution becomes clear
- Add 5 µl ethidium bromide (Sigma, E1510) and pure into the form. Cool down till the gel becomes solid.

2.3. Loose-seal cell-attached recording

2.3.1. Rationale

Cell-attached recording is suited for recording the activity of (or stimulating) neurons in brain slices without perturbing the cell membrane (for review see Perkins, 2006). As with the whole-cell recording configuration, there are two modes of recording, i.e. voltage-clamp and current-clamp recording. Voltage-clamp mode is particularly suited for monitoring firing activity of neurons, whereas current-clamp mode is suitable for measuring resting membrane potentials and synaptic potentials. By adjusting the magnitude of seal resistance, i.e. resistance between the glass pipette tip and the cell membrane, two different types of experiments are possible: loose-seal voltage-clamp recording enables monitoring of firing activities of neurons by measuring current flow induced by action potentials; tight-seal current-clamp recording provides a way to evoke action potentials in the attached cell and to measure resting membrane and synaptic potentials. Since the aim of the present study is to monitor firing activities of neurons, loose-seal cell-attached recording in the voltage-clamp mode was used for the above-mentioned reason.

2.3.2. Preparation of dorsal raphe slices

Slice preparation procedures followed those described in Gutknecht et al., 2012. Female mice were not checked for their estrus cycle because previous studies showed that regulation of DRN firing through 5-HT_{1A} receptors does not change due to the cycle (Bouali et al., 2003 as in Loucif et al., 2006). Mice (28 to 80 days old) were anaesthetised with isofluorane and decapitated. The whole head was immediately immersed in ice-cold artificial cerebrospinal fluid (ACSF) comprising the following (mM): 124 NaCl; 2.75 KCl; 1.25 NaH_2PO_4; 26 $NaHCO_3$; 1.3 $MgCl_2$; 2 $CaCl_2$; 11 D-Glucose. The ACSF was continuously bubbled with a gas composed of 95% O_2 and 5% CO_2 (pH 7.4). Then the brain was rapidly isolated and placed in a Petri dish for dissection, completely submerged by oxygenated, ice-cold, ACSF. About one third of the rostral brain and the cerebellum were cut off to leave the block of brain containing the raphe nucleus. The caudal edge of this brain block was glued onto the metal plate of the vibratome (DSK-1000, Dosaka Co. Ltd, Kyoto, Japan), whose chamber was filled with ice-cold ACSF, bubbled with a gas mixture. Brainstem slices with 200 µm thickness (typically F66-72 of Mouse Brain Atlas by Paxinos and Franklin, 2001) were made with the vibratome and transferred to a multi-well incubation chamber filled with bubbled ACSF, which was preheated at 33 ℃ and left at a room temperature after slice transfer.

2.3.3. Electrophysiological recording

Recording procedures followed those described in Gutknecht et al., 2012. After at least 1.5 h of recovery time, a single slice was transferred to the recording chamber and perfused with ACSF, bubbled and warmed up (34-35 ℃), at the rate of 2 ml/min. ACSF was supplemented with a mixture of neurotransmitter blockers to isolate a single neuron from the influence of surrounding neurons. The composition of neurotransmitter blockers are as follows (μM): 10 DNQX; 20 D-APV; 10 strychnine; 1 CGP-55845 (in DMSO); 10 SR-95531. In a living mouse, spontaneous firing of serotonergic neurons is mediated by noradrenergic input (Levine and Jacobs, 1992). To mimic this noradrenergic input, α_1 adrenoceptor agonist phenylephrine (10 μM) was added to facilitate firing of serotonergic neurons.

Neurons were visualised by infrared differential interference contrast video microscopy with a Newicon C2400-07 camera (Hamamatsu, Hamamatsu City, Japan) mounted to an Axioskop microscope (Zeiss, Göttingen, Germany). Electrophysiological recordings were made with PatchMaster software on an EPC10 patch clamp amplifier (HEKA, Lamberecht, Germany). Patch pipettes were pulled from thick-walled borosilicate glass capillaries with a P-97 Brown-Flaming electrode puller (Sutter Instruments, Novato, CA, USA). The pipette had a resistance of 2-6 MΩ when filled with a HEPES-based ACSF-like solution (filtered by 0.2 μm pore filter) containing (mM): 125 NaCl, 10 HEPES, 2.75 KCl, 2 $CaCl_2$, 1.3 $MgCl_2$ (pH 7.4 with NaOH). The pipette was placed in an electrode holder containing a Cl-coated silver wire mounted on the headstage of the EPC10 amplifier. Slight positive pressure was given to the recoding pipette and lowered onto the surface of a neuron under the visual guidance using a motorised micromanipulator (Luigs and Neuman, Ratingen, Germany). Loose-seal (5-20 MΩ resistance) between the pipette and the cell surface was formed when the positive pressured was released and gentle negative pressure was applied to the pipette. Since the experiments depend largely on the synthesis of endogenous 5-HT, neurons located at least 50 μm below the surface of the slice were targeted.

Recordings were made in a voltage-clamp mode with pipette potential maintained at 0 mV. Signals were digitised at 10 kHz and filtered at 3 kHz. For action current shape measurement, signals were acquired at 100 kHz and filtered at 8 kHz. Recordings were aborted if firing rate was sensitive to changes in pipette holding potential or if shape of action current changed. Acquired data were analysed using Clampfit 9.2 (Molecular Devices, Foster City, CA, USA) and GraphPad Prism version 5.04 (GraphPad Software, San Diego, CA, USA). One

experiment was done per one slice to avoid aftereffect of any chemical application from the previous experiment.

2.3.4. Drugs

SR-95531 (= gabazine; $GABA_A$ antagonist >98%), D-AP5 (NMDA glutamate site antagonist >99%), DNQX (AMPA / kainate antagonist >99%) were purchased from Ascent Scientific Ltd. (Bristol, UK). CGP-55845 hydrochloride (selective $GABA_B$ antagonist) and R-8-hydroxy-2-(di-n-propylamino)tetralin (R-8-OH-DPAT; abbreviated as R8HD) were purchased from Tocris Bioscience (Bristol, UK). Strychnine (glycine and acetylcholine antagonist) and L-Phenylephrine-HCl were obtained from Sigma-Aldrich S.r.l. (Milan, Italy).

2.3.5. Identification of serotonergic neurons

i). Morphological & histological criteria

Serotonergic neurons have large cell bodies (15 – 35 μm) and have various forms; fusiform, triangular or multipolar (Li et al., 2001), while non-serotonergic neurons have relatively small cell bodies (~ 10 μm) (Liu et al., 2002), typically with a bipolar shape. Immunohistochemical study demonstrated that most serotonergic neurons are located along the midline of the DRN, whereas non-serotonergic neurons (such as GABAergic neurons) are distributed lateral to the midline and rather scattered (Brown et al., 2008; Day et al., 2004). In the rostral DRN, catecholaminergic neurons are also expressed along the midline but to a much lesser extent than serotonergic neurons (Day et al., 2004). Thus, it can reasonably be said that neurons with large soma located along the midline of DRN are highly likely to be serotonergic neurons.

ii). Electrophysiological criteria

Neurons were identified according to electrophysiological criteria (Vandermaelen and Aghajanian, 1983; Allers and Sharp, 2003). Neurons were classified as serotonergic if: 1) neurons displayed, during at least 5 min-long control period at the beginning of the recording, slow and steady firing rate of 1 to 4 Hz with coefficient of variation (COV) less than 15%; 2) the shape of action current had an asymmetric shape (ratio of upstroke to downstroke > 3.5) (Brown et al., 2002) with long Up-to-Downstroke Interval (UDI. see **Fig. 2.1**; >0.85 ms. Mathematically, this corresponds to action potential half-height width (HHW) measured in the whole-cell configuration. See (Bean, 2007)). Typically, serotonergic neurons have clear two downstrokes and UDIs are measured based on the second downstroke (referred to as UD_2I). Whenever possible, a 5-HT_{1A} receptor agonist R8HD (30 nM) was applied at the end

of experiment to see the cessation of firing to confirm that the recorded neuron was serotonergic.

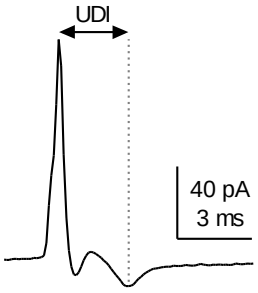

Fig. 2.1. Illustration of Up-to-Downstroke Interval (UDI) of action current measured in loose-seal cell-attached recording. Serotonergic neurons typically have clear two downstokes and thus UDIs are often expressed as UD_2I.

2.3.6. Quality control

i). Amount of inter-spike current (ISC)

In ideal loose-seal cell-attached configuration, the membrane of recorded neuron remains intact, reflected as minimal ISC (**Fig. 2.2.A**). Occasionally, as the time lapses during recording, ISC increases. In the present study, only recordings showing stable ISC or minimal changes were accepted, with an end amplitude of ISC below ~10 pA. Recordings showing greater ISC amplitude were discarded because an increase in ISC indicates possible deterioration of cell membrane under the recording, which may influence the firing rate of the recorded neuron. **Fig. 2.2.B** shows a non-ideal situation where the amplitude of ISC exceeds an acceptable range. The large ISC and thus damaged cell membrane results in unstable monitoring of cell firing rate, as shown in **Fig. 2.3**.

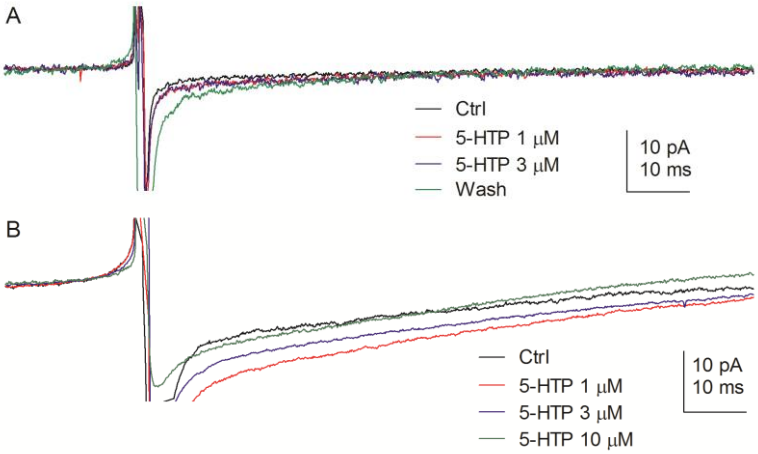

Fig. 2.2. Ideal and non-ideal recording configuration judged by the amplitude of inter-spike current (ISC). **A**) Ideal recording configuration with minimum ISC. ISC increased at a later stage of recording (Wash) but still in an acceptable range. **B**) Non-ideal configuration where ISC is notably large from the beginning of recording. Note that ISC became larger during the recording (5-HTP 1 μM, 5-HTP 3 μM) and later recovered to the control level (5-HTP 10 μM). Ctrl: control.

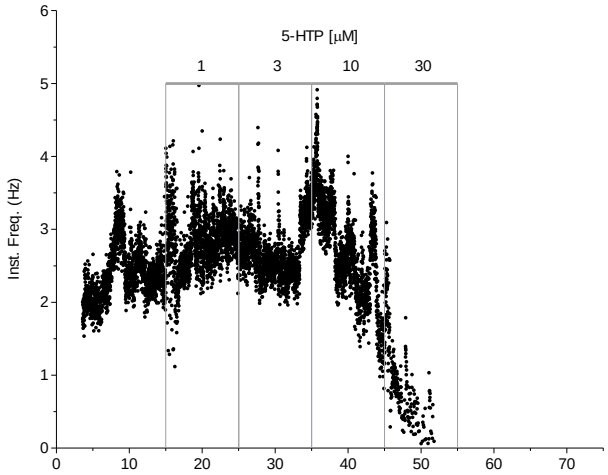

Fig. 2.3. Time course of firing rate change from the same neurons as in Fig. 2.2.B. Firing rate fluctuates substantially, which hinders stable measurement of firing rate. Note also that there is no recovery of firing after 20 mins of washing-out of 5-HTP.

ii). Action current shape should remain the same

As loose-seal cell-attached recording monitors firing rate from a neuron with intact cell membrane, the action current shape observed should keep the same shape throughout the experiment. **Fig. 2.4.A** shows a case where the action current shape changed significantly. The change in action current shape indicates that the integrity of cell membrane is not maintained and therefore the firing rate measured might have been affected by experimental manipulation. As shown in **Fig. 2.4.B**, loss of the membrane integrity results in unstable firing, especially evident in the control period (i.e. before tryptophan application) in this case.

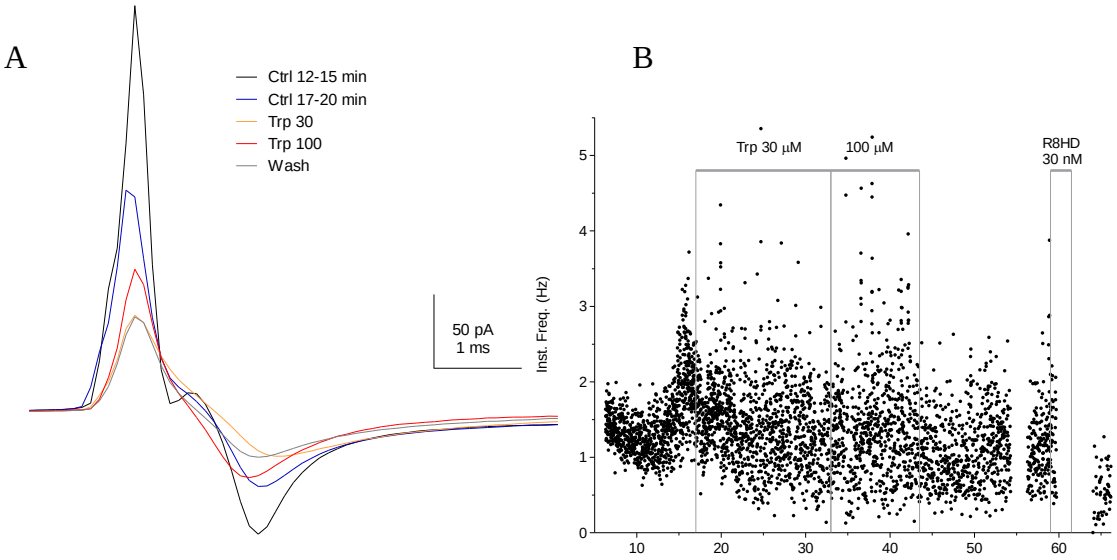

Fig. 2.4. Change in action current shape. **A)** Average action current shapes from each experimental procedure. Unless otherwise stated, traces were obtained by averaging traces in the last 3 mins of each experimental epoch (Trp 30 µM, 100 µM application etc.). **B)** Time course of firing rate change recorded from the same neuron as A. Trp: tryptophan. Ctrl: control. R8HD: R-8-OH-DPAT.

iii). Action current amplitude should be monitored to avoid false positive effects of drugs

In the present study, drugs applied all work on 5-HT_{1A} receptors either directly (R8HD) or indirectly (Trp, 5-HTP). Stimulation of 5-HT_{1A} receptors reduces/stops firing of a neuron by its hyperpolarising effect. Thus, decrease/cessation of firing should not be concomitant with decrease in action current amplitude (**Fig. 2.5**). In fact, due to its hyperpolarising effect, the action current of a recorded neuron tends to increase (**Fig. 2.6**). In the case of **Fig. 2.5**, firing frequency also accelerated despite the presence of increasing concentrations of R8HD, indicating that the healthiness of the recorded neuron started to deteriorate. When the decrease in firing rate is concomitant with decrease in action current, it is often the case that firing does not recover even after a long-duration washout of 5-HT_{1A} receptor agonists (**Fig. 2.5**).

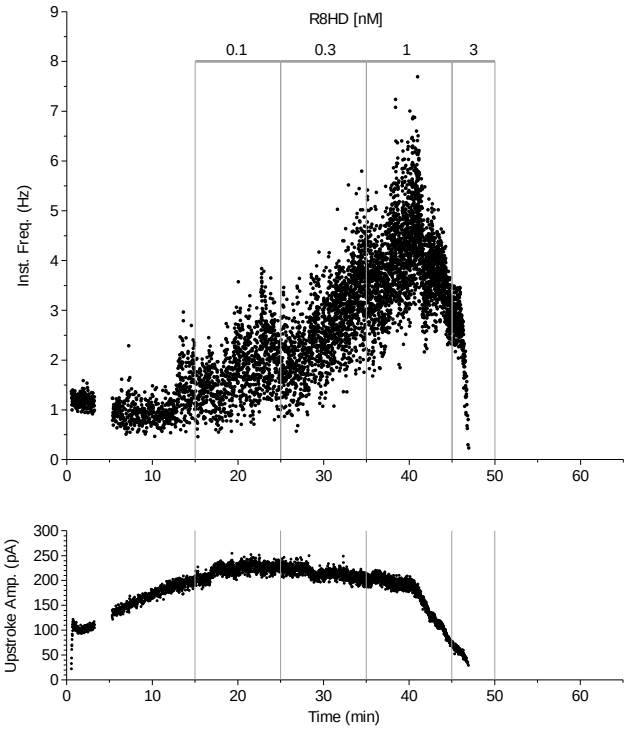

Fig. 2.5. Ceasing of firing was accompanied by decrease in action current amplitude. At around 41minutes of recording, firing frequency started to decrease, accompanied by gradual decrease in action current amplitude. Cessation of firing therefore is therefore likely to be due to the deterioration of cell healthiness. Note that firing did not recover even after 20 minutes of washout.

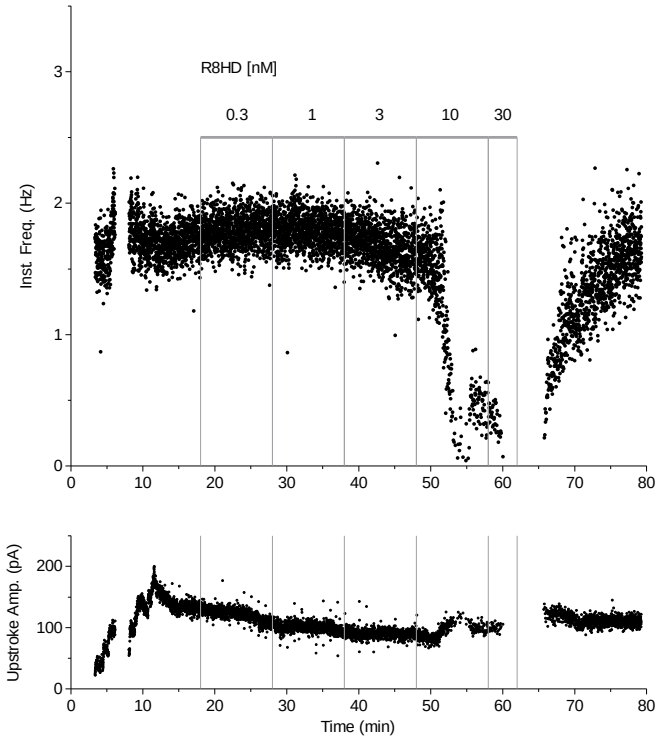

Fig. 2.6. Hyperpolarising effects of 5-HT_{1A} receptor agonist can be manifested as increase in action current amplitude. At around 51 minutes of recording, firing frequency started to decrease. This decrease in the firing rate is accompanied by a sudden increase in action current amplitude, reflecting the time when 5-HT_{1A} receptor agonists started to take their effects.

iv). I_{hold} should be monitored to prevent pipette current from affecting firing frequency

For recording spontaneous firing activities of neurons in the voltage-clamp mode of the loose-seal cell-attached recording configuration, the command potential should be adjusted so that no current flows through the recording pipette. This is because the current flowing through the pipette can change the membrane potential of the neuron being recorded and thus may affect the firing rate of the neuron. As a default, the command potential is set at 0 mV so that no current flows through the pipette. To assure that no current was actually flowing, the baseline current (I_{hold}) was continuously monitored (**Fig. 2.7**). In case the I_{hold} substantially deviated from 0 pA as a result of changes in the recording status, e.g. contact between the pipette and the cell membrane, healthiness of the neuron etc., the offset voltage of the voltage-clamp was adjusted so that no current flew. This was achieved by interrupting the recording for a few seconds and utilising the auto-offset function of the recording software (PatchMaster, HEKA).

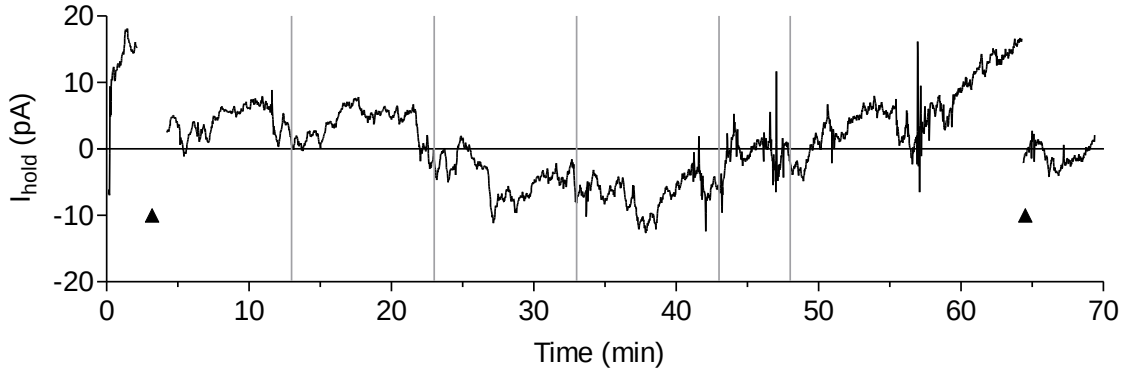

Fig. 2.7. The baseline current (I_{hold}) is kept near 0 pA. The figure shows the time course of I_{hold} change. ▲ indicates the time when the recording was interrupted and the I_{hold} was offset to 0 pA by adjusting the offset voltage of the voltage-clamp recording.

2.4. Whole-cell (WC) recording

2.4.1. Rationale

Whole-cell patch clamp recording is particularly suited to measure subtle electrophysiological properties of a single neuron in a detailed manner. In the whole-cell recording configuration, the voltage of the patched cell is under tight control, enabling experimenters to interpret conductance changes of a recorded neuron at given voltages. Such precise control of voltage results from formation of tight seal between the glass pipette rim and the cell membrane, commonly called "gigaseal" due to its resistance in the range of gigaohms (10^9 Ω). This is in a clear contrast to the sharp electrode recording technique, in which the electrode penetrates the cell and the seal around the pipette results imperfect, as revealed by the lower total membrane resistance measured compared with that found with whole-cell recording in the same type of neurons. In the whole-cell recording, on the other hand, the patch pipette seals onto the cell membrane, producing much less damage to the cell and inducing much smaller leakage currents at the electrode-membrane interface. For the formation of the whole-cell configuration, patched cell membrane is ruptured by negative suction applied by the experimenter. This inevitably involves exchange of the pipette solution with the cell interior. This has both advantages and disadvantages. One advantage is that the experimenter can control the interior of the cell and record from different neurons under the same cell interior condition. One of the major disadvantages is that the exchange of the cell interior may "washout" cellular components which may sustain biological phenomena under investigation.

For example, exchange of the cell interior with pipette solution may disrupt spontaneous firing of serotonergic neurons or formation of LTP. Reasonably enough, the use of whole-cell patch clamp in the present study was thus limited to the measurement of biophysical properties of neurons within a rather short recording time to minimise this washout effect. The sensitivity of the whole-cell recording for measuring electrophysiological parameters in a detailed manner at the same time renders this methodology sensitive to potential errors. From the perspective of circuitry, the whole-cell recording uses a single electrode for continuously measuring voltage from the cell and injecting current into the cell to maintain the membrane potential at the intended value. This is in contrast to two-electrode voltage clamp or single-electrode switch clamp, which, respectively, uses two electrodes for voltage measurement and current injection or one electrode does two duties intermittently. The circuitry of the whole-cell recording therefore introduces an unknown and unstable source of voltage drops across the so-called "series resistance" which is comprised from both the electrode resistance and the resistance derived from the pipette's access to the cell. For this reason, a particular attention was paid to the quality of the recording data, in order to draw reliable conclusions obtained from the experimental data.

I). Dorsal raphe nucleus recording

2.4.2. Materials and Methods

i). Animals

Dorsal raphe serotonergic neurons from WT and Tph2 KO mice, aged at P21~P32, were used for the investigation.

ii). Preparation of dorsal raphe nucleus slices

The procedure follows that of cell-attached recording.

iii). Electrophysiological recording

The overall recording procedure and setup follows that of loose-seal cell-attached recording. The temperature of perfusing ACSF was set at 29-30 ℃ and supplemented with a mixture of neurotransmitter blockers (see method for cell-attached recording) without phenylephrine. Patch-pipettes were filled with the following intracellular solution (IC) (mM): 120 K-gluconate, 15 KCl, 2 $MgCl_2$, 0.1 EGTA-K_4, 10 HEPES, 10 Na_2-phosphocreatine, 0.3 Na_3-GTP, 4 Mg-ATP (pH 7.3 with KOH, 320-330 mOsm, filtered by 0.2 μm pore filter). The

pipette had a resistance of 2-4 MΩ. Gigaseal (> 1 GΩ resistance) between the pipette and the cell surface was formed when the positive pressured was released and gentle negative pressure was applied to the pipette. Data were acquired through PatchMaster software on an EPC10 patch-clamp amplifier (HEKA, Lamberecht, Germany) and analysed by Clampfit 9.2 software (Molecular Devices, Foster City, CA, USA).

2.4.3. Parameters measured

From each whole-cell recording, the following parameters were measured to characterise electrophysiological properties of a single neuron.

i). Resting membrane potential (V_m)

V_m's were measured several times during the recording whenever a current-clamp protocol without current injection was applied. Such protocols were intentionally executed (named "C-Rest") or were present at the beginning of protocols intended to measure some other parameters, e.g. "C-Search", "C-IV-Dep", "C-IV-Hyp", and "C-Hyp 5x" protocols (see below for the meanings of these protocols). The first V_m was measured immediately after the break-in of the cell (initial V_m). Other V_m´s were measured some time after the break-in of the cell when the cell interior was already equilibrated with the pipette solution. The latter was obtained not immediately after the break-in but from a relatively early stage of the recording (from 20 sec up to 4 mins after the break-in). Preferably, it was obtained immediately before/after the "V-Access" protocol where R_m, C_m and R_s values were obtained (see the next section). Normally V_m values except the initial V_m were more or less the same since the cell interior was already dialysed and therefore a single representative V_m value was chosen as "stable V_m". The initial V_m reflects a resting membrane potential before cell dialysis and therefore indicates V_m at the minimum experimental manipulation. On the other hand, the stable V_m reflects the stabilised resting membrane potential after cell dialysis and also most of the following measurements were done under this V_m value. Therefore both the initial and stable V_m's are of relevance to characterise an electrophysiological property of a single recorded neuron and thus both were taken into account to see differences between genotypes.

ii). Series resistance (R_s), membrane resistance (R_m), cell capacitance (C_m), time constant (τ)

These parameters were measured in a voltage-clamp mode by applying a square-shaped voltage pulse (ΔV = -10mV, 100 ms, called "V-Access" protocol) from a holding potential (V_{hold}) of -65mV. Since a cell membrane works as a capacitor, current flow becomes large when the voltage change is large. This is expressed as the following equation.

$$I = C \frac{\Delta V}{\Delta t}$$

(I: current (A), C: capacitance (F), V: voltage (V), t: time (second))

In the case of the protocol adopted in the present study (V-Access), the voltage change is the biggest at the beginning and the end of the voltage pulse. Therefore large current flows are observed at these time points (denoted as I_{in}). In the middle of the voltage pulse, current response becomes steady (I_{ss}) (**Fig. 2.8**). In the actual protocol used, this square-shaped voltage pulse was given five times (inter-pulse interval of 4 seconds) and responses were aligned at the baseline and averaged. I_{in} and I_{ss} values were taken from the averaged trace. I_{in} values for measurement were taken only from the first I_{in}, i.e. when the current injection was started. I_{ss} values were taken as the average response at the last 40 ms of the voltage pulse.

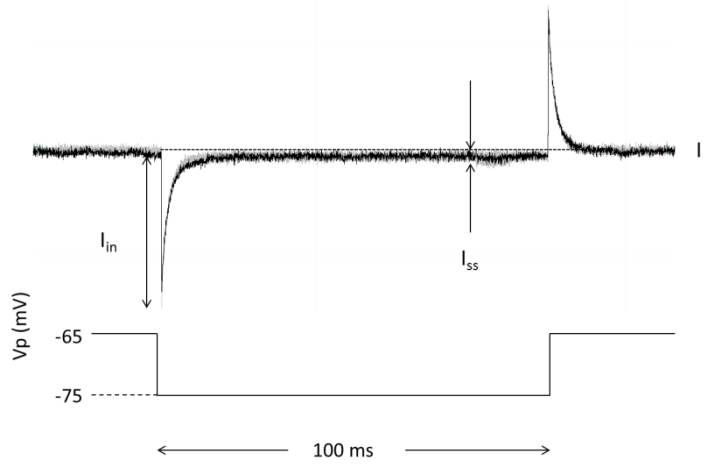

Fig. 2.8. Capacitive current in response to a voltage step. The recorded neuron was voltage-clamped at its resting membrane potential (-65 mV). A voltage step of -10 mV was applied for 100 ms to see the capacitive current. Current flow at the beginning and end of the voltage step is termed "instantaneous current" (I_{in}). Current during the voltage step is named "steady state current" (I_{ss}). V_p: commanded pipette voltage.

Theoretically, R_s and R_m are calculated as follows:

$$R_s = \Delta V / I_{in}$$

$$R_m = \Delta V / I_{ss}$$

On the other hand, τ of mono-exponential decay is defined as the time required for I_{in} to decay to 37 % (= 1/*e*) of its peak value. (**Fig. 2.9**)

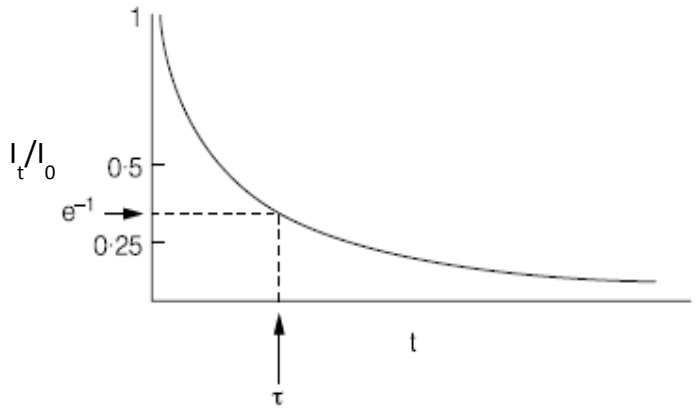

Fig. 2.9. Time course of current decay with time t (I_t). The time constant (τ) of the decay is defined as a time required for the current to reach 37% (= 1/*e*) of its initial value (I_0).

τ is experimentally measurable and is mathematically approximated as follows:

$$\tau = R_s \cdot C_m$$

Therefore, C_m can be calculated. Alternatively, C_m values can be obtained as C-Slow values from the auto compensation function of HEKA PatchMaster software by pressing "C-Slow Auto" button during the recording.

iii). Action potential (AP) shape (amplitude, HHW, τ of AHP)

If the neuron was spontaneously firing in a current-clamp mode without current injection, action potential shapes were measured from the averaged traces of those spontaneous spikes. If the neuron was not spontaneously firing, stepwise current injection (called "C-Search" protocol; start from 5 pA with the increment of 5 pA, 10 seconds duration) was conducted until repetitive firing of minimum 4-5 spikes occurred (firing rate must fall in approximately 0.4-1.5 Hz). In order to measure the action potential shape under similar conditions between different cells, either positive or negative current was injected at the resting state prior to the step-wise current injection so that the resting membrane potential is set at around -65 mV. Action potential shapes were measured from the averaged traces of the repetitive firings at the minimum current injection. When averaging the traces, the first two action potentials were excluded (in case there were only a few spikes in total, only the first one was excluded) because they had a different shape from the other action potentials. Since current injection affects the shape of action potentials, firing frequency in response to current injection was

noted as an indication of intensity of current for the cell. If the spike shape was averaged from the third to the last spike during current injection, interval between the second to the last spike was measured and divided by the number of intervals to calculate average inter-spike interval where action potential shape was taken out. Then the firing frequency was expressed as the inverse of this average inter-spike interval.

Action potential amplitude is defined as a voltage difference between the highest voltage of the upstroke and the lowest voltage of the downstroke (**Fig. 2.10**). HHW is defined as the width of the action potential at its medial amplitude (in ms). HHW is dependent on the kinetics of the repolarising phase of an action potential: when the repolarisation is fast, HHW becomes short, when slow, becomes long. Since the repolarising phase of an action potential results from inactivation of voltage-gated Na^+ channels and activation of voltage-gated, delayed rectifier K^+ channels, differences in HHW may indicate changes in these channel functions. For this reason, HHW serves as a useful parameter to compare electrophysiological properties of neurons between genotypes. Moreover, for recording from neurons in the DRN, HHW serves as a criteria to differentiate serotonergic neurons from non-serotonergic neurons. Namely, serotonergic neurons have larger HHW, typically more than 1 ms, whereas non-serotonergic neurons have narrower HHW.

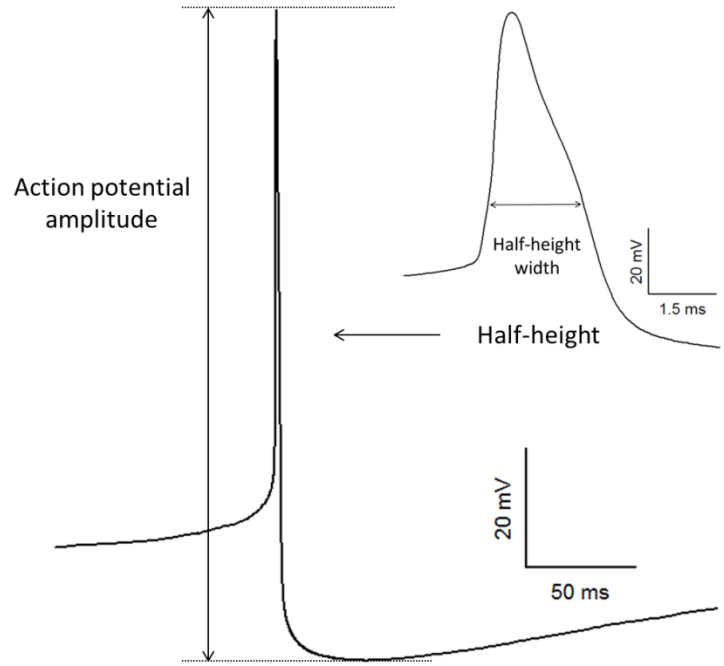

Fig. 2.10. Parameters measured from an action potential shape. Action potential amplitude is defined as the difference between the peak and the anti-peak of an action potential. Action potential half-height width (inset) is defined as the width of the action potential at its medial amplitude (in ms).

Time constant (τ) of AHP is measured by fitting two-component exponential curves. This is because one-component fit did not produce any reliable fit and there was no noticeable improvement in fitting when the number of components was increased to three. The starting point of AHP is set at the end of a shoulder of an action potential. To specify this time point objectively, the starting point of AHP was set at the time defined as "peak-time of AP + HHW". Mathematically, HHW corresponds to UD_2I of the first derivative of APs as is experimentally observed in the cell-attached recoding configuration. Since the upstroke in the first derivative corresponds to the peak of AP and the two downstrokes in the first derivatives derive from the shoulder of AP measured in the WC mode, the time at "peak-time of AP + HHW" reliably corresponds to the end of shoulder. On the other hand, the end point of AHP fitting was set at 90% of the anti-peak. Setting the end point at the peak of AHP is vulnerable to potential errors because the voltage trace near the peak is flat and slight fluctuation of voltage, either biological or by noises, influences the position of absolute peak. Moreover, at the peak of AHP, it is possible that the recovering process from AHP has already started and this may affect time constants measured when the end point was set to the peak of AHP. For these reasons, it was considered reasonable to set the end point of AHP time constant measurement at 90% of absolute peak value.

From each exponential fit to the AHP, two values were noted for comparison among cells: the time constant of the first component (τ_1) and the time constant of the second component (τ_2).

iv). Maximum firing rate

Maximum firing rate was measured in a current-clamp mode in response to step-wise current injection (called "C-IV-Dep" protocol; start from 20 pA, 20 pA increments, 3000 ms duration, 12-second intervals between current injections; **Fig. 2.11**). In order to measure the maximum firing rate under similar conditions between different cells, either positive or negative current was injected at the resting state prior to the step-wise current injection so that the resting membrane potential is set at around -65 mV. Normally, when the amount of current injection was increased, the number of action potential during the current injection increased accordingly. However, the action potential number near the end of current injection becomes constant (i.e. saturated) after some amount of current injection and the increase in the number of action potentials was observed only at the beginning of current injection. Of interest was the highest action potential frequency of this constant phase near the end of current injection, which was termed "maximum sustained firing rate". The maximum sustained firing rate was calculated in the following way. First, the time span between the first and the last action

potential within the one-second segment was measured (ms). Then this time span was divided by the number of inter-spike intervals (= the number of action potentials -1) to calculate the mean inter-spike interval in time (ms). Then, the maximum sustained firing rate was calculated as the inverse of this averaged inter-spike interval.

The maximum sustained firing rate was calculated in this way rather than simply counting the number of action potentials in the last one-second segment. This is because, in the latter way, the firing frequency will be affected by where the first or the last action potential is positioned in this one-second segment. In other words, if the action potential is located slightly outside of this one-second segment, it substantially reduces the firing frequency because the next action potential comes with a certain interval. This is purely artificial outcome imposed by setting the one-second segment, and therefore this method for calculating the maximum frequency was discarded.

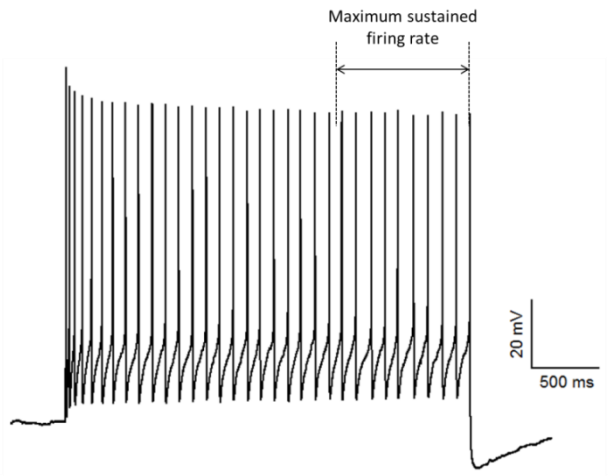

Fig. 2.11. The maximum sustained firing rate. The maximum sustained firing was obtained from the last 1 second segment of step-wise current injection where the firing rate reached a plateau after a certain amount of current injection (typically 200 pA).

v). Sag amplitude

When a neuron is hyperpolarised by a negative current injection, I_h current flows through HCN channels and exhibits counteracting depolarisation. This is called "sag" (**Fig. 2.12**). A sag was induced by injecting hyperpolarising current (3000 ms duration) which reaches a voltage between -110 and -120 mV from a holding potential between -60 and -70 mV ("C-Hyp 5x" protocol). In practice, current injection was repeated five times and the measurement was made on the averaged trace. Prior to this protocol, stepwise negative currents were injected (called "C-IV-Hyp" protocol) to know the amplitude of hyperpolarising current required to reach the aimed voltage (-110/-120 mV). A sag (V_{sag}) was defined as a difference between the voltage at the end of current injection (V_{end}) and the maximum negative voltage reached (V_{peak}) as a result of current injection. Thus,

$$V_{sag} = V_{end} - V_{peak}$$

For each sag measurement, amount of current injected during the protocol as well as resting membrane potential (average of 5 traces) and associated current injection prior to the initiation of the protocol were noted.

Both maximum sustained firing rate and sag serve as parameters to identify serotonergic neurons (see the following section).

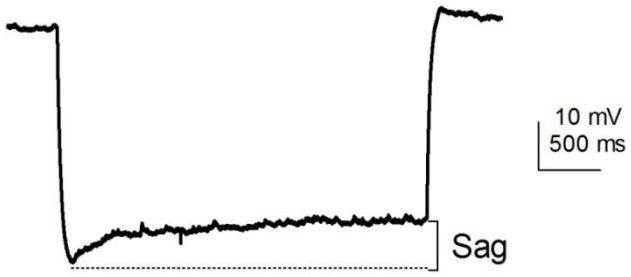

Fig. 2.12. Sag in response to negative current injection. Sag is defined as voltage difference between the negative peak voltage and the voltage at the end of current injection.

2.4.4. Identification of serotonergic neurons in the WC recording configuration

i). Morphological & histological criteria

The same criteria as for the cell-attached recording apply here.

ii). Electrophysiological criteria

Putative serotonergic neurons were identified when the cell had 1) a long-duration action potential (HHW > ~ 1 ms) with a 'shoulder' during the repolarising phase, 2) high input resistance (> 200 MΩ), 3) large and slow (10-20 mV, 200-800 ms) AHP, 4) pacemaker firing activity either at rest or in response to current injection (Liu et al., 2002; Sharkey et al., 2008; Ohliger-Frerking et al., 2003). Mathematically, HHW corresponds to UDI as measured in cell-attached recording. Therefore, a wide action potential HHW is in accordance with long UDI, which is used as a criterion to recognise serotonergic neurons in cell-attached recording.

On the other hand, non-serotonergic neurons have fast-spiking, short-duration action potential comprising HHW of 0.3-0.5 ms (Liu et al., 2002) or up to 0.9 ms (observation in the laboratory of Prof Corradetti) with shorter AHPs. In response to high current injection, non-serotonergic neurons often cease firing, unlike serotonergic neurons, which maintain its regular firing patterns (reflected as "maximum sustained firing rate"; observation in the laboratory of Prof Corradetti).

Another feature of serotonergic neurons is that they express rich population of 5-HT_{1A} receptors. This leads to the high sensitivity of these neurons to a selective 5-HT_{1A} receptor agonist, such as R8HD. Typically, 30 nM of R8HD is sufficient to cease the firing of serotonergic neurons by its hyperpolarising action, whereas non-serotonergic neurons do not respond to such low concentration of R8HD.

Moreover, while non-serotonergic neurons express HCN channels, serotonergic neurons lack or if at all present only weakly express HCN channels. As a result, when hyperpolarising current is injected, non-serotonergic neurons exhibit prominent sags (Li et al., 2001; Brown et al., 2008), which may sometimes exceed 10 mV (observation in the laboratory of Prof Corradetti).

2.4.5. Quality control

Since the whole-cell recording measures subtle electrophysiological changes, the quality of recording is crucial. Here is the list of criteria to be considered for most of the cases (Molleman, 2003).

i). The series resistance should not exceed ~20 MΩ throughout the recording.

Since the resistance of the pipette itself does not change during the recording, a high series resistance indicates a high access resistance. This causes a serious problem in the voltage-clamp because the access resistance and the membrane resistance in succession work as a voltage divider and the command voltage will be divided to two resistances proportionally to their resistance values. In other words, if the access resistance is high, it is not possible to clamp the potential of a cell to the intended value. Increase of the access resistance often occurs as a result of gradual resealing of the patch after breakthrough of the membrane and manifested as the decrease of I_{in} in the "V-Access" protocol mentioned above. If this was the case, subtle suction was applied to reduce the access resistance. In the present study, data with series resistance of at around 25 MΩ were sometimes included but in any case no data were included when the series resistance exceeded 30 MΩ.

ii). The membrane potential should be more negative than -50 mV

This is the case when high-potassium intracellular solution as in the current study (K-gluconate) is used. Resting membrane potential more positive than -50 mV may indicate a disrupted patch and thus all the subsequent measurements become unreliable.

iii). Membrane capacitance and resistance must be stable at the steady state

Together with access resistance, these two parameters were repeatedly monitored throughout the recording by applying the "V-Access" protocol several times. "Spontaneous" decrease in cell membrane resistance indicates that the neuron has become "leaky". This implies that either the integrity of the cell membrane was disrupted, or that a set of channels was durably open, due to change of their properties or to the action of an unknown endogenous substance/neurotransmitter occasionally released following experimental manipulations. Experiments showing unexpected decrease in cell membrane resistance were discarded. Decrease in the membrane resistance can be observed as an increase in the ohmic current, i.e. I_{ss} value in response to a voltage step (see the preceding section "parameters measured").

II). Hippocampus recording

Overall procedures are the same as those of DRN recording. Here only those that differ from DRN recording are mentioned.

2.4.6. Animals

CA1 pyramidal hippocampal neurons from three different genotypes of 5-Htt transgenic mice, i.e. WT, Het, and KO, aged at P14~P18, are used for the investigation.

2.4.7. Preparation of hippocampal slices

Brain slices of 300μm thickness (typically F46-52 of Mouse Brain Atlas by Paxinos and Franklin, 2001) are made with a vibratome (VT1000S, Leica, Nussloch, Germany) in ice-cold sucrose based artificial cerebrospinal fluid (Sucrose-ACSF (mM): 210 sucrose, 26 $NaHCO_3$, 1.3 $MgSO_4$, 1.2 KH_2PO_4, 2 $MgCl_2$, 2 KCl, 2 $CaCl_2$, 10 glucose), equilibrated with carbogen (95% O_2／5% CO_2). Slices were then transferred to ACSF (124 NaCl, 26 $NaHCO_3$, 2 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 2 $CaCl_2$, and 10 glucose; pH 7.4), equilibrated with carbogen and incubated for one hour at 32℃.

2.4.8. Electrophysiological recording

Slices were then transferred to a recording chamber, constantly perfused with ACSF, equilibrated with carbogen. The whole-cell patch clamp technique is applied with a borosilicate glass pipette of 3~5MΩ, filled with the following intracellular solution (IC) (mM): 140 K-gluconate, 5 NaCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 10 HEPES, 2 K_2-ATP, 0.5 Na-GTP, pH 7.2. 0.2% Neurobiotin (Vector Laboratories, Inc., Burlingame, CA, USA) was added to the IC for the later morphological reconstruction. Pyramidal neurons in the CA1 area were differentiated from interneurons primarily based on their location, i.e. stratum pyramidale. Moreover, under infrared DIC microscopy, pyramidal neurons were often visible with apical dendrites passing through the stratum radiatum, whereas interneurons typically have dendrites projecting in many directions (Nishikawa and MacIver, 2000). Electrophysiologically, pyramidal neurons exhibit spike frequency adaptation in response to depolarising current injection with gradual decrease in spike amplitude. On the contrary, interneurons have action potentials of short duration and sustain high frequency firing with minimum reduction in amplitude in response to depolarising current injection (Nishikawa and Maclver, 2000; Zhang et al., 2012; Frazier et al., 1998). In the present study, depolarising step-wise current was injected to count action potential numbers and to measure action potential thresholds. The identity of pyramidal neurons was at the same time confirmed by the presence of spike frequency adaptation.

In order to measure excitatory postsynaptic current (EPSC), the Schaffer collateral/commissural pathway from CA3 to CA1 was stimulated with a concentric bipolar electrode (SNE-100X100 mm, Rhodes Medical Instruments (RMI), Woodland Hills, CA, USA). Stimuli were triggered by PULSE software (HEKA, Lamberecht, Germany) and delivered by a stimulus isolator (ISO-FLEX+, A.M.P.I., Jerusalem, Israel). Stimulus amplitude was gradually increased and the maximum EPSC amplitudes were compared among genotypes. In addition to absolute EPSC amplitude, paired-pulse ratio was calculated as a ratio of the second EPSC to the first EPSC when the two stimuli were given at a short interval (50 ms). Since paired-pulse ratio was relatively constant across different stimulus amplitudes, several responses at different stimulus amplitude were averaged as a representative value. Data acquisition and analysis were conducted with PULSE/PULSEFIT software on an EPC9 or EPC10 patch-clamp amplifier (HEKA, Lamberecht, Germany).

2.4.9. Staining procedures

After the recording, slices are fixed overnight at 4^{o}C in 0.1 M phosphate buffered saline (PBS; **Table 5**) containing 4% paraformaldehyde. After fixation, slices are washed several times in PBS and this is done between each step afterwards. Slices are then pretreated by 1% H_2O_2 for 30 mins to neutralise endogenous peroxidase. Slices are incubated for 1 day at room temperature in 1/100 avidin-biotin peroxidase complex (Vector Laboratories, Inc., Burlingame, CA, USA) diluted in PBS containing triton 0.3%. Slices are incubated in PBS containing 0.04% 3-3'-diamino-benzidine-HCl (DAB; Sigma) and 0.006% H_2O_2. Slices are placed onto glass slides and covered with coverslips using Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA). Coverslips were then fixed by nail varnish.

Table 5. PBS (0.1 M = 10X)

- 12.4 g $Na_2HPO_4.2H_2O$
- 3.8 g $NaH_2PO_4. H_2O$
- 800 ml distilled water
- Adjust the pH to 7.4
- Add distilled water till 1000 ml

2.5. Extracellular field potential recording

2.5.1. Rationale

Extracellular field potentials are electrical fields measured in the extracellular space, which originated from the activity of single neurons or groups of neurons. The activity of neurons is typically evoked by synaptic or antidromic stimulation. The histological arrangement of the hippocampus makes this neuronal region particularly suited for the application of extracellular field potential recording. Namely, the dense laminated packaging of cell bodies, the approximate parallel positioning of apical dendrites, ant the ease with which neurons can be synchronously activated (Andersen et al., 2007). When recordings are made from the CA1 region of the hippocampus, stimulation of the Schaffer collateral/commissural pathway produces an EPSP in the dendrites. Extracellularly recorded form of this signal (field EPSP; fEPSP) possesses negativity due to the current influx into the activated dendrite (current sink). If the synaptic stimulation is sufficiently strong, this causes an action potential in the soma, called a population spike (pSpike). The pSpike then induces current influx into the soma. Due to the dipole nature of the soma and the dendrite, current influx in the soma results in the

outflow of the current in the dendrite (current source). Extracellularly, this is observed as positivity in the field potential. The summation of fEPSP and pSpike often results in a tri-phasic shape, positive deflection in the middle of negatively going fEPSP. For this reason, it is common to use the slope of fEPSP as an index of the strength of synaptic communication since the absolute amplitude of fEPSP tends to be contaminated by pSpike.

In the extracellular field potential recording, neurons are not directly impaled as in the intracellular recording nor are the intracellular components exchanged as in the whole-cell patch clamp. Therefore, extracellular field potential recording enables stable and long-duration recording of synaptic activity, which is a prerequisite for investigating LTP. Taken together, the highly laminated structure of the hippocampus, and the stability and reliability of field potential recording make this method ideal for investigating LTP.

2.5.2. Preparation of hippocampal slices

Slice preparation procedures followed those described in Morini et al. (2011). Mice (from 6 weeks up to 4 months) from both 5-Htt KO and Tph2 KO lines were anesthetised with isoflurane and decapitated. The whole head was rapidly immersed in ice-cold artificial cerebrospinal fluid (ACSF) comprising the following (mM): 124 NaCl; 2.75 KCl; 1.25 NaH_2PO_4; 26 $NaHCO_3$; 1.3 $MgSO_4$; 2 $CaCl_2$; 11 D-Glucose. The ACSF was continuously bubbled with a gas composed of 95% O_2 and 5% CO_2 (pH 7.4). After taken out from the skull, the whole brain was cut into two hemispheres. First, the fimbria was cut by a surgical knife. Then, a plastic scalpel was inserted below the hippocampus and scraped it out from the cortex. The hippocampus was then placed on an agar bed on a chopping plate. Transversal hippocampal slices of 400 µm thickness were made with a Mcllwain tissue chopper (Gomshall, UK) from the ventral pole. Typically, the first two slices were discarded and from the third to ~tenth slices were taken and transferred to a multi-well incubation chamber filled with bubbled ACSF at a room temperature. Slices were incubated at least 1.5 h before the recording. Typically, the fourth to the eighth slices were used for the experiments.

2.5.3. Electrophysiological recording

After the incubation time, a slice was transferred to a recording chamber consisting of a nylon mesh, continuously superfused by oxygenated ACSF at 33 °C (2 ml/min). The composition of ACSF for recording follows that of ACSF used for dissection of brain and for incubating brain slices after cutting but the concentration of Ca^{2+} is increased to 2.5 mM. A cut was made

between the CA1 and CA3 area to prevent recurrent propagation of action potentials (**Fig. 2.13**). A stimulus electrode (twisted bipolar nichrome electrode) was placed on the Schaffer collateral/commissural pathway and electric pulses (80 μs duration; 15 s interpulse interval) were given through a stimulus isolation unit (DS2, Digitimer, Welwyn Garden City, UK), triggered by PC running WinLTP software (Anderson and Collingridge, 2007) to elicit postsynaptic responses of CA1 pyramidal neurons (**Fig. 2.13**). Prior to the recording, the tip of an electrode filled with NaCl was broken by touching a nod of the nylon mesh so that the duration of the stimulus artefact becomes less than 1 ms (~0.8 ms) (2-10 MΩ resistance). Field EPSP was recorded by placing this glass electrode in the distal third of the stratum radiatum. The magnitude of fEPSP was determined as the slope of the 30-60 % segment of the falling phase of fEPSP. Recorded signals were amplified with Neurolog NL 104 amplifiers (Digitimer, Welwyn Garden City, UK), digitised with TL-1 interface (Molecular Devices, Foster City, CA, USA) at the sampling rate of 10 kHz. At the beginning of each recording, a stimulus-response curve (SRC) was made by gradually increasing stimulus intensity (1-6 V (1V increment), 8, 10, 12, 15, 20 V) to know the maximum slope one can obtain from the respective recording. The stimulus intensity of test pulses was then adjusted to evoke fEPSP that has a slope corresponding to 25-40 % of the maximum slope and was kept constant throughout the experiment. Before commencing LTP induction, baseline responses were collected at least for 20 min. LTP was induced by theta-burst stimulation consisting of 5 bursts of 5 stimuli (100 Hz intraburst frequency, 5 Hz burst frequency), called TB5. 45 min after LTP induction, SRC was made again and those recordings in which the maximum slope changed more than 10 % before and after LTP induction, indicating probable artificial changes in recording conditions, were discarded. Then, 10 bursts of 5 stimuli (the same intra- and inter- burst frequency as TB5), called TB10, were given three times with 20 sec interval to know the maximally inducible LTP in each experiment.

This theta-rhythm-based LTP protocol used in the present study, i.e. TB5 and TB10, is based on physiological patterns of activation in the CA1 pyramidal neurons during learning-correlated behaviour (Morini et al., 2011 and references therein). Compared to the classical high-frequency stimulation protocol, the theta rhythm-based protocol requires significantly lower levels of activation of synaptic afferents, reflecting a facilitatory role of hippocampal theta oscillation in memory acquisition (Morini et al., 2011). The theta-patterned stimulation used in the present study made it possible to detect subtle potential changes in synaptic plasticity that could have been masked if protocols producing maximal LTP had been applied. This is because the magnitude of LTP induced by theta-patterned burst stimulation can be

graded based on the number of theta bursts (i.e. TB5 or TB10), and TB5 induces sub-maximal levels of LTP, allowing for quantification of LTP inducibility.

The summary of experimental protocol is illustrated as **Fig. 2.14**. Data were acquired through WinLTP software (Anderson and Collingridge, 2007) and analysed by WinLTP and GraphPad Prism 5 version 5.04 (GraphPad Software, San Diego, CA, USA).

2.5.4. Quality control of LTP data

Data which did not satisfy the following criteria were discarded from further analyses:

- Linear regression of baseline measurement (BSL) should indicate that its slope is not statistically different from zero.
- Stimulus artefacts and afferent volley (see **Fig. 3.34**) should always be constant
- Maximum response in the SRC before/after TB5 should not change more than 10%
- BSL should be 25-40% of Maximum response.

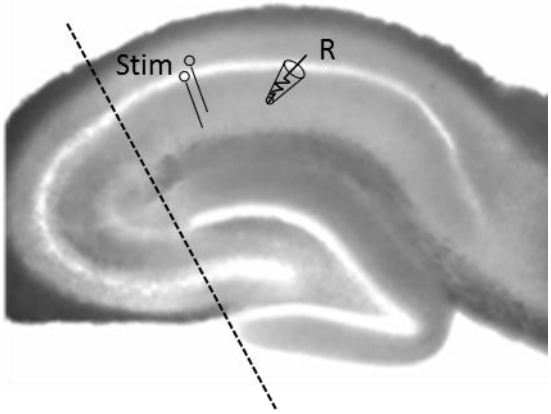

Fig. 2.13. Arrangement of the stimulus electrode (Stim) and recording electrode (R). Stimulus electrode was placed on the Schaffer collateral/commissural pathway and the recording electrode was positioned in the stratum radiatum of the CA1 field. The dashed line indicates a cut made between CA3 and CA1 area to prevent recurrent propagation of action potentials.

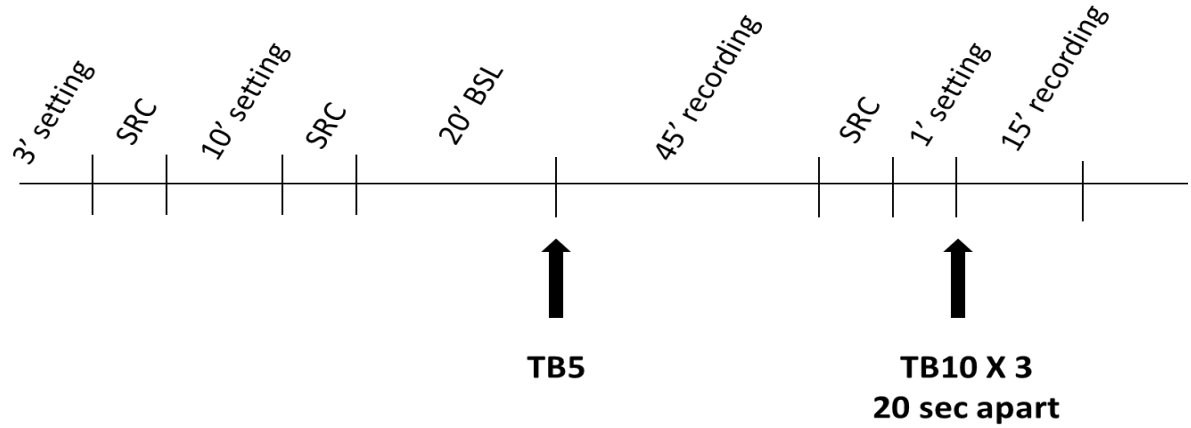

Fig. 2.14. Illustration of recording procedure. Immediately after starting recording, 3 mins of steady state recoding was conducted to stabilise the whole recording system (3' setting). Then, 1st Stimulus Response Curve (SRC) was conducted and repeated after 10 mins of setting time. After the 2nd SRC, 20 mins of Baseline measurement (BSL) was conducted, followed by TB5 stimulation. 45 mins of steady state recording was done to see the magnitude and maintenance of LTP induced by TB5. SRC was repeated after this to see if the max response remained the same. After 1 min of setting time, TB10 was delivered three times with 20 second intervals to induce the maximum LTP. 15 mins of stable recording followed this to see the time course of maximum LTP induced.

2.6. A dose-response curve and EC_{50}

2.6.1. Rationale

i). A dose-response curve

A dose-response curve (DRC) shows the relationship between drug dose or concentration and response to such drug treatment. In *in vitro* experiments, experimenters normally know the concentrations of drugs they applied, so the term "concentration-response curve" is also used as an alternative for DRC. Typically, DRC is used to describe actions of agonists (or antagonists) when they are bound to their receptors. DRC typically have a standard sigmoid curve (when plotted on a semi-log scale), which is quite similar to the plot describing relationship between agonist concentration and receptor occupancy (receptor binding curves). It should be noted, however, that DRC is not the same as receptor binding curves since the relationship between agonist binding and its response is not necessarily directly proportional in biological systems. This is typically the case when the response is mediated by a second messenger system. This applies to 5-HT_{1A} receptor action, too (see Introduction). In practice, however, when the production of second messengers is proportional to receptor occupancy and then the binding of second messengers to their effectors is proportional to second

messenger concentrations, the resulting DRC still has a typical sigmoidal shape as a receptor binding curve. Sensitivity to a drug acting at a specific, saturable receptor typically spans several orders of magnitude. For this reason, DRCs are normally semi-logarithmic, i.e. concentrations of drugs are converted to their logarithmic values, resulting in a typical sigmoidal curve. To be equally spaced on a logarithmic scale, doses of agonists typically have such values as 1, 3, 10, 30, and 100 μM, which when covered to their logarithms produce 0.0, ~0.5, 1.0, ~1.5, and 2.0. (Motulsky and Christopoulos, 2003)

ii). EC_{50}

A standard DRC is defined by the following four parameters: the maximum response (Top), the baseline response (Bottom), the slope (Hill slope), and EC_{50}. EC_{50} is defined as the dose of agonist that produces a response half way between the Bottom and the Top. In the present study, the term "EC_{50}" was used in the sense of "median effective concentration" regardless whether it is excitatory or inhibitory. If the data is normalised by its maximum response and its baseline is subtracted, EC_{50} corresponds to the concentration that gives 50% of the maximum response. If there is baseline activity which is not subtracted, this is not necessarily the case, e.g. if the Bottom is 20%, EC_{50} corresponds to 60% of the maximum response. EC_{50} is one of the most commonly used measures to describe agonist's potency since it defines the location of the DRC for a particular drug. The Hill slope on the other hand, defines the shape of DRCs; the higher the Hill slope, the steeper the curve. In the case of antagonist action, the Hill slope has a negative value (Motulsky and Christopoulos, 2003).

2.6.2. Procedure

DRCs were produced for R8HD and 5-HTP application in the cell-attached recording, using GraphPad Prism version 5.04 (GraphPad Software, San Diego, CA, USA). From each single experiment, the number of firing at the last one minute segment of each experimental epoch, i.e. baseline, R8HD 0.3 nM, 1 nM etc., was counted and divided by time (i.e. 60 sec) to produce average firing rates of each epoch. These values were then plotted against logarithms of agonist concentration. The following equation (called "four-parameter logistic equation" a.k.a. "variable slope sigmoid equation") was fit to the data to find the best fit curve:

$$Y = Bottom + \frac{(Top - Bottom)}{1 + 10^{(LogEC_{50} - X) * HillSlope}}$$

(Y: Response, X: concentration of agonists)

The fitted curve is the curve that minimises the sum-of-squares of the distance along Y axis from each data point. This equation gives the best fit value of the $LogEC_{50}$, instead of the EC_{50} itself. This is more useful when interpreting data since 95% confidence interval (CI) becomes symmetrical around the best fit value. Moreover, curve fitting with variable slopes is more reliable than fitting with constant slopes when there are multiple data points because curve fitting finds also the best fit slope value. The Bottom was most of the cases set as 0 since the high dose of agonists stopped firing of the neuron (does not apply to DRCs for 5-Htt KO mice).

For creating average fits for each genotype, data were first normalised by dividing average firing rates of each epoch by those of baseline value, hence setting Top as 1.0. Then normalised response firing rates were averaged for each experimental epoch and curve fitting was done on these average data points for each genotype. To test whether the curves are statistically different among genotypes "Extra sum-of-squares F test" was conducted. Since this test only tells whether the curves are statistically different or not, detailed statistical analysis was done on law $LogEC_{50}$ values taken from each experiment (not normalised) using standard statistical tests as described in the following section.

2.7. Statistics

All the statistical tests were performed by GraphPad Prism version 5.04 (GraphPad Software, San Diego, CA, USA). Unless otherwise stated, genotype effects were tested by first checking data for their normality by D'Agostino & Pearson omnibus normality test and for their equal variances by Bartlett's test for equal variances. When these conditions were satisfied, data were analysed by one-way ANOVA (expressed as $F(_{df1}, _{df2})$ values) followed by Tukey's post hoc test for multiple group comparison. If not, data were analysed by Kruskall-Walis test (expressed as $H(_{df})$ values) with Dunn's post hoc test for multiple group comparison. When the dependent variable was under the influence of two factors, two-way ANOVA followed by Bonferroni multiple comparison test was conducted.

When data were compared between only two groups, e.g. WT and KO, as in the analysis of DRN WC data, data were first checked for their normality by D'Agostino & Pearson omnibus normality test. When data were normally distributed, unpaired t-test (two-tailed) was applied; otherwise Mann-Whitney test (two-tailed) was applied. If data were normally distributed but had unequal variances, results from unpaired t test were corrected by Welch's correction.

For testing effects of tryptophan in comparison to respective baseline, data (change in firing rate with baseline set as 1) were analysed by Wilcoxon Signed Rank test (two-tailed).

In all cases, $p \leq 0.05$ was considered statistically significant and indicated by stars as follows: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. P value of $0.05 < p \leq 0.10$ was considered to be a trend of significance.

3 . Results

3.1. Loose-seal cell-attached recording from serotonergic neurons in the DRN of 5-Htt and Tph2 KO mice (Study 1)

3.1.1. Rationale

For maturation and specification of a particular neuronal population, acquisition of neuronal subtype-specific electrophysiological properties is critical. To investigate whether the deficiency of Tph2 activity and subsequent lack of 5-HT affects the normal development and retention of serotonergic neuron-specific electrophysiological characteristics, we recorded the spontaneous firing of serotonergic neurons in the DRN at baseline and in response to various compounds related to serotonin synthesis to verify the extent of deletion of Tph2 activity and the possible presence of autoinhibitory mechanisms in Tph2 KO mice. In the case of 5-Htt KO mice, life-long deficiency of 5-HT reuptaking proteins and consequent elevation of extracellular 5-HT may have affected functions of 5-HT receptors and autoinhibition mediated by such receptors. Thus potential alterations induced by genetic knockout on these properties of serotonergic neurons were assessed by measuring their firing rate as the functional probe for quantitative appraisal of serotonergic neuron sensitivity to appropriate pharmacological tools.

3.1.2. Identification of serotonergic neurons

For identification of serotonergic neurons, standard criteria for wildtype animals were adopted as mentioned in Materials and Methods. There is, however, possibility that genetic manipulation of serotonergic systems renders these criteria inapplicable to serotonergic neurons from knockout mice. To assess if the standard criteria are still applicable to distinguish serotonergic from non-serotonergic neurons and to establish precise criteria particularly suited to the present study, distribution of baseline mean instantaneous firing frequency (i.e. firing frequency based on two adjacent spikes) and UDI of all the neurons recorded was first plotted (all the genotypes pooled) (**Fig. 3.1**). As shown here, UDI clearly separates two distinct groups of neurons, those with UDI < 0.85 and those with UDI > 0.85. According to the standard criteria neurons with larger UDI are serotonergic and those with smaller UDI are non-serotonergic. This was actually the case for the present study as well since neurons with UDI > 0.85 decreased firing rates or stopped firing in response to agonists of 5-HT_{1A} receptors either directly by R8HD or indirectly by 5-HT synthesis through Trp or

5-HTP. Neurons with UDI < 0.85 lacked such responses (**Fig. 3.2**). Since UDI around 0.85 is a border line between serotonergic and non-serotonergic, a neuron was classified as serotonergic only if it responded to 5-HT_{1A} receptor agonists. If there is no such pharmacological confirmation, those neurons were left as unclassified (??? in **Fig. 3.1**). As a consequence, two neurons whose UDI fell in the range of 0.84-0.85 were not possible to be categorised either serotonergic or non-serotonergic. As for mean instantaneous firing frequency, there was a substantial overlap between serotonergic and non-serotonergic neurons: serotonergic neurons had a frequency of ~0.5-4.5 Hz; non-serotonergic ~1-5 Hz. For this reason, it is not possible to classify neurons based on mean instantaneous firing frequency alone unless a serotonergic neuron had a low firing frequency (< ~ 1 Hz) or a non-serotonergic neuron had a high firing frequency (> ~ 4.5 Hz). Thus it was concluded that neurons with UDI > 0.85 are safely classified as serotonergic. Typical action current shapes of serotonergic and non-serotonergic neurons are shown in **Fig. 3.3**. All the following experiments were done on serotonergic neurons identified this way.

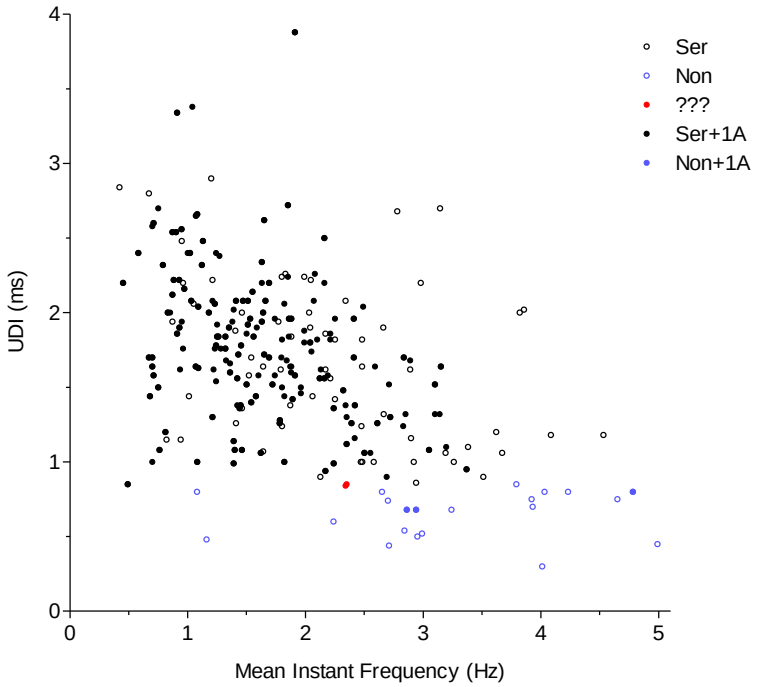

Fig. 3.1. Distribution of Up-to-downstroke interval (UDI) and mean instantaneous frequency of all the neurons recorded (all the genotypes pooled). Ser = serotonergic; Nonser = non-serotonergic; ??? = unknown cell type; Ser+1A = Serotonergic and verified by decrease/ceasing of firing in response to 5-HT_{1A} receptor agonist, either directly (R8HD) or indirectly through serotonin synthesis (Trp, 5-HTP). Non+1A = Non-serotonergic and verified by the absence of deceasing of firing in response to 5-HT_{1A} receptor agonist, either directly (R8HD) or indirectly through serotonin synthesis (Trp, 5-HTP).

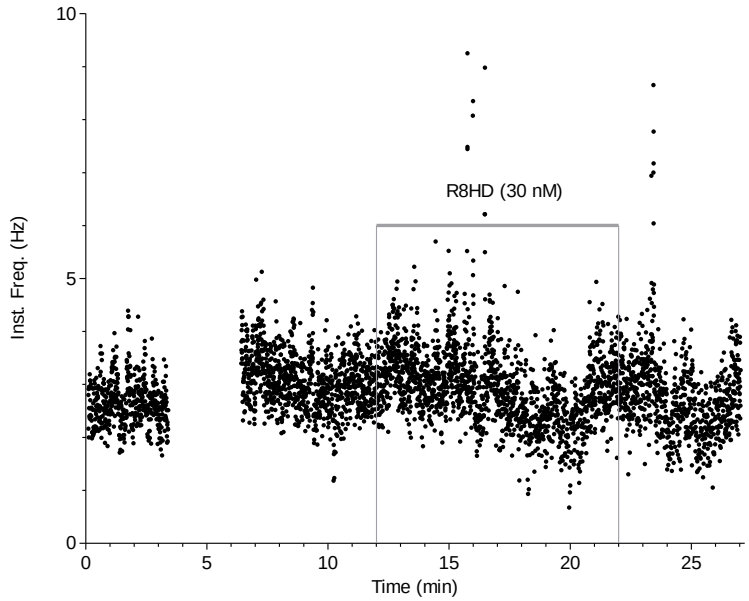

Fig. 3.2. Non-serotonergic neurons do not respond to R8HD. In this experiment, 30 nM of R8HD was applied during 12-22 mins of the recording.

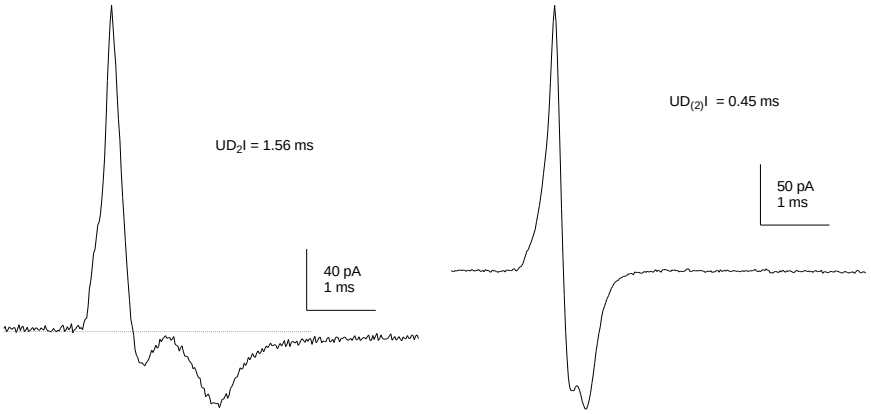

Fig. 3.3. Typical action current shapes of serotonergic and non-serotonergic neurons. Serotonergic neurons have a long UD_2I (Left; UD_2I = 1.56 ms). Non-serotonergic neurons have a short $UD_{(2)}I$ (Right; $UD_{(2)}I$ = 0.45 ms). Note also that serotonergic neurons have clear first and second downstrokes, while they are not clear in non-serotonergic neurons.

3.1.3. Comparison of baseline firing rates

Here Baseline firing rates of serotonergic neurons among genotypes were compared to see if there are intrinsic differences before assessing the effects of 5-HT_{1A} receptor agonists or 5-HT precursor application on decease in firing rates. **Fig. 3.4** shows distribution of baseline firing rates of all the neurons identified as serotonergic (n = 250). Kruskal-Wallis test followed by Dunn's multiple comparison test showed statistically significant differences ($H_{(4)}$ = 16.67, p = 0.002) for 5-Htt-KO vs. Tph2-KO (**p ≤ 0.01) and WT vs. Tph2-KO (*p ≤ 0.05). Mean firing rate (Hz) ± Standard Deviation (SD): Tph2-KO, 1.609 ± 0.817; Tph2-Het, 1.902 ±

0.665; WT, 1.968 ± 0.687; 5-Htt-Het, 1.853 ± 0.736; 5-Htt-KO, 2.123 ± 0.749. WT data are pooled data from both Tph2 WT and 5-Htt WT mice.

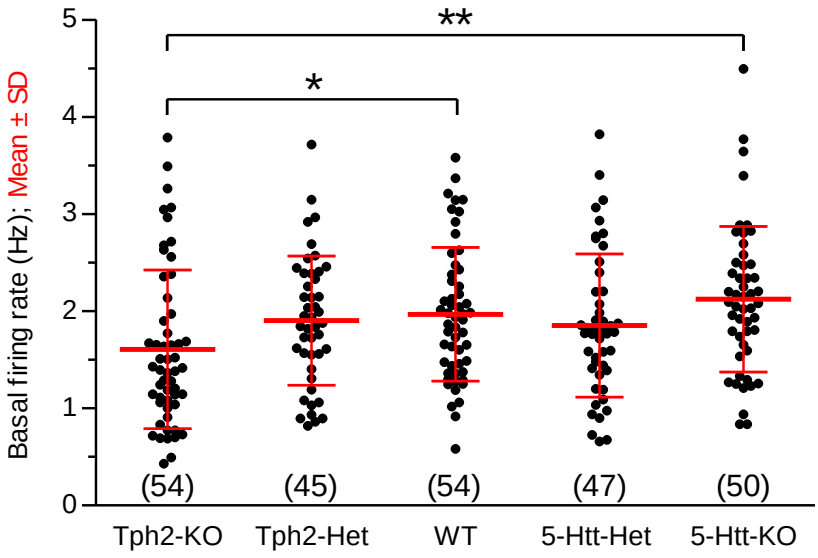

Fig. 3.4. Summary plot of baseline firing rates. Figure shows distribution of baseline firing rates (Hz) of all the serotonergic neurons recorded from the DRN in brainstem slices. Serotonergic neurons were defined based on the Up-to-Downstroke Interval (UDI; see the preceding section). Data are shown as mean ± SD (number of cells shown in parentheses). Kruskal-Wallis test followed by Dunn's multiple comparison test showed statistically significant differences ($H_{(4)} = 16.67$, $p = 0.002$) for 5-Htt-KO vs. Tph2-KO and WT vs. Tph2-KO.

3.1.4. Comparison of UDI among genotypes

We also compared the UDI of action current among genotypes to address the possibility that action current shapes were altered due to genetic manipulation. **Fig. 3.5** shows the summery plot of UDI distribution obtained from serotonergic neurons defined by the above-mentioned method (see section 3.1.2.). Kruskal-Wallis test showed no statistically significant differences among genotypes ($H_{(4)} = 1.550$; $p = 0.8178$). Mean UDI ± SD: Tph2-KO, 1.772 ± 0.527; Tph2-Het, 1.755 ± 0.580; WT, 1.735 ± 0.493; 5-Htt-Het, 1.778 ± 0.494; 5-Htt-KO, 1.635 ± 0.393; WT data were pooled from Tph2 WT and 5-Htt WT).

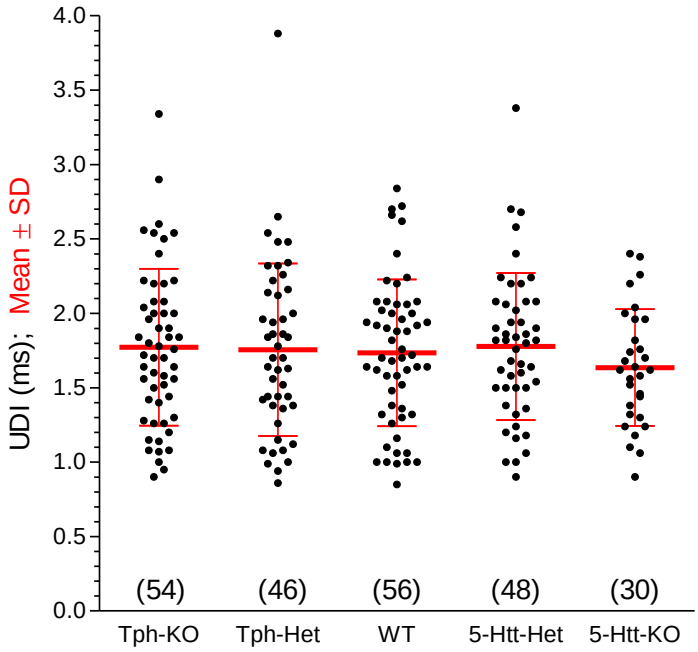

Fig. 3.5. Distribution of Up-to-Downstroke Interval (UDI) of serotonergic neurons from all the genotypes recorded. Data are shown as mean ± SD (number of cells shown in parentheses). Kruskal-Wallis test followed by Dunn's multiple comparison tests showed no statistically significant differences among genotypes ($H_{(4)} = 1.550$; $p = 0.8178$).

3.1.5. Assessment of 5-HT$_{1A}$ receptor function by a selective agonist R8HD

We first tested the functional response of serotonergic neurons to 5-HT$_{1A}$ receptor agonist R8HD in a dose-dependent manner in different genotypes where genetic alteration of serotonergic system functions may have altered autoinhibition exerted by 5-HT through 5-HT$_{1A}$ receptor activation. **Fig. 3.6** illustrates typical experiments done on Tph2 WT (**A**) and KO mice (**B**). Bath-applied R8HD activates autoinhibitory 5-HT$_{1A}$ receptors and reduces the firing rate of serotonergic neurons. **Fig. 3.6 C** shows the change in firing rate in response to increasing concentration of R8HD, i.e. DRC, corresponding to **Fig. 3.6 A** and **B**. The concentration of R8HD which reduces firing of a serotonergic neuron to 50% of its baseline is calculated from the DRCs (EC_{50} (nM)).

Fig. 3.7 shows logarithm of EC_{50} among five different genotypes including both Tph2 and 5-Htt KO mice. Compared to WT controls (pooled from Tph2 WT and 5-Htt WT), the response to application of R8HD showed slightly higher effectiveness of R8HD in Tph2 KO mice, lower effectiveness in 5-Htt Het mice, and weak effectiveness in 5-Htt KO mice. One-way ANOVA followed by Tukey's multiple comparison test showed statistically significant differences ($F_{(4,53)} = 48.38$, $p \leq 0.0001$) between 5-Htt KO and all the other four phenotypes

(***p ≤ 0.001) (log EC_{50} ± SD: Tph2$^-$KO, -8.821 ± 0.290; Tph2$^-$Het, -8.519 ± 0.185; WT, -8.516 ± 0.246; 5-Htt$^-$Het, -8.223 ± 0.269; 5-Htt$^-$KO, -7.172 ± 0.420). Differences between Tph2-KO vs. WT (*p ≤ 0.05) and Tph2-KO vs. 5-Htt-Het (***p ≤ 0.001) also reached statistical significance. All the other multiple comparisons did not reach statistical significance. **Fig. 3.8** shows the DRC from averaged responses of the Tph2 KO mice line (**left**) or 5-Htt mice line (**right**).

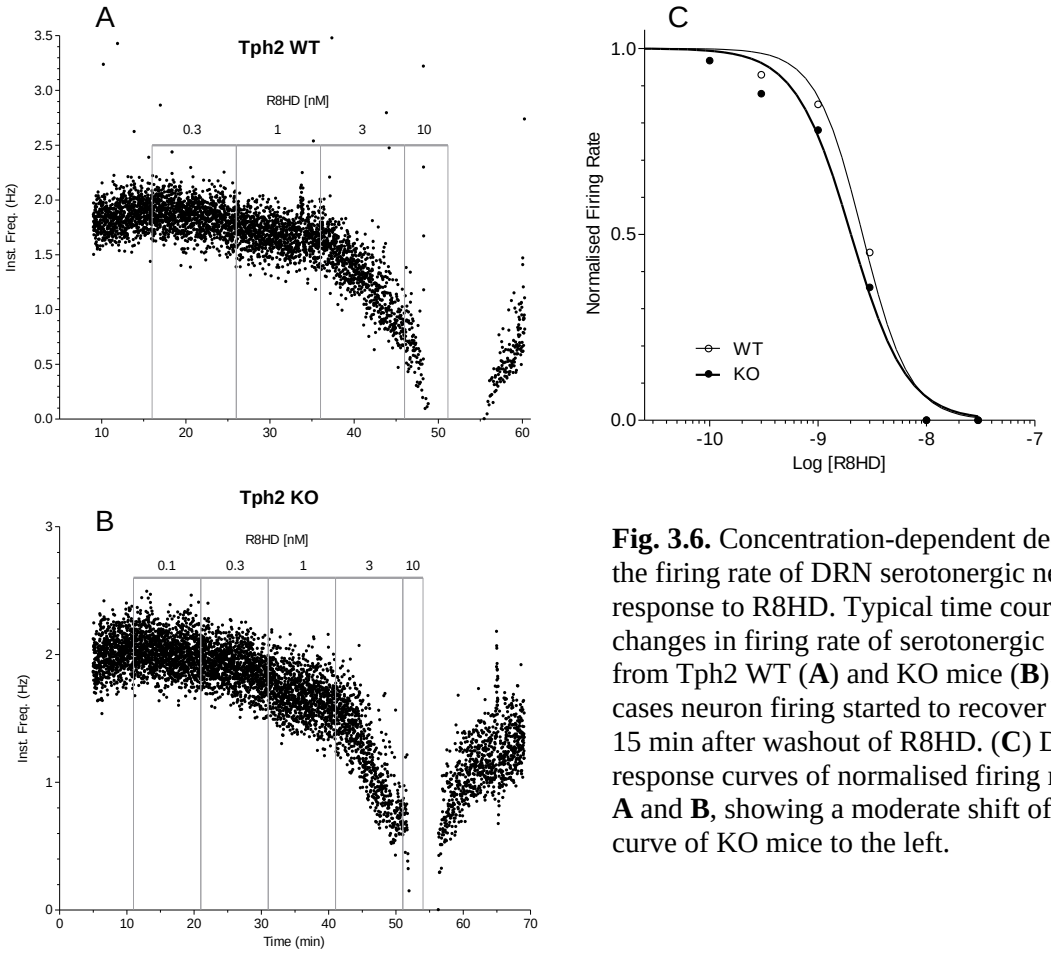

Fig. 3.6. Concentration-dependent decrease in the firing rate of DRN serotonergic neurons in response to R8HD. Typical time courses of changes in firing rate of serotonergic neurons from Tph2 WT (**A**) and KO mice (**B**). In both cases neuron firing started to recover within 15 min after washout of R8HD. (**C**) Dose-response curves of normalised firing rate from **A** and **B**, showing a moderate shift of the curve of KO mice to the left.

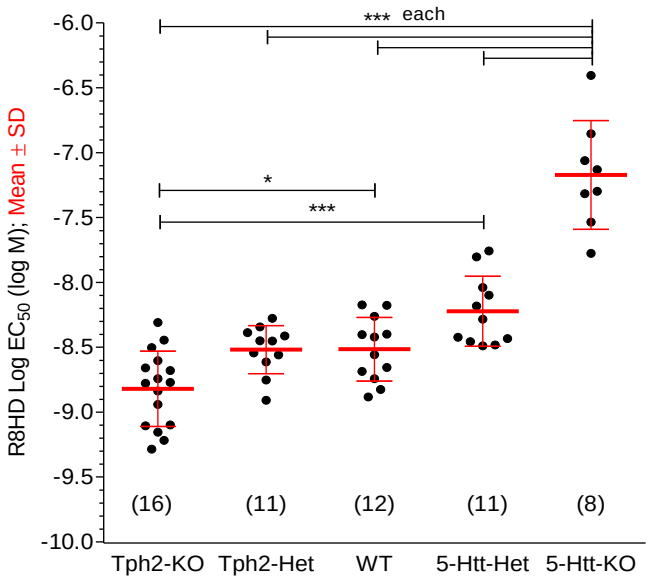

Fig. 3.7. Scatter plot of log EC_{50} values for R8HD in inhibiting the firing rate of DRN serotonergic neurons in all the genotypes tested. Each data point corresponds to the EC_{50} value obtained from a full dose-response curve in a single neuron. The number of recorded neurons from brain slices of each genotype is shown in parentheses. Data are shown as mean ± SD. Data from Tph2 WT and 5-Htt WT mice are pooled together. One-way ANOVA followed by Tukey's multiple comparison test showed statistically significant differences ($F_{(4,53)}$ = 48.38, $p \leq 0.0001$) between 5-Htt KO and all the other four phenotypes. Differences between Tph2-KO vs. WT and Tph2-KO vs. 5-Htt-Het also reached statistical significance.

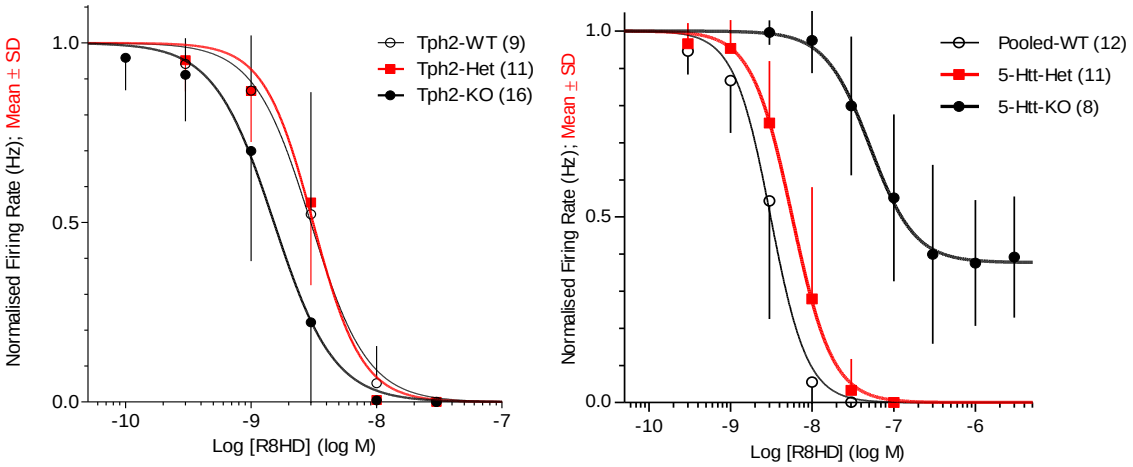

Fig. 3.8. Dose-response curves for R8HD in DRN serotonergic neurons. Graphs show firing rate changes of DRN serotonergic neurons in response to increasing concentrations of R8HD (log M). For each neuron, the firing rate was normalised to the mean firing rate during baseline measurement. Data were obtained from brain slices of Tph2 KO (left) and 5-Htt KO (right) mice and compared to those obtained by the respective Het and WT counterparts. Each data point corresponds to the mean ± SD (number of cells shown in parentheses). Note that the dose-response curve of 5-Htt KO does not reach the bottom and therefore the EC_{50} value of 5-Htt KO mice is only indicative for comparisons with other genotypes. Note also that some error bars are shown only in one direction for clarity.

The results obtained from this R8HD application experiment confirmed the presence of functional 5-HT_{1A} receptors in all the genotypes tested including Tph2 KO mice. Therefore, the presence and magnitude of autoinhibition was then tested by applying 5-HT precursor,

Trp. Application of Trp in brain slices from wildtype animals is known to lead to decrease of serotonergic neuron firing rate through the *de novo* synthesised 5-HT, which work on autoinhibitory 5-HT_{1A} receptors (Liu et al., 2005; Mlinar et al., 2005; Evans et al., 2008). Such autoinhibition should be absent in Tph2 KO mice, which lack the enzyme converting Trp into 5-HT. Apart from Tph2 KO mice, one could reasonably expect from the functional sensitivity of 5-HT_{1A} receptors, which has been revealed by the R8HD application experiment mentioned above, that autoinhibition level is unchanged in Tph2 Het, slightly decreased in 5-Htt Het, and greatly impaired in 5-Htt KO mice compared to wildtype controls. We tested this assumption in the following section.

3.1.6. Tryptophan: Testing 5-HT synthesis by applying a 5-HT precursor

i). Complete absence of 5-HT synthesis in Tph2 KO mice (published in Gutknecht et al., 2012)

Previous studies have shown that application of Trp in brain slices induces synthesis of 5-HT. Subsequent increase in extracellular 5-HT then activates somatodendritic 5-HT_{1A} autoreceptors and inhibits serotonergic neuron firing as a feedback mechanism (Liu et al., 2005; Evans et al., 2008; Mlinar et al., 2005). Importantly, endogenous paracrine 5-HT release in the absence of Trp is low or absent in DRN slices (Johnson et al., 2002 as in Cornelisse et al., 2007). Thus, decrease in firing rates is solely due to the *de novo* synthesised 5-HT in response to exogenously applied Trp, allowing reliable quantitative comparisons of autoinhibitory function across different slices. Here, the effect of 30 and 100 µM Trp application on the firing rate of serotonergic neurons was investigated as a functional assay to know whether serotonergic neurons in Tph2 KO mice are able to produce 5-HT. Since it has been demonstrated that 5-HT_{1A} receptors in Tph2 KO mice are functionally active (see the previous section), it was possible to test whether serotonergic cells were able to synthesise 5-HT from Trp, using the decrease of firing rate as a detector of 5-HT in the extracellular compartment. The 30 µM concentration of Trp is based on the K_m of Tph2 for Trp (20-50 µM) and the physiological concentrations of extracellular Trp in the brain previously reported (10-30 µM) (Mlinar et al., 2005; Evans et al., 2008). Additionally, 100 µM concentration of Trp was tested to see if there were further effects of Trp at a high concentration.

The result presented here confirmed the complete absence of Tph activity and thus 5-HT synthesis ability in serotonergic neurons in the DRN of Tph2 KO mice since the application

of Trp did not decrease the firing rate of these neurons, whereas the same treatment led to the evident decrease in the firing rate in WT and Het counterparts (**Fig. 3.9.A**). In contrast, application of 5-HTP (the by-product of Tph2 enzyme and the direct precursor of 5-HT; see introduction) in Tph2 KO mice reversibly stopped serotonergic neuron firing, indicating that the 5-HT synthesis pathway downstream of Tph2 is retained and serotonergic neurons in these mice can respond to endogenous 5-HT once it is produced (**Fig. 3.9.A**). At the end of each experiment, 30 nM R8HD was applied to see suppression of firing, confirming serotonergic identity of the recorded neuron. Summary histogram of % decrease in the firing rate is shown in **Fig. 3.9.B**. Both concentrations of Trp (30 and 100 µM) did not change the firing rate of serotonergic neurons from Tph2 KO mice, but significantly reduced serotonergic neuron firing rates in Tph2 WT and Het mice ($p \leq 0.05$, Wilcoxon Signed Rank test). Comparison across genotypes revealed that the responses of serotonergic neurons from Tph2 KO were statistically different from those of WT and Het mice both for Trp 30 µM ($H_{(2)} = 16.28$, $p < 0.0003$) and 100 µM ($H_{(2)} = 10.43$, $p < 0.0054$, Kruskal-Wallis, followed by Dunn's multiple comparison test). 100 µM of Trp did not evidently produce further decrease in firing rates compared to Trp 30 µM (**Fig. 3.9.A, B**).

ii). Comparison of Trp-driven 5-HT synthesis and autoinhibition among genotypes

The magnitude of autoinhibition in response to 30 µM Trp application was then measured across all the genotypes including the 5-Htt KO mice line, except Tph2 KO mice, for which absence of 5-HT synthesis has already been demonstrated (**Fig. 3.9**). As shown in **Fig. 3.10**, 30 µM of Trp reduced firing rates of serotonergic neurons in all the genotypes tested to a similar extent. Wilcoxon Signed Rank test showed that decrease in firing rate was significantly different from zero ($p \leq 0.05$) in all the genotypes tested (% decrease in firing rates ± SD: Tph2-Het, 25.62 ± 15.37; WT, 25.55 ± 19.87; 5-Htt-Het, 17.51 ± 12.99; 5-Htt-KO, 22.03 ± 17.00; WT data were pooled from Tph2 WT and 5-Htt WT mice). Kruskal-Wallis test showed that responses across four genotypes were not statistically different ($H_{(3)} = 3.336$, $p = 0.3427$).

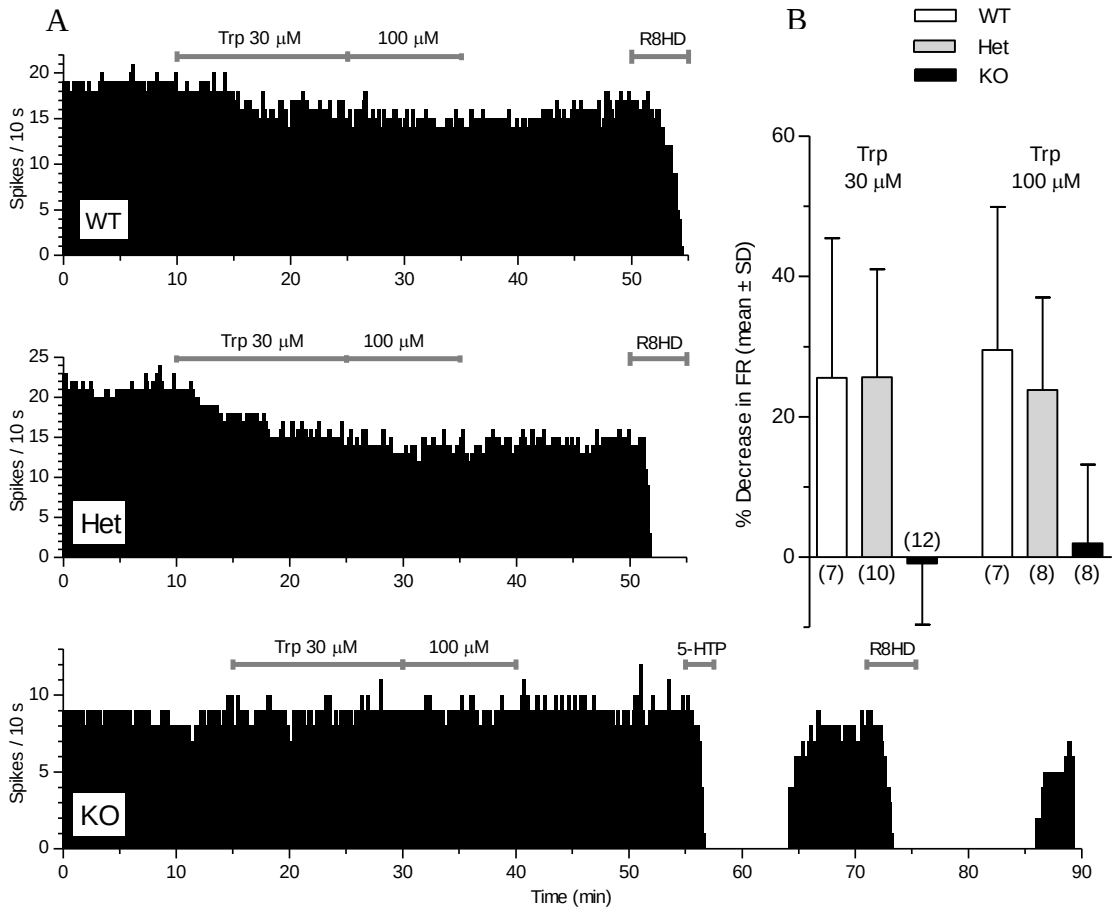

Fig. 3.9. Time-course of firing rate and effect of tryptophan on firing rate. **A)**Time-course of firing rate changes in response to tryptophan (Trp; 30 and 100 μM) and R-8-OH-DPAT (R8HD; 30 nM) of serotonergic neurons in slices obtained from WT (upper panel), Tph2 Het (middle panel) and Tph2 KO (lower panel) mice. The lower panel also demonstrates response to the application of 30 μM L-5-hydroxytryptophan (5-HTP) in serotonergic neurons from Tph2 KO mice. 5-HTP stopped firing of serotonergic neurons in WT and Tph2 Het mice, too (data not shown). **B)** Histogram summarising % decrease in the firing rate (FR) in response to 30 μM (left) and 100 μM (right) tryptophan. Data are shown as mean ± SD. Numbers in parentheses indicate the number of tested cells. Figure modified from Gutknecht et al, 2012. Impacts of brain serotonin deficiency following Tph2 inactivation on development and raphe neuron serotonergic specification. PLoS One 7:e43157.

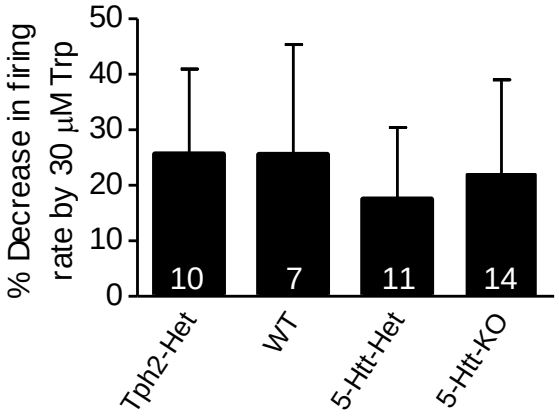

Fig. 3.10. Histograms of % decrease in firing rates of serotonergic neurons in the DRN in response to 30 µM tryptophan (Trp). Data are shown as mean ± SD and the total number of recorded neurons from brain slices of each genotype is shown in at the bottom of histograms. WT data were pooled from Tph2 WT and 5-Htt WT mice. Wilcoxon Signed Rank test showed that decrease in firing rate was significantly different from zero ($p \leq 0.05$) in all the genotypes tested. Kruskal-Wallis test followed by Dunn's multiple comparison test showed no statistically significant differences among genotypes ($H_{(3)} = 3.336$, $p = 0.3427$).

3.1.7. Test of 5-HT synthesis from 5-HTP

5-HTP is the immediate precursor of 5-HT and is converted into 5-HT by 5-hydroxy-tryptophan decarboxylase (and amino acid decarboxylase; AADC) (Carlsson et al., 1972). This step resides downstream of the Tph2-dependent step in the enzymatic pathway leading to 5-HT synthesis. 5-HTP, when converted into 5-HT, is expected to decrease the firing rate of 5-HT neurons via 5-HT_{1A} receptors. As shown previously, 5-HTP caused a strong and reversible inhibition of 5-HT neuron firing in Tph2 KO mice (see **Fig. 3.9**). Therefore application of 5-HTP allowed assessment of autoinhibition across all the genotypes studied here including Tph2 KO mice. Since 5-HTP had relatively stronger effects compared to Trp, it also allowed monitoring dose-dependent responsiveness of autoinhibition. As opposed to Trp application, Tph2 KO mice may exhibit higher responsiveness to 5-HTP application due to a mild functional sensitisation of 5-HT_{1A} receptors as demonstrated in response to R8HD application (**Fig. 3.7**; **Fig. 3.8**).

Fig. 3.11 shows logarithm of EC_{50} in response to 5-HTP application among five different genotypes comprising of both Tph2 and 5-Htt KO mice. Kruskal-Wallis test followed by Dunn's multiple comparison test showed statistically significant differences ($H_{(4)}=19.00$, $p = 0.0008$) only for 5-Htt-KO vs. WT (***$p \leq 0.001$) and 5-Htt-KO vs. Tph2-Het (*$p \leq 0.05$) (log EC_{50} ± SD: Tph2-KO, -5.509 ± 0.410; Tph2-Het, -5.292 ± 0.304; WT, -5.165 ± 0.204; 5-

Htt-Het, -5.481 ± 0.356; 5-Htt-KO, -5.761 ± 0.125; WT data pooled from both Tph2 WT and 5-Htt WT mice). All the other comparisons did not show any statistically significant differences. **Fig. 3.12** shows the DRC from averaged responses of the Tph2 KO mice line (**left**) or 5-Htt mice line (**right**). As with R8HD, the highest concentration of 5-HTP did not always stop firing of neurons in 5-Htt KO mice.

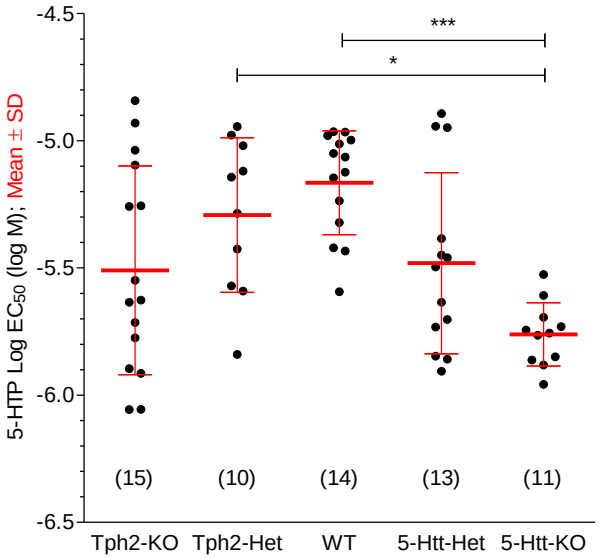

Fig. 3.11. Scatter plot of log EC_{50} values of serotonergic neurons in the DRN in response to 5-HTP. Each data point corresponds to the EC_{50} value obtained from a full dose-response curve in a single neuron and the total number of recorded neurons from brain slices of each genotype is shown in parentheses. Data are shown as the mean ± SD. Tph2 WT and 5-Htt WT mice are pooled together. Kruskal-Wallis test followed by Dunn's multiple comparison test showed statistically significant differences ($H_{(4)} = 19.00$, $p = 0.0008$) only for 5-Htt-KO vs. WT and 5-Htt-KO vs. Tph2-Het.

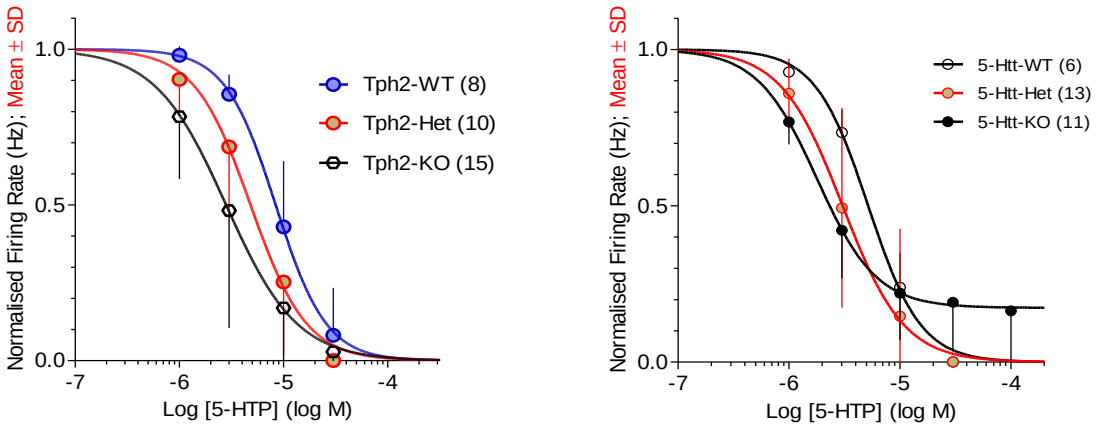

Fig. 3.12. Dose-response curve of 5-HTP application. Normalised firing rate change of serotonergic neurons in the DRN in response to increasing concentration of 5-HTP (log M). Data were obtained from brain slices of the Tph2 KO line (left) and the 5-Htt KO line (right). Each data point corresponds to the mean ± SD (number of cells shown in parentheses). Note that the dose-response curve of 5-Htt KO does not reach the bottom and therefore the EC_{50} value of 5-Htt KO mice is only indicative for comparisons with other genotypes. Note also that some error bars are shown only in one direction for clarity.

3.2. WC recording from serotonergic neurons in the DRN of Tph2 KO mice (Study 2)

3.2.1. Static membrane properties

i). Resting membrane potential

Fig. 3.13 shows initial resting membrane potential (**A**; initial V_m) and stable resting membrane potential (**B**; stable V_m), as were defined in the Materials and Methods. Unpaired t test revealed no statistically significant differences between WT and KO both for initial V_m (initial V_m (mV) ± SD: WT, -67.66 ± 9.00; KO, -64.85 ± 7.47) and stable V_m (stable V_m (mV) ± SD: WT, -66.33 ± 8.38; KO, -67.65 ± 9.22).

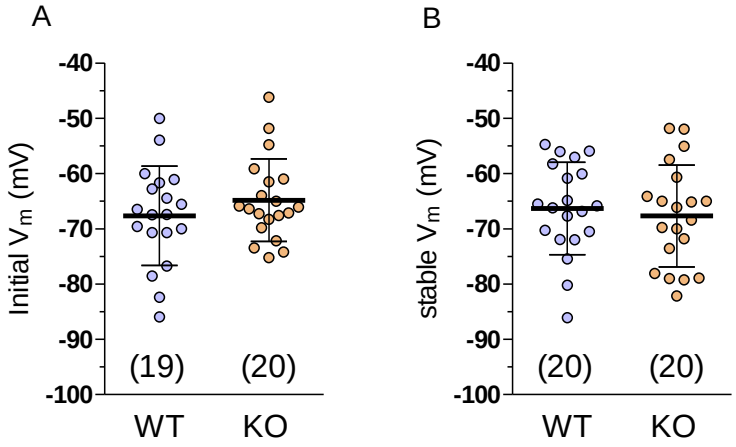

Fig. 3.13. Summary plots of resting membrane potential (V_m). **A**) initial resting membrane potential measured immediately after the whole-cell recording configuration was established. **B**) stable resting membrane potential measured some time after the whole-cell configuration was established, when the cell interior was supposed to be equilibrated with the pipette solution. For precise procedure to measure resting membrane potentials, see Materials and Methods. Data are shown as mean ± SD. Numbers in the parentheses indicate the number of neurons recorded. Unpaired t test revealed no statistically significant differences between the two genotypes.

ii). Membrane resistance and capacitance

Fig. 3.14.A shows a scatter plot of membrane resistances. Unpaired t test showed that there was a statistically highly significant difference between WT and KO ($p = 0.0007$) (R_m (MΩ) ± SD: WT, 961.3 ± 204.5; KO, 752.2 ± 151.5). **Fig. 3.14.B**, on the other hand, shows membrane capacitance (C_m) of the same neurons from which membrane resistances were measured. Mann-Whitney test indicated no statistically significant differences ($p = 0.1293$) between genotypes (C_m (pF) ± SD: WT, 45.46 ± 6.90; KO, 53.39 ± 15.70).

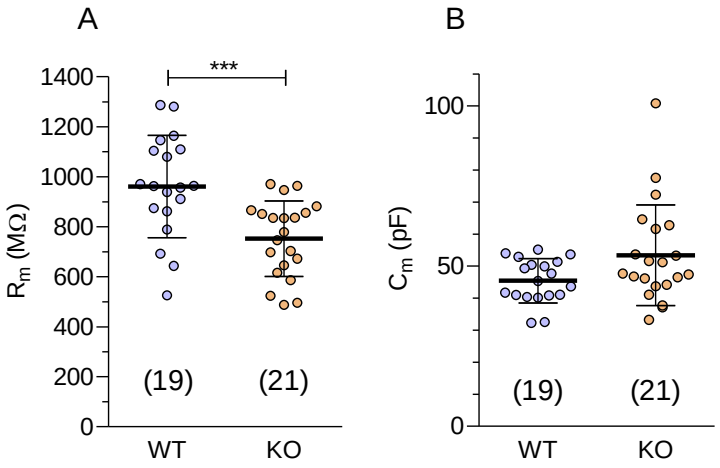

Fig. 3.14. Comparison of **A**) membrane resistance (R_m) and **B**) membrane capacitance (C_m). In both cases, data are shown as mean ± SD. Numbers in the parentheses indicate the number of neurons recorded. Unpaired t test showed that there was a statistically significant difference in R_m between WT and KO (p = 0.0007).

iii). Membrane conductance

Significantly lower R_m as shown in **Fig. 3.14.A** indicates that membrane conductance (G (pS); defined as the inverse of membrane resistance) of KO mice is higher than that of WT. Indeed, as in **Fig. 3.15.A**, comparison of membrane conductance showed statistically highly significant differences (p=0.0012; Mann-Whitney test) between WT and KO mice (G (pS) ± SD: WT, 1095 ± 282.7; KO, 1389 ± 315.6). To account for potential differences in cell sizes, the conductance was then normalised by membrane capacitance, i.e. G/C_m (**Fig. 3.15.B**). Mann-Whitney test showed no statistically significant differences (p = 0.1042) between the two genotypes (G/C_m (pS/pF) ± SD: WT, 24.58 ± 7.36; KO, 27.05 ± 6.05). The result may indicate that the higher conductance of neurons from KO mice may be partially attributable to their larger size, which is in part reflected as a higher C_m (**Fig. 3.14.B**), although the difference did not reach statistical significance.

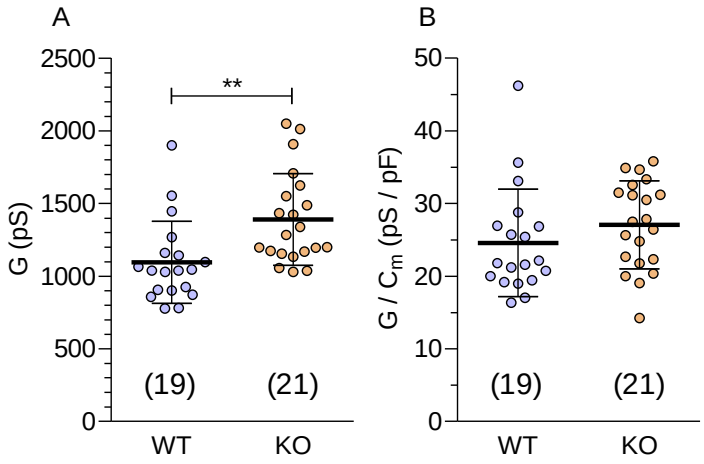

Fig. 3.15. Membrane conductance and normalised membrane conductance. **A**) Membrane conductance (G) calculated as the inverse of membrane resistance. **B**) Membrane conductance was normalised by membrane capacitance (G/G_m) to account for differences in the cell size. Data are shown as mean ± SD. Numbers in the parentheses indicate the number of neurons recorded. Mann-Whitney test showed that the membrane conductance of neurons from KO mice was significantly higher than that of WT mice ($p = 0.0012$), but the difference became non-significant when normalised.

iv). Series resistance and holding current

As a control for recording quality, series resistance (R_s) of the same group of neurons, from which the membrane resistance, capacitance, and conductance were derived, were checked (**Fig. 3.16.A**). The amount of current flow when the neuron was voltage-clamped at -65 mV (I_{-65mV}) was also checked (**Fig. 3.16.B**). Since -65 mV is near the resting membrane potential, the value of I_{-65mV} should be close to 0 in an ideal patch condition. Unpaired t test showed that there was no statistically significant difference in R_s between two genotypes ($p = 0.2647$) (R_s (MΩ) ± SD: WT, 16.71 ± 4.55; KO, 15.02 ± 4.82). I_{-65mV} for both genotypes remained near 0pA (Mean I_{-65mV} (pA) ± SD: WT, 0.942 ± 7.090; KO, 1.918 ± 11.23), which was ideal, and there was no statistically significant difference between two genotypes ($p = 0.7472$).

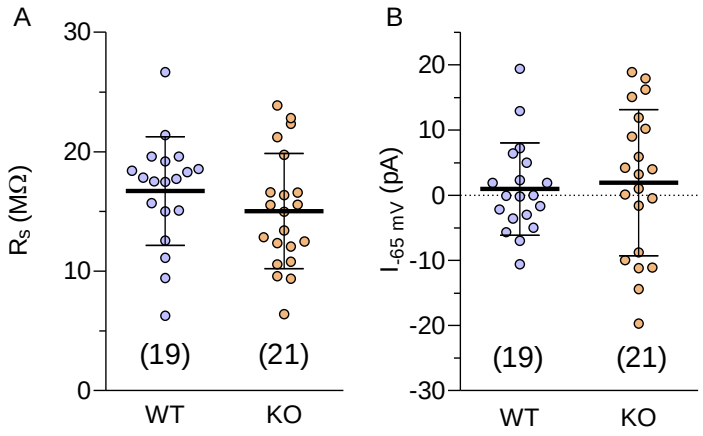

Fig. 3.16. Scatter plots of **A**) series resistance (R_s) and **B**) holding current at -65 mV ($I_{-65\ mV}$). Data are shown as mean ± SD. Numbers in the parentheses indicate the number of neurons recorded. There was no statistically significant difference in both parameters between genotypes.

v). Sag amplitude

Most of the neurons recorded had no sags or sags of relatively small amplitude, corresponding to the nature of serotonergic neurons (**Fig. 3.17**). Mann-Whitney test showed that there was no statistically significant difference (p = 0.227) between the two genotypes (mean sag (mV) ± SD: WT = 1.63 ± 2.55; KO = 2.43 ± 2.31).

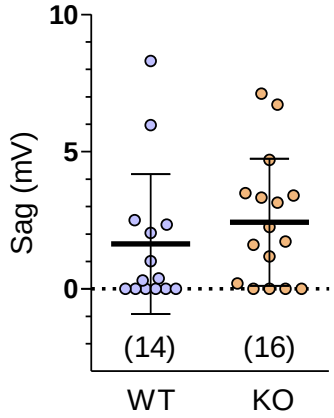

Fig. 3.17. Comparison of sag amplitude induced by negative current injection. Data are shown as mean ± SD. The number of recorded neurons is shown in the parentheses. Mann-Whitney test showed that there was no statistically significant difference (p = 0.227) between the two genotypes.

3.2.2. Active membrane properties

i). Action potential properties

Active membrane properties were then compared between genotypes including action potential shapes and firing frequency measured in the current-clamp mode (**Fig. 3.18. A-C**). Comparison of action potential amplitude (AP amp) showed trend of statistical significance (p = 0.0569; unpaired t test) (Mean AP amp (mV) ± SD: WT, 116.0 ± 6.66; KO, 112.2 ± 6.19). Mann-Whitney test showed that there was no significant difference (p = 0.2609) in action potential HHW (AP HHW (ms) ± SD: WT, 1.958 ± 0.317; KO, 1.858 ± 0.391). Comparison of maximum sustained firing rate also did not show any statistically significant difference (p = 0.9512; unpaired t test) ($FR_{max\text{-}sus}$ (Hz) ± SD: WT, 10.29 ± 2.44; KO, 10.23 ± 3.31).

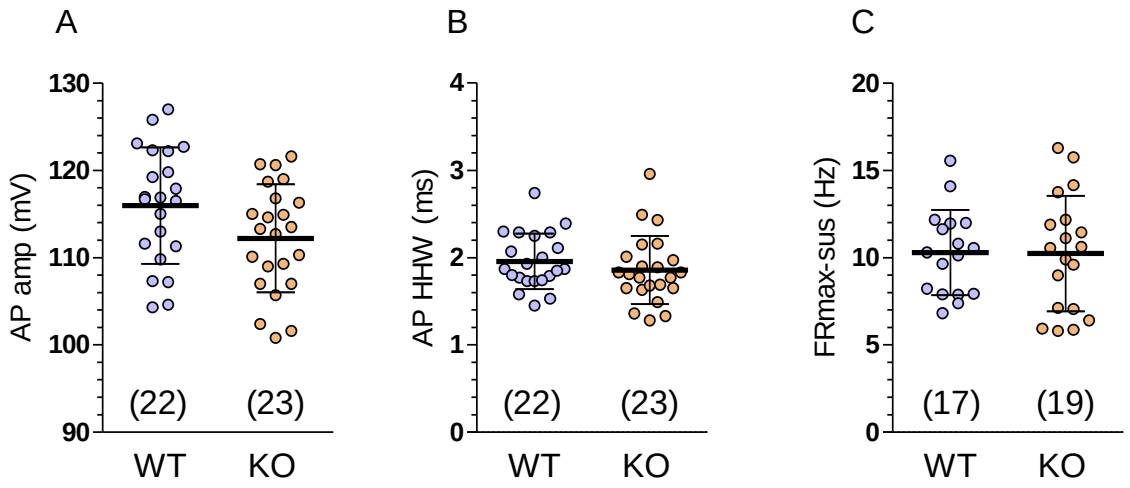

Fig. 3.18. Comparison of action potential (AP) properties. **A**) AP amplitude and **B**) AP half-height width (HHW) were measured from an averaged action potential. **C**) maximum sustained firing rate ($FR_{max\text{-}sus}$) was obtained in response to depolarising current injection. Data are shown as mean ± SD. Numbers in the parentheses indicate the number of neurons recorded. None of the parameters showed statistically significant differences between genotypes although AP amplitude showed trend of statistical significance (p = 0.0569; unpaired t test).

Fig. 3.19 shows comparison of time constants derived from a two-component exponential fit to the action potential AHP. For the time constant of the first component (τ_1), Mann-Whitney test showed no statistically significant differences (p = 0.837) between WT and KO mice (Mean ± SD: WT, 4.51 ± 1.97; KO, 4.70 ± 1.94). Comparison of the time constant of the second component (τ_2) also revealed no statistical differences (p = 0.443; unpaired t test) between the two genotypes (Mean ± SD: WT, 0.42 ± 0.05; KO, 0.40 ± 0.06).

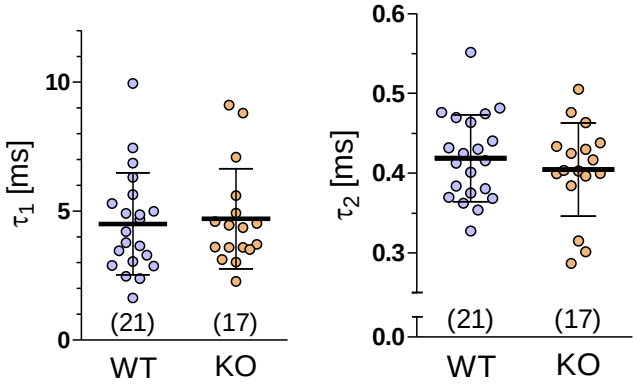

Fig. 3.19. Time constants (τ) of action potential afterhyperpolarisation (AHP). (left) time constant of the first component of the exponential fit to the AHP (τ_1). (right) time constant of the second component of the exponential fit to the AHP (τ_2). The number of recorded neurons is shown in parentheses.

ii). Conductance in response to voltage ramp

Fig. 3.20.A shows average current flow in response to negative voltage ramp from 0 mV to -125 mV. **Fig. 3.20.B** shows distribution of conductances obtained by fitting linear regression to the current response trace between -113 and -98 mV. Mann-Whitney test showed a trend of statistically significant differences (p = 0.0569) between WT and KO mice (mean G (nS) ± SD: WT, 1.574 ± 0.513; KO, 2.645 ± 1.663).

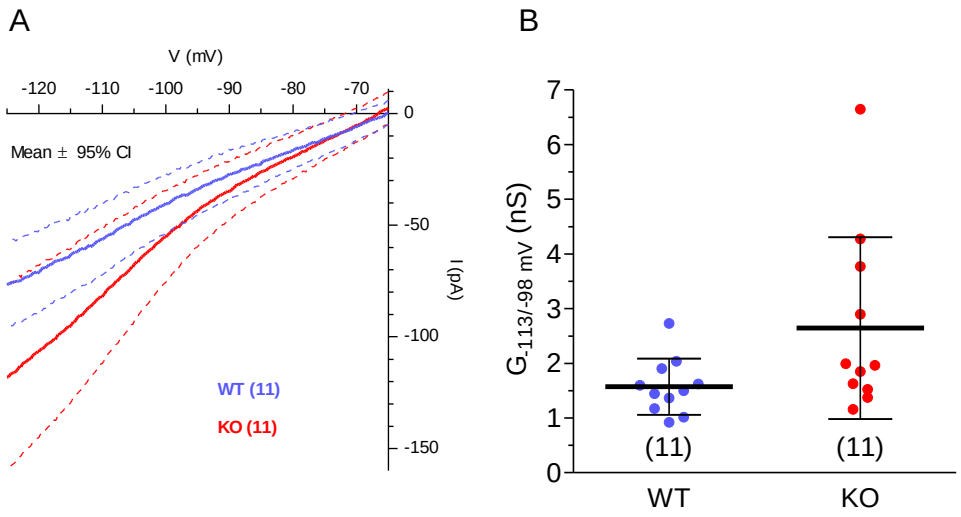

Fig. 3.20. Conductance in response to voltage ramp. **A**) Inward current induced by negative voltage ramp. Data were shown as mean ± 95% confidence interval (CI). **B**) Summary plots of conductance calculated by linear regression in the voltage range of -113 and -98 mV ($G_{-113/-98\ mV}$). Data are shown as mean ± SD. In both **A** and **B**, numbers in the parentheses indicate the number of neurons recorded. Mann-Whitney test showed a trend of statistically significant differences in $G_{-113/-98\ mV}$ (p = 0.0569) between WT and KO mice.

3.3. WC recording from hippocampal CA1 pyramidal neurons in 5-Htt KO mice (Study 3)

Using the whole-cell patch clamp, detailed basic membrane properties and action potential characteristics were investigated on pyramidal neurons in the CA1 area of the hippocampus from the 5-Htt KO mice line. Since the hippocampus has a highly laminated structure, synaptic communication between the Schaffer collateral/commissural pathway and CA1 pyramidal neurons was also investigated. Additionally, morphological analysis of the recorded neurons was conducted using the Neurobiotin tracer and Neurolulcida software. The motive for investigating this neuronal area is, as mentioned in the introduction, that the hippocampus is one of the target regions of the serotonergic innervation and thus lack of 5-Htt may have altered basic electrophysiological properties of neurons in this area. Such potential alterations may have close relevance to behavioural and molecular characteristics of 5-Htt KO mice that have been previously reported. Investigating electrophysiological properties may therefore further contribute to the understanding of phenotypes of 5-Htt KO mice.

3.3.1. Static membrane properties

As static membrane properties, the following three parameters were measured: resting membrane potential (V_m; only stable V_m was measured), membrane resistance (R_m), and membrane capacitance (C_m) (**Fig. 3.21**). Among these, difference in V_m (mean V_m (mV) ± SD: WT, -66.39 ± 3.63; Het, -64.29 ± 6.83; KO, -62.93 ± 5.24) and C_m (mean C_m (pF) ± SD: WT, 67.63 ± 29.04; Het, 81.28 ± 45.22; KO, 72.36 ± 18.96) did not show any statistical significance ($H_{(2)} = 3.386$, $p = 0.184$; $H_{(2)} = 0.1952$, $p = 0.907$, respectively; Kruskal-Wallis test followed by Dunn's multiple comparison test). Difference in R_m reached statistical significance ($F_{(2,30)} = 9.413$, $p = 0.0007$; One-way ANOVA). Tukey's multiple comparison test showed the difference became highly significant ($p \leq 0.01$) between WT vs. Het and WT vs. KO (Mean R_m (MΩ) ± SD: WT, 374.5 ± 172.1; Het, 215.6 ± 74.88; KO, 177.5 ± 36.19). Since series resistance (R_s) affects measurements of membrane resistance, the magnitude of series resistance was also monitored (**Fig. 3.22**). One-way ANOVA showed no statistically significant differences ($F_{(2,30)} = 1.023$, $p = 0.372$) in R_s among genotypes (Mean R_s (MΩ) ± SD: WT, 17.65 ± 3.37; Het, 16.92 ± 3.67; KO, 15.59 ± 3.09).

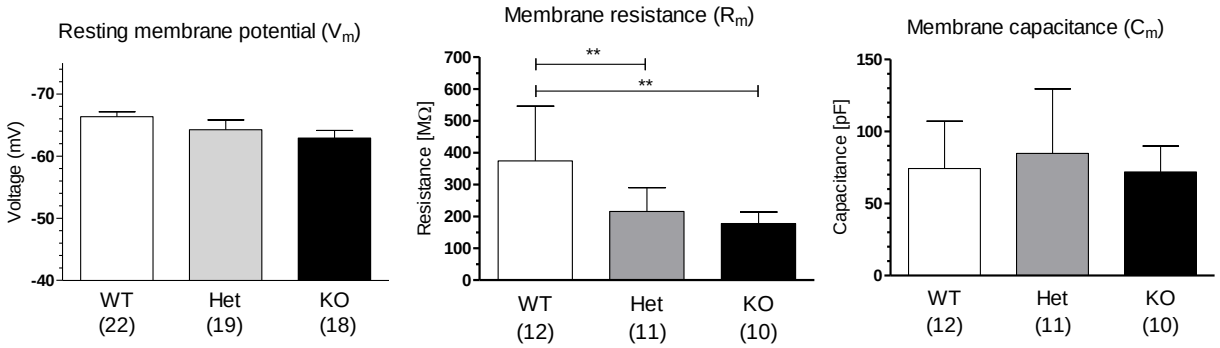

Fig. 3.21. Static membrane properties of CA1 pyramidal neurons. Data were obtained by the whole-cell configuration from the 5-Htt KO mice line. Numbers at the bottom of each column indicates the number of neurons recorded. Data are shown as the mean ± SD. Cell membrane resistance is significantly lower in KO and Het compared to WT (One-way ANOVA followed by Tukey's multiple comparison test, WT vs. Het, $p \leq 0.01$; WT vs. KO, $p \leq 0.01$).

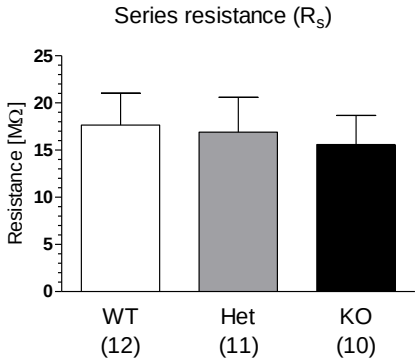

Fig. 3.22. Series resistance of whole-cell recording in CA1 pyramidal neurons among three genotypes of the 5-Htt KO mice line. Numbers at the bottom of each column indicates the number of neurons recorded. Data are shown as the mean ± SD. One-way ANOVA showed no statistically significant differences among three genotypes.

3.3.2. Action potential properties

To characterise action potentials, the following four parameters were quantified (**Fig. 3.23**): action potential amplitude (AP amp; mean (mV) ± SD: WT, 88.03 ± 8.99; Het, 88.73 ± 10.02; KO, 90.77 ± 7.72), action potential HHW (AP HHW; mean (ms) ± SD: 2.57 ± 0.44; Het, 2.15 ± 0.43; KO, 2.37 ± 0.74), action potential threshold (AP threshold; mean (mV) ± SD: WT, -49.58 ± 4.09; Het, -47.62 ± 4.51: KO, -45.27 ± 4.37), and minimum current injection to cause the first action potential (threshold current to cause AP: mean (pA) ± SD: 36.00 ± 17.24; Het, 35.71 ± 13.42; KO, 39.17 ± 20.65). Kruskal-Waillis test showed no statistical difference in AP amp among genotypes ($H_{(2)} = 0.8032$, $p = 0.669$). One-way ANOVA showed no statistically significant difference in AP HHW, AP threshold, and threshold current to cause AP among genotypes ($F_{(2,32)} = 1.828$, $p = 0.178$; $F_{(2,31)} = 2.711$, $p = 0.082$; $F_{(2,38)} = 0.158$, $p = 0.854$, respectively).

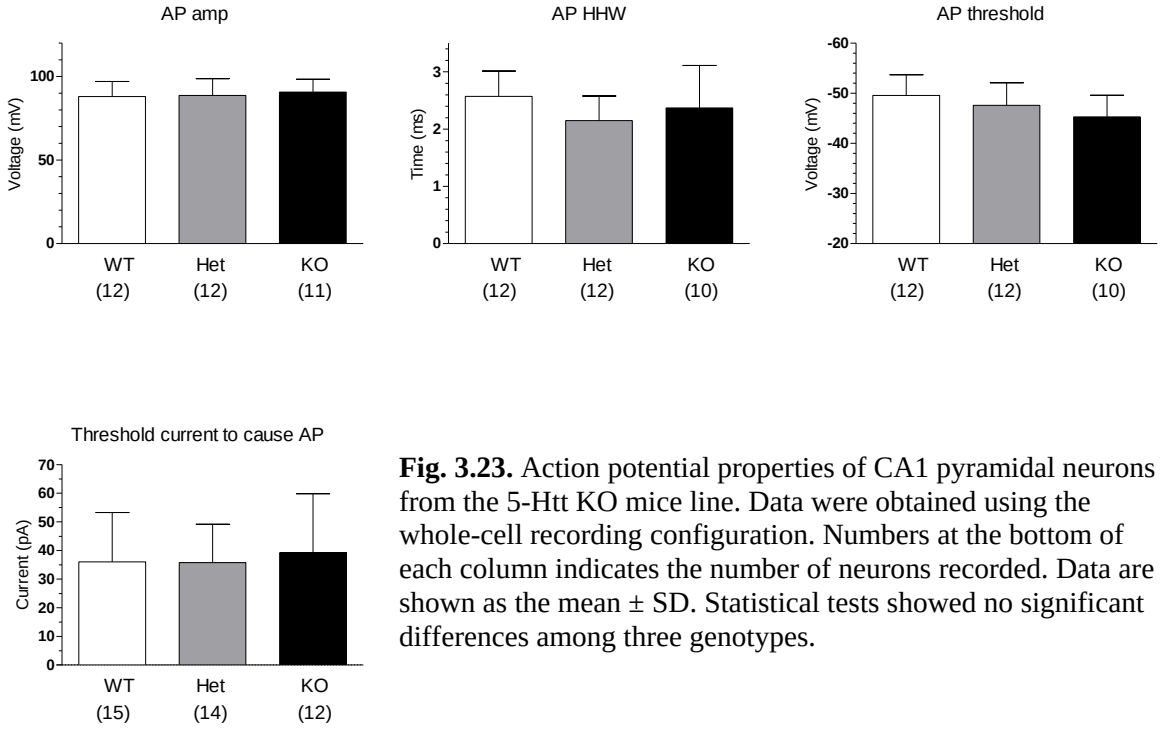

Fig. 3.23. Action potential properties of CA1 pyramidal neurons from the 5-Htt KO mice line. Data were obtained using the whole-cell recording configuration. Numbers at the bottom of each column indicates the number of neurons recorded. Data are shown as the mean ± SD. Statistical tests showed no significant differences among three genotypes.

Fig. 3.24 shows voltage changes in response to step-wise current injection of 800 ms duration. Action potential numbers were counted from this protocol with respect to different amounts of current injection. **Fig. 3.25** shows the summary of the number of action potentials from three genotypes. 5-Htt Het and KO mice had a lower number of action potentials than WT mice across the different amounts of current injection. Two-way repeated measure ANOVA followed by Bonferroni multiple comparison test showed statistical significance between Het and WT at 50 and 60 pA injection ($p \leq 0.05$) (main effect of current $F_{(8)} = 82.04$, $p \leq 0.0001$; main effect of genotype $F_{(2)} = 3.820$, $p = 0.031$; current by genotype interaction $F_{(16)} = 1.845$, $p = 0.025$).

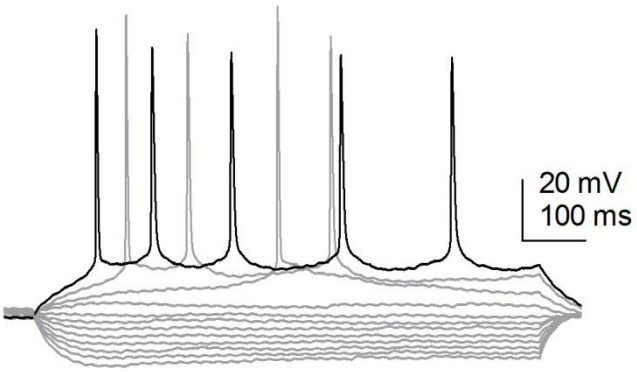

Fig. 3.24. Action potentials in response to step-wise current injection. The example shows voltage response to step-wise current injection ranging from -70 pA to 40 pA. Traces expect the response to 40 pA injection are dimmed. The number of action potentials was counted from each trace.

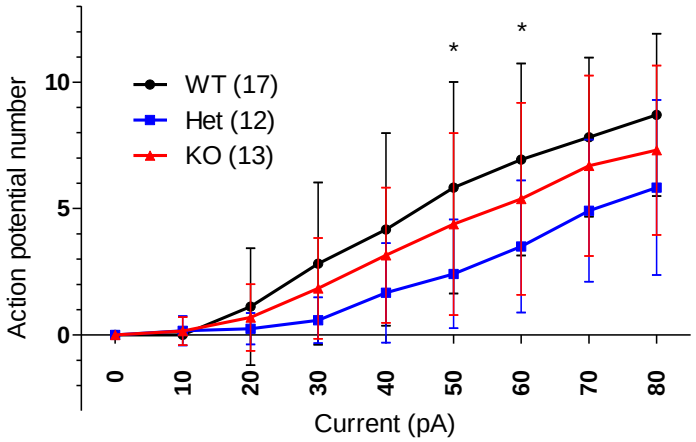

Fig. 3.25. Number of action potentials caused by increasing amount of current injection. The number of neurons recorded is shown in parentheses next to the name of genotypes. Data are shown as the mean ± SD. Two-way repeated measure ANOVA followed by Bonferroni multiple comparison test showed statistical significance between Het and WT at 50 and 60 pA injection ($p \leq 0.05$).

3.3.3. Synaptic transmission

To assess the efficacy of synaptic transmission, the amplitude of EPSC in response to the Schaffer collateral/commissural pathway stimulation was measured (**Fig. 3.26 left**). In addition, the ratio of the second EPSC to the first was calculated when two stimuli were given at a short time interval (Paired-pulse ratio; **Fig. 3.26 right**). Kruskal-Wallis test showed no differences in EPSC amplitude among genotypes ($H_{(2)} = 4.287$, $p = 0.117$) although the average EPSC amplitude recorded from Het and KO mice were about 100 pA larger than that from WT mice (mean EPSC (pA) ± SD: WT, 210.8 ± 160.1; Het, 304.9 ± 225.0; KO, 294.1 ± 153.1). One-way ANOVA showed that there is also no statistically significant difference ($F_{(2,26)} = 0.298$, $p = 0.745$) in paired-pulse ratio among genotypes (mean paired-pulse ratio ± SD: WT, 1.61 ± 0.25; Het, 1.54 ± 0.23; KO, 1.54 ± 0.18).

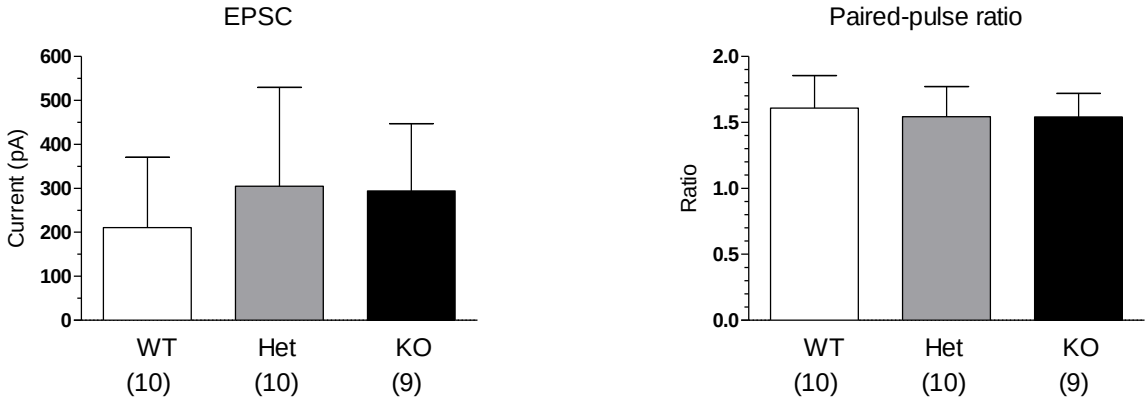

Fig. 3.26. Synaptic transmission properties measured from CA1 pyramidal neurons in the 5-Htt KO mice line. Synaptic transmission was induced by stimulating the Schaffer collateral/commissural pathway. (left) amplitude of excitatory postsynaptic current (pA). (right) Paired-pulse ratio (see Materials and Methods for details). Data are shown as the mean ± SD. Numbers at the bottom of each column indicates the number of neurons recorded. Statistical test showed no significant differences among genotypes.

3.3.4. I/V relationship

Fig. 3.27 illustrates how the I/V relationship, i.e. current-voltage relationship, was measured in the voltage-clamp mode. The membrane potential was changed from -130 mV to 30 mV in 10 mV increments for 200 ms. The current response was measured at the end of each voltage step, where the current response became steady. The amount of this steady-state current (i.e. current not associated with action potentials) at various voltages is plotted in **Fig. 3.28**. Two-way repeated measure ANOVA showed statistically highly significant differences ($p \leq 0.01$) between WT and Het at the voltage range of 10 to 30 mV (main effect of voltage $F_{(16)} = 240.1$, $p \leq 0.0001$; main effect of genotype $F_{(2)} = 2.743$, $p = 0.081$; voltage by genotype interaction $F_{(32)} = 2.637$, $p \leq 0.0001$). When the membrane potential was depolarised above the action potential threshold (c.a. above -50 mV), action potentials were generated, mostly at the beginning of voltage steps (**Fig. 3.27**). The amount of inward current accompanying action potentials is plotted in **Fig. 3.29**. Two-way repeated measure ANOVA indicated significant differences between WT and KO at the voltage range of -40 to 0 mV and between WT and Het at -50 to -20 mV (main effect of voltage $F_{(16)} = 84.93$, $p \leq 0.0001$; main effect of genotype $F_{(2)} = 6.314$, $p = 0.006$; voltage by genotype interaction $F_{(32)} = 3.982$, $p \leq 0.0001$).

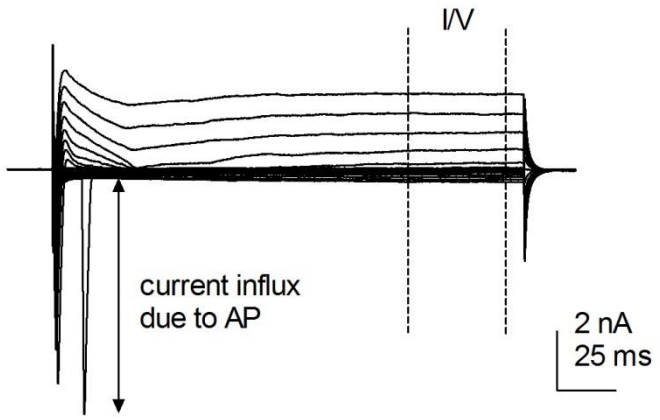

Fig. 3.27. Current measurement in response to voltage steps. The example shows current responses in the voltage-clamp mode where the membrane potential was changed from -130 mV to 30 mV (Δ 10 mV) for 200 ms duration. I/V relationship was obtained from the segments at the end of each voltage step where current responses become stable. When the voltage steps exceeded the action potential (AP) threshold, action potentials were generated at the beginning of each voltage step. The amount of current influx due to action potentials was measured.

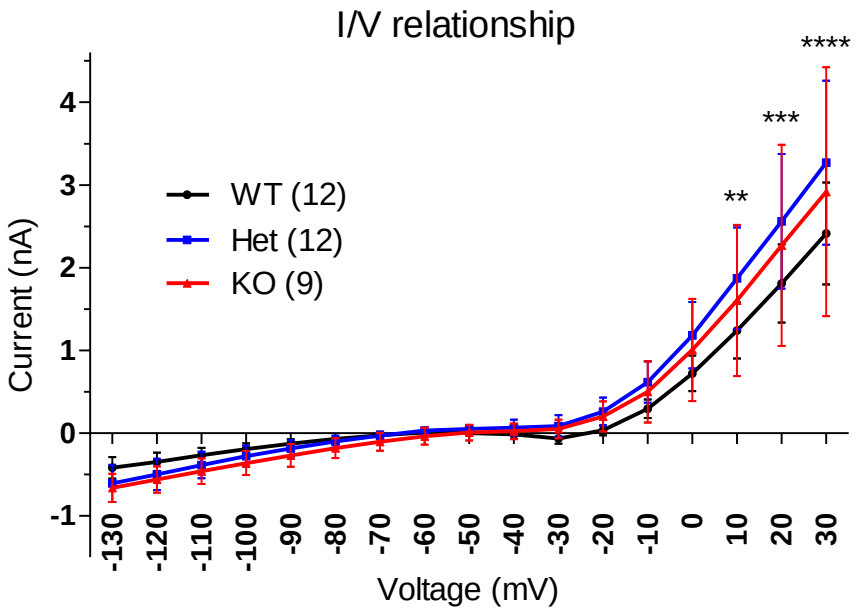

Fig. 3.28. Current (I) / Voltage (V) relationship of CA1 pyramidal neurons in the 5-Htt KO mice line. Data were obtained by the whole-cell recording configuration at the voltage-clamp mode. Each curve represents the average response of several neurons, whose number is indicated in the parentheses for each genotype. Error bars show SD. Two-way repeated measure ANOVA showed statistically significant differences between WT and Het at the voltage range of 10 to 30 mV.

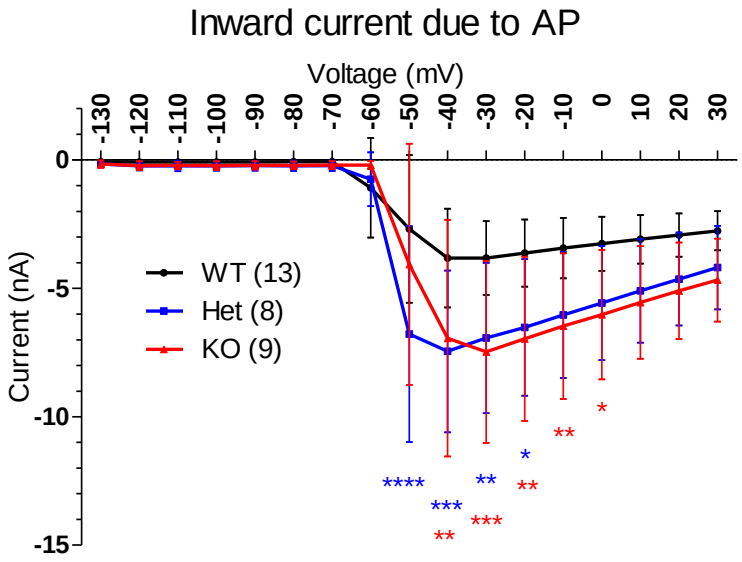

Fig. 3.29. Magnitude of inward current induced by action potentials (APs). Recordings were made in the whole-cell configuration at the voltage-clamp mode from CA1 pyramidal neurons of the 5-Htt KO mice line. Data are presented as mean ± SD. The number of recorded neurons from each genotype is shown in parentheses next to genotype names. Two-way repeated measure ANOVA indicated significant differences between WT and KO at the voltage range of -40 to 0 mV and between WT and Het at -50 to -20 mV.

3.3.5. Morphological analysis

Dendritic arborisation patterns of Neurobiotin-stained neurons were traced by Neurolucida software (Microbrightfield, Inc., Williston, VT, USA). Dendritic parameters to be analysed included total length of dendritic material, total number of branch points of all apical/basal dendrites, and spine density. For 3D analysis, Sholl analysis was conducted (**Fig. 3.30**). In this analysis, spheres of increasing radii (20 μm steps) are applied around the soma of neurons, and such parameters as the number of intersections between each sphere and dendritic tree, the total dendritic tree extension (i.e. the largest sector intersected), the length of dendritic material within each spherical sector are collected (Nietzer et al., 2011). No differences concerning total length, total number of nodes and total branch packing were found. Concerning Sholl analysis, there were some differences in one single sector (200 - 220 μm around the soma; **Fig. 3.31**). KO neurons have a significantly shorter radius length in this particular sector compared to WT or Het neurons (Median in μm): WT (n=12) 103.05, Het (n=3) 120.4, KO (n=6) 54.15; p value (Kruskal-Wallis test): WT-Het 0.734, WT-KO 0.014, Het-KO 0.024). However, since the number of Het samples was not enough, only the comparison between WT and KO was taken into account.

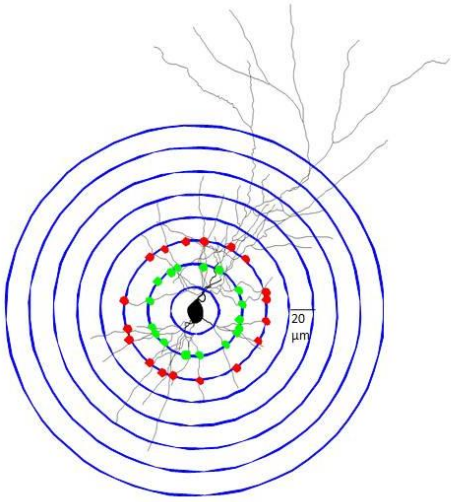

Fig. 3.30. Schematic of Sholl analysis. Scholl analysis applies concentric circles around the soma with equal inter-circle distance (in this case 20 µm). Green and red dots indicate points where dendritic arborisation intersects circles. Such parameters as the number of intersects, the dendritic length between each circle etc. are quantified.

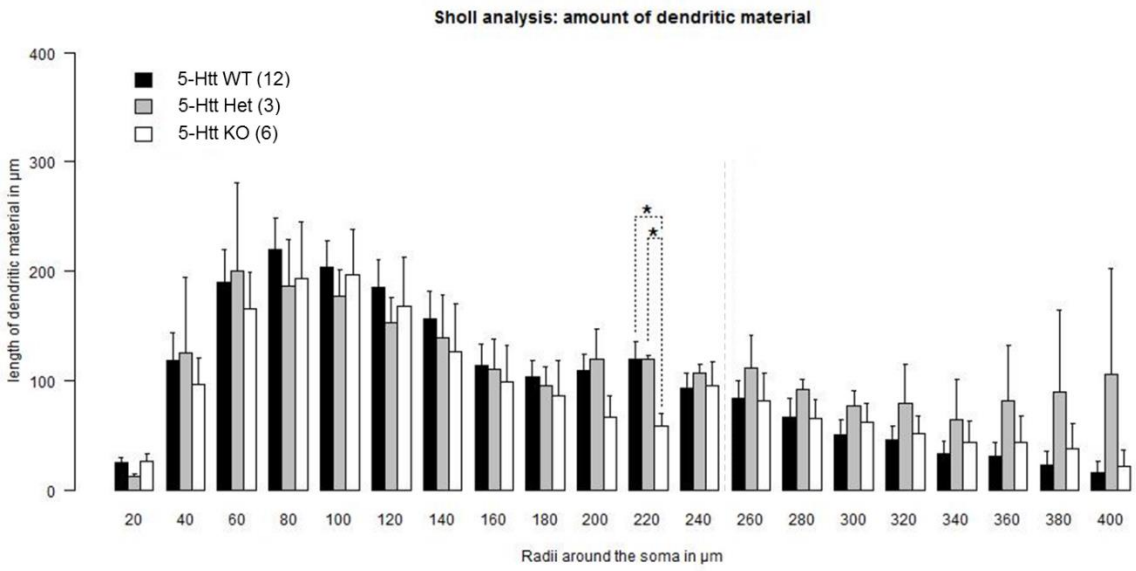

Fig. 3.31. Sholl analysis of Neurobiotin-stained hippocampal CA1 pyramidal neurons. Data are shown as mean ± Standard Error of the Mean (SEM). The number of neurons analysed is shown in the parentheses in the legends. Kruskal-Wallis test revealed that neurons from KO mice have significantly shorter dendritic material length compared to WT and Het neurons in 200 - 220 µm segment.

3.4. Extracellular field potential recording of long-term potentiation (LTP) (Study 4)

3.4.1. Time course of fEPSP change

Fig. 3.32 shows the time course of fEPSP slope change. The figure shown here represents an ideal experiment which satisfies criteria for quality control as mentioned in Materials and Methods. First, the last 15 mins of BSL measurement before the delivery of TB5 remained flat. Second, the maximum slope as revealed in the SRC before and after the TB5 protocol (c.a. -30 mins and 55 mins of recording, respectively) remained the same. Third, the stimulus amplitude was set properly to produce the BSL response which is within 25-40% of maximum slope.

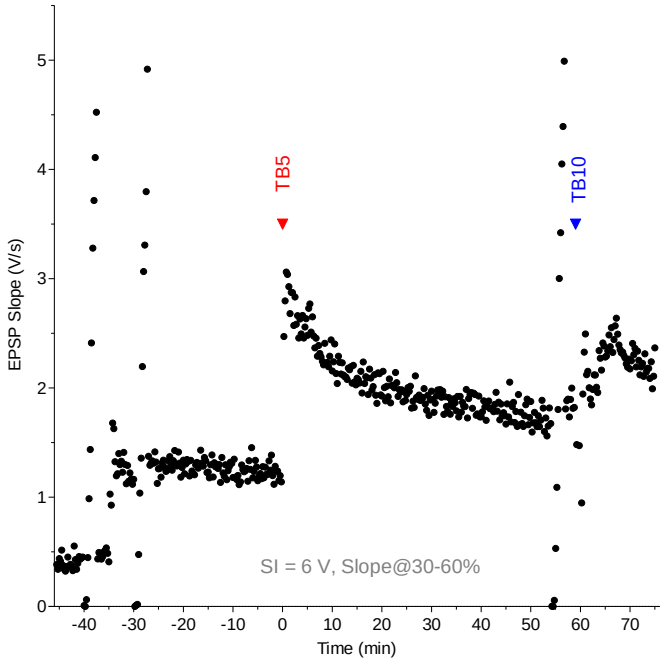

Fig. 3.32. Typical example of time course of fEPSP slope change. Each dot corresponds to slope measured every 15 seconds. Stimulus intensity was set at 6V, slope was measured at 30-60 % of the slope amplitude. See Materials and Methods for the description of TB5 and TB10 protocols. SI: stimulus intensity.

3.4.2. Shape of fEPSP

Fig. 3.33 shows fEPSP measured before and after TB5 (left) and TB10 (right) potentiation protocols. In each case, the amplitude and slope of fEPSP increased after the respective potentiation stimulus. It should be noted that even though the fEPSP response increased in amplitude and slope, their stimulus artefacts and afferent volleys (See **Fig. 3.34**) remained the

same, satisfying the criteria for quality control mentioned in Materials and Methods. **Fig. 3.34** describes details of the fEPSP shape.

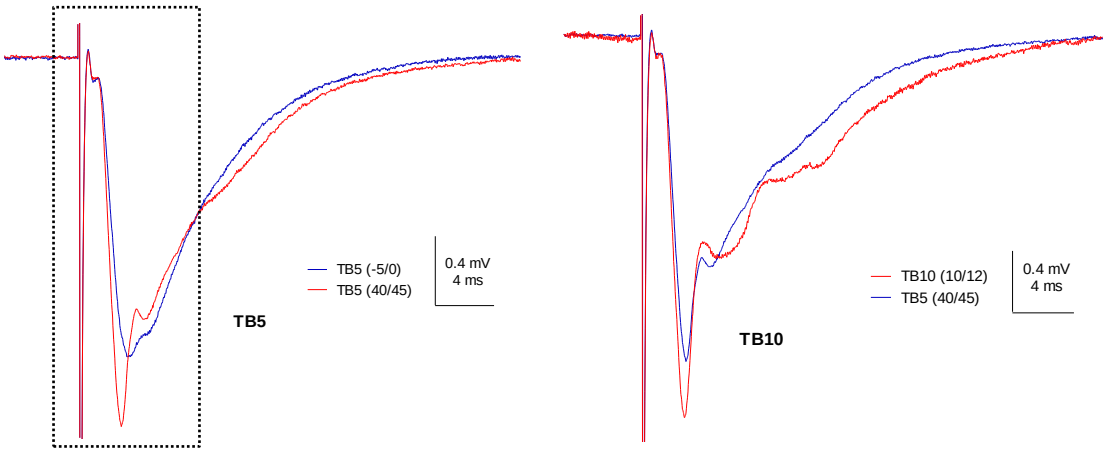

Fig. 3.33. Change in the amplitude and slope of fEPSP before/after TB5 (left) and TB10 (right) potentiation protocols. Blue lines represent average fEPSP prior to TB5/TB10 protocols and red lines represent averaged fEPSP after TB5/TB10 protocols. (left) TB5 (-5/0): average fEPSP obtained from 5 mins interval prior to TB5; TB5 (40/45): average fEPSP obtained at 40-45 mins interval following TB5. (right) TB5 (40/45): average fEPSP obtained at 40-45 mins interval following TB5 (the same as in the left figure); TB10 (10/12): average fEPSP obtained at 10-12 mins interval following TB10. Note that in both cases of TB5 and TB10, amplitude and slope of fEPSP increased compared to those prior to the potentiation stimulus. Figure within the hatched square on the left is further explained in **Fig. 3.34**.

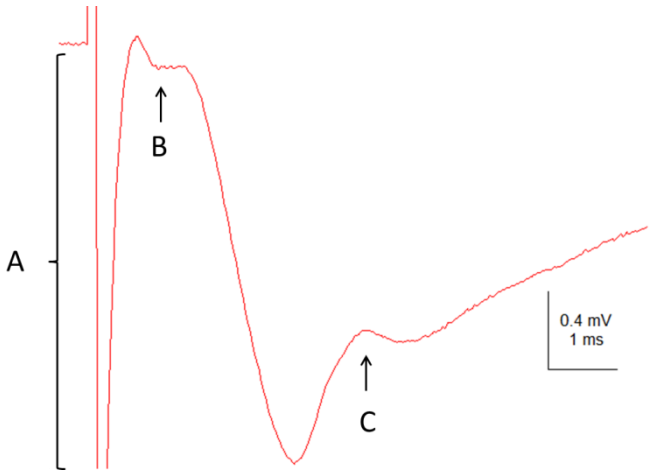

Fig. 3.34. Description of fEPSP shape. The figure corresponds to that enclosed by a hatched square in Fig. 3.33 left. A) Stimulus artefact. This is an electrical signal derived directly from the stimulus electrode. B) Field potential associated with action potentials in presynaptic axons, called an afferent volley. C) Upward deflection caused by a population spike in the pyramidal cell layer.

3.4.3. Comparison of normalised fEPSP across different genotypes

Fig. 3.35 shows normalised fEPSP slope change from the Tph2 KO mice line, where the average of baseline slope value was set as 1. TB5 was given at time 0 and the baseline was taken from 15 mins-segment preceding TB5 (i.e. -15 - 0 mins). In all the genotypes, augmentation of slope was observed. **Fig. 3.36** shows the same parameters in the 5-Htt KO mice line.

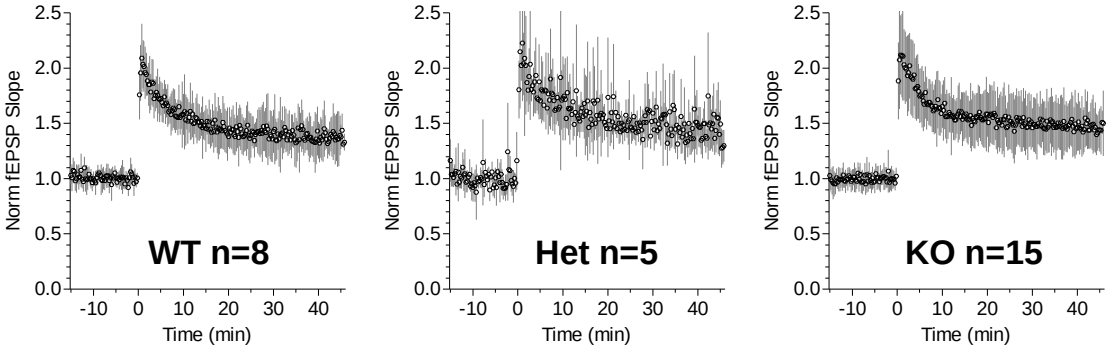

Fig. 3.35. Normalised fEPSP slope obtained from ventral hippocampi of the Tph2 KO mice line. TB5 was given at time 0. Number of recorded slices from each genotype is shown as "n=". Data are shown as mean ± SD.

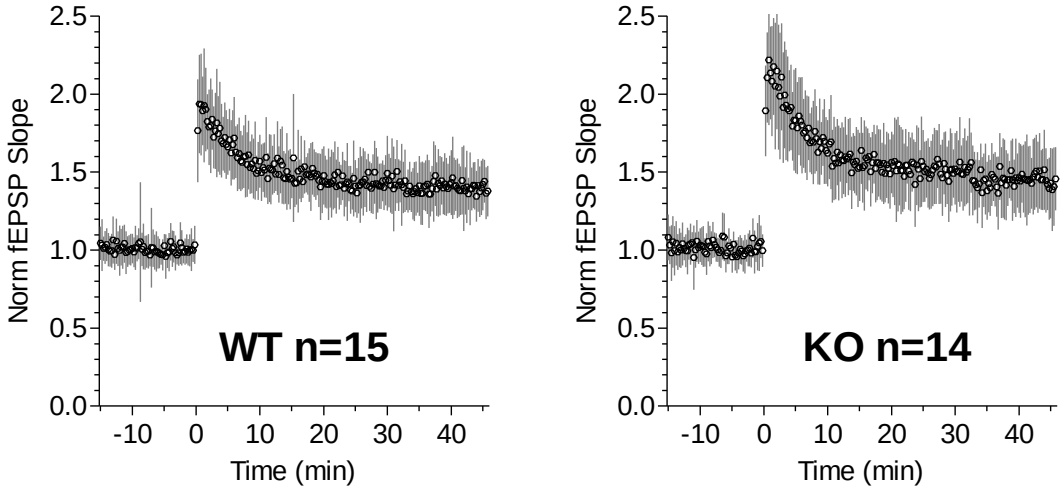

Fig. 3.36. Normalised fEPSP slope obtained from ventral hippocampi of the 5-Htt KO mice line. TB5 was given at time 0. Number of recorded slices from each genotype is shown as "n=". Data are shown as mean ± SD.

3.4.4. Superposition of fEPSP traces and comparison of % potentiation

Fig. 3.37 shows the time course of normalised fEPSP slope from Tph2 KO and 5-Htt KO mice. To facilitate comparison among genotypes, traces were overlaid and error bars were omitted for clarity. As shown here, no clear differences were visible among three genotypes of the Tph2 KO mice line. In the case of the 5-Htt KO mice line, KO mice exhibited higher initial fEPSP slope increase immediately after the TB5. However, after 45 mins of recording following the TB5, fEPSP slope declined to the same level as WT mice. As a definition of LTP, this indicates that LTP inducibility between KO and WT mice is not different.

To quantify the magnitude of LTP inducibility, distribution of % potentiation compared to respective baseline is shown as a scatter plot (**Fig. 3.38**) (mean ± SD; Tph2-KO, 46.33 ± 18.73; Tph2-Het, 47.27 ± 15.98; Tph2-WT, 37.30 ± 15.08; 5-Htt-WT, 40.18 ± 13.67; 5-Htt-KO, 45.85 ± 16.47). Kruskal-Wallis test showed that there was no statistically significant difference among genotypes ($H_{(4)} = 3.615$, $p = 0.461$).

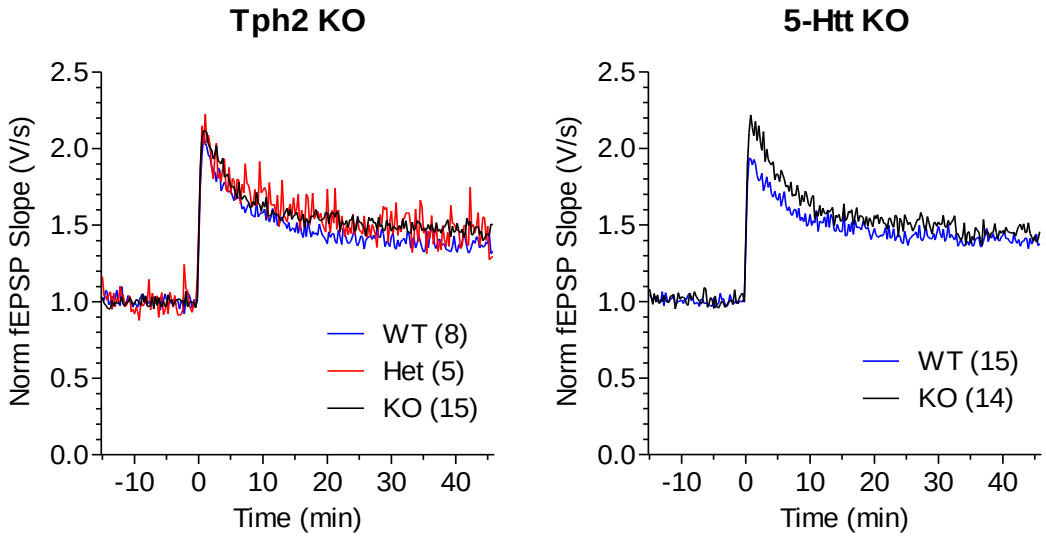

Fig. 3.37. Overlay of normalised fEPSP slope obtained from ventral hippocampi of the Tph2 KO mice (left) and 5-Htt KO mice line (right). TB5 was delivered at the time 0. Number of recorded slices from each genotype is shown in parentheses. For clarity, error bars are omitted.

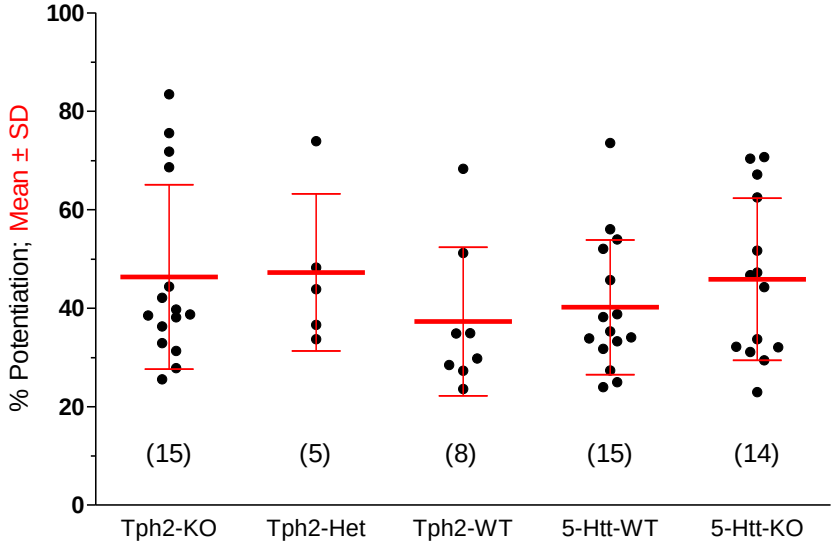

Fig. 3.38. Summery plot of % potentiation in response to TB5 stimulation in ventral hippocampi across all the genotypes measured. Data are shown as mean ± SD. Number of recorded slices from each genotype is shown in parentheses. Kruskal-Wallis test showed that there was no statistically significant difference among genotypes ($H_{(4)} = 3.615$, $p = 0.461$).

4 . Discussion

4.1. Loose-seal cell-attached recording from serotonergic neurons in the DRN of 5-Htt and Tph2 KO mice (Study 1)

4.1.1. Baseline pacemaker firing activity was preserved in KO mice

In the present series of studies, firing rate changes in response to different pharmacological interventions, i.e. R8HD, 5-HTP, and Trp, were monitored. There is a possibility that baseline firing rates were already altered by genetic manipulation itself even before any drug application. To address this possibility, baseline firing rates of all the neurons identified as serotonergic were compared across genotypes (**Fig. 3.4**). We observed similar baseline firing rates among genotypes except that Tph2 KO mice had slightly lower baseline firing rates compared to other genotypes. As a consequence, differences in firing rates reached statistical significance only between Tph2-KO vs. 5-Htt-KO and Tph2-KO vs. WT. It is known that in bran slice preparations, there is very little, if any, 5-HT_{1A} receptor-mediated suppression of firing rates when the superfusing ACSF lacks 5-HT precursor, Trp (Liu et al., 2005; Mlinar et al., 2005; Evans et al., 2008). This is different from baseline firing rates typically reported in *in vivo* studies, where 5-HT_{1A} receptor-mediated autoinhibition as well as other mechanisms to control firing rates of serotonergic neurons exist, which holds in particular for recordings in non-anaesthetised animals. The baseline firing rates presented here, on the contrary, are not correlated with autoinhibition of serotonergic neuron firing. Nonetheless, they indicate that the basic characteristics of intrinsic pacemaker firing activity of serotonergic neurons are preserved independently from autoinhibition. The slight decrease in baseline firing rates observed in Tph2 KO mice may indicate that, in the chronic absence of 5-HT, neurons adapt their membrane properties, e.g. conductance, to substitute for absent autoinhibition and homeostatically keep pacemaker firing activity constant.

4.1.2. Serotonergic neurons in Tph2 KO mice and 5-Htt KO mice retain normal electrophysiological properties

The results presented here demonstrate that serotonergic neurons in Tph2 KO mice and 5-Htt KO mice retain gross electrophysiological properties as found in serotonergic neurons of WT counterparts, consisting of a wide action potential width (manifested as a wide UDI of action

current in the cell-attached recording configuration) and a slow and regular firing rate. The result was rather surprising considering that 5-HT has been proposed to play a crucial role in the development of the CNS (for review see Daubert and Condron, 2010).

As mentioned in Introduction, several types of ion conductance are involved in the formation of a typical shape of action potential generated by serotonergic neurons: low threshold Ca^{2+} current, high threshold Ca^{2+} current, Ca^{2+}-activated K^+ outward current, and I_A current. Accordingly, several ion channels have been implicated in the formation of such conductance, i.e. T channels seem to account for the low-threshold Ca^{2+} current whereas at least three different Ca^{2+} channel types (including N- and L-type) seem to be involved in the generation of high-threshold Ca^{2+} current (Pineyro and Blier, 1999). The results from the present study may imply that genetic manipulation of the serotonergic system did not affect normal expression of those ion channels that take part in the generation of serotonergic-like action potential shapes. Further investigation is needed to elucidate the mechanism which enables serotonergic neurons in Tph2 KO mice and 5-Htt KO mice to retain typical serotonergic characteristics in spite of the lack of endogenous 5-HT and in the presence of excessive extracellular 5-HT, respectively.

4.1.3. Responsiveness of 5-HT$_{1A}$ receptors were shifted in the opposite direction in Tph2 KO and 5-Htt KO mice

The results obtained from R8HD application demonstrated mild sensitisation and marked desensitisation of 5-HT_{1A} receptors in Tph2 KO mice and 5-Htt KO mice, respectively. The slight increase in responsiveness to 5-HT_{1A} receptor agonists in Tph2 KO mice is consistent with a previous immunohistochemical study where increase in 5-HT_{1A} receptor density was observed (Gutknecht et al., 2012). Similarly, no change in responsiveness of serotonergic neurons from Tph2 Het mice to R8HD is in accordance with only slight, non-significant increase in 5-HT_{1A} levels previously reported (Gutknecht et al., 2012). The results obtained from 5-Htt KO mice are also in agreement with previous immunohistochemical studies demonstrating downregulation of 5-HT_{1A} receptors (Fabre et al., 2000; Li et al., 1999, 2000) and electrophysiological studies (Gobbi et al., 2001; Mannoury la Cour et al., 2001). Moreover, it was shown in previous electrophysiological studies that chronic SSRI treatment, which mimics the effects of constitutive *5-Htt* KO, leads to a 2- to 3-fold shift to the right of the DRC of 5-HT autoreceptor agonist application (Blier, 2010 and references therein). *In vitro* electrophysiological recordings on brain slices made by Mannoury la Cour et al. (2001)

demonstrated ~55-fold shift in EC_{50} values in response to a selective 5-HT_{1A} receptor partial agonist ipsapirone (EC_{50}-WT = ~63.1 nM vs. EC_{50}-KO = ~3.5 μM) whereas ~6-fold shift in response to a non-selective 5-HT_{1A} receptor full agonist 5-CT (EC_{50}-WT = ~9.05 nM vs. EC_{50}-KO = ~52.7 nM). Moreover, in the same study, it was shown that ipsapirone completely blocked firing of serotonergic neurons in 5-Htt WT and Het mice at the concentration of 1 μM, while 100 μM of ipsapirone was needed to stop firing of serotonergic neurons in KO mice. In the present study, EC_{50} values was shifted ~20-fold in response to R8HD application (EC_{50}-WT = ~3.0 nM vs. EC_{50}-KO = ~67.6 nM). In our study, firing of serotonergic neurons in 5-Htt KO mice did not further decrease in response to R8HT at the concentration of 300 nM or higher. The difference between the current study and that of Mannoury la Cour et al. (2001) seems to derive from the difference in recording configurations as well as types of 5-HT_{1A} receptor agonists. It should be noted that R8HD is a selective full agonist against 5-HT_{1A} receptors, although moderate affinity (≤ 100 nM) for 5-HT_7 receptors is also reported (To et al., 1995). It should also be emphasised, however, that the electrophysiological data presented in the present study are more robust than previous electrophysiological studies because EC_{50} values were obtained from the average of several neurons, each producing the full DRCs. The magnitude of desensitisation in 5-Htt KO mice was larger than that of sensitisation in Tph2 KO mice. In fact, even high concentrations (>300 nM) of R8HD did not completely stop firing of serotonergic neurons from 5-Htt KO mice. This is probably because, in Tph2 KO mice, the complete lack of brain 5-HT does not produce any functional consequences regardless of the sensitivity of 5-HT_{1A} receptors, thus there is no need for intense sensitisation of 5-HT_{1A} receptors. In 5-Htt KO mice, on the other hand, there is an impending need for intense desensitisation to counteract excessive amounts of extracellular 5-HT, which may otherwise stimulate 5-HT_{1A} receptors too strongly. Our data also showed that heterozygous mice of each KO line, i.e. Tph2 Het and 5-Htt Het, are no different from their WT counterparts, although there is a trend of desensitisation in 5-Htt Het mice. This would indicate that deleting one of the alleles encoding either Tph2 or 5-Htt is not enough to produce functionally relevant changes in 5-HT_{1A} receptor sensitivity, probably due to an as-yet-unknown biological compensatory mechanism. Moreover, one should bear in mind that data for 5-HT_{1A} receptor responsiveness in Tph2 KO mice should be treated separately when inferring to changes in autoinhibition because in these mice serotonergic neurons do not produce 5-HT and therefore *in vivo* the 5-HT_{1A} receptors are unlikely to be physiologically stimulated.

4.1.4. Lack of 5-HT synthesis from Trp in Tph2 KO mice and presence of autoinhibition in 5-Htt KO mice

The result obtained from Trp application experiments confirmed the complete lack of Tph2 reactivity/5-HT synthesis and autoinhibition in Tph2 KO mice when supplied with 5-HT precursor Trp (**Fig. 3.9**). In contrast, application of 5-HTP readily decreased firing of serotonergic neurons in these mice, indicating that the enzymatic pathway of 5-HT synthesis downstream of Tph2-dependant rate-limiting step is intact in Tph2 KO mice. The latter result, together with the result obtained from direct R8HD application, also demonstrates that 5-HT_{1A} autoreceptors are functional in Tph2 KO mice. This further reinforces the result obtained from Trp application, verifying that the lack of response to Trp is not due to the lack of functional 5-HT_{1A} autoinhibitory receptors but indeed the lack of 5-HT synthesis. Moreover, the preserved capability to produce 5-HT from 5-HTP, which may *in vivo* enter the brain from peripheral sources, could explain the trace amount of 5-HT found in the brain of Tph2 KO mice (Gutknecht et al., 2012). It should be noted that responsiveness to Trp application in brain slices from Tph2 Het mice was not different from that of WT mice; both led to reduction in firing rate to a similar extent. This may reflect the fact that 5-HT_{1A} receptor sensitivity, measured as responsiveness to R8HD, was not different in Tph2 Het mice compared to WT mice. Alternatively, the lack of difference in Tph2 Het mice implies that gene dose-dependent reduction in serotonin synthesis cannot be directly translated into functional difference. In fact, Tph2 Het mice were shown to have only 20-25% reduction in 5-HT content, and the reduction was limited to the rostral raphe (Gutknecht et al., 2012).

To our surprise, 5-Htt Het and KO mice retained normal autoinhibitory function as seen in wildtype counterparts despite the decrease in functional response of 5-HT_{1A} receptors (**Fig. 3.10**). Indeed, the magnitude of desensitisation of 5-HT_{1A} receptors was quite intense as was shown by direct stimulation of 5-HT_{1A} receptors by R8HD. Here, even the high concentration (>300 nM) of R8HD did not stop firing of serotonergic neurons (**Fig. 3.8**). For this reason, the argument that the magnitude of 5-HT_{1A} receptor desensitisation was not so intense as to impair the auto-inhibitory mechanism may not apply. Admittedly, there is a possibility that autoinhibition after Trp or 5-HTP application in 5-Htt KO mice might have been mediated by receptors other than 5-HT_{1A} receptors. To address this possibility, a selective 5-HT_{1A} receptor antagonist, WAY-100635 (50 nM), was applied on a slice from a 5-Htt KO mouse to see if autoinhibition is still present. As shown in **Fig. 4.1**, WAY-100635 completely abolished autoinhibition following Trp (30 μM) application, confirming that autoinhibition in 5-Htt KO

mice is also mediated by 5-HT_{1A} autoreceptors. The same effect of WAY-100635 was observed in two other experiments done on brain slices obtained from 5-Htt KO and two other experiments from 5-Htt Het mice (data not shown).

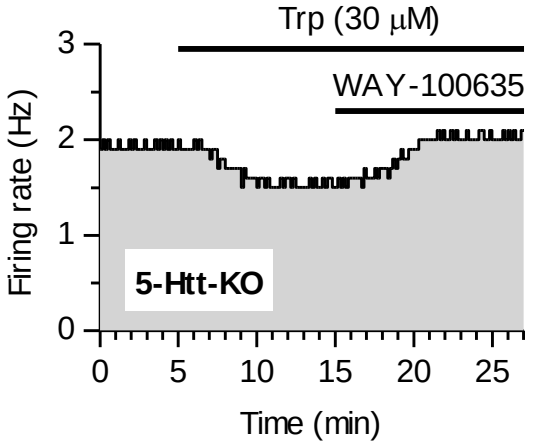

Fig. 4.1. Autoinhibition is mediated by 5-HT_{1A} autoreceptors also in 5-Htt KO mice. Supplementation of Trp (30 μM) on a brain slice obtained from a 5-Htt KO mouse induced synthesis of 5-HT, which then led to autoinhibition of serotonergic neuron firing, seen as a decrease in the firing rate. Application of a selective 5-HT_{1A} receptor antagonist, WAY-100635 (50 nM) abolished autoinhibition and brought back the firing rate to its baseline level, confirming that autoinhibition was mediated by 5-HT_{1A} autoreceptors.

It can be assumed that in 5-Htt Het and 5-Htt KO mice, desensitised 5-HT_{1A} receptors allow these mice to maintain normal autoinhibitory function of serotonergic neurons in the presence of elevated/excessive amounts of extracellular 5-HT, which is attributed to the lesser expressed or complete lack of 5-Htt. As opposed to R8HD and 5-HTP application, quantitative analysis of responsiveness of serotonergic neurons to Trp, i.e. dose-response curve, was not conducted. This is because serotonergic neurons in Tph2 KO do not respond to Trp at all and the effect of Trp is relatively small and even application of a higher concentration (100 μM) did not further decrease serotonergic neuron firing (**Fig. 3.9**).

4.1.5. Serotonergic neurons of Tph2 KO and 5-Htt KO mice are more responsive to 5-HTP than those of wildtypes

Plots of logarithm of EC_{50} in response to 5-HTP application among five different genotypes (**Fig. 3.11**) resulted in a bell-shape distribution; EC_{50} values decreased both in Tph2 KO mice and 5-Htt KO mice with each respective heterozygous mice showing intermediate responses. The reduction in EC_{50} in Tph2 KO mice is probably due to mild sensitisation of 5-HT_{1A}

receptors as was observed in response to R8HD application. Alternatively, it may reflect potential up-regulation of AADC as a compensatory mechanism to the complete absence of Tph2 in Tph2 KO mice. This possibility was not further investigated in the present study. Another possibility is that in Tph2 KO mice, levels of 5-Htt decreased in order to increase extracellular 5-HT and thus enhance 5-HT mediated autoinhibition. This is not likely to be the case, however, since no change in 5-Htt expression levels was found previously (Kriegebaum et al., 2010). Moreover, past studies indicate that 5-Htt expression levels are relatively insensitive to the level of extracellular 5-HT, although they are sensitive to blockade by SSRI (Jacobsen et al., 2012b). Therefore it is rather unlikely that 5-Htt levels decreased in response to reduced extracellular 5-HT in Tph2 KO mice.

The results obtained from 5-Htt Het and KO mice were quite opposite to our expectation. Despite the fact that 5-HT_{1A} receptors are mildly and significantly desensitised in 5-Htt Het and KO mice, respectively, serotonergic neurons in 5-Htt Het and KO mice decreased firing at a lower concentration of 5-HTP compared to WT mice, with the difference reaching statistical significance in the case of 5-Htt KO mice. However, while high concentration of 5-HTP stopped firing in 5-Htt WT and Het mice, it did not always stop firing in 5-Htt KO mice, reaching saturation in decrease in firing rates. At first sight, this is consistent with the possibility that 5-HT_{1A} receptor density/function is decreased as demonstrated in response to R8HD. Another possible interpretation is that, in 5-Htt KO mice, the level of extracellular 5-HT becomes so high in the presence of a high concentration of 5-HTP, even higher than normal because of the complete lack of serotonin-reuptaking proteins, that it circumvents desensitisation of 5-HT_{1A} receptors to reduce firing of serotonergic neurons, which would be biologically sufficient to maintain homeostatic regulation of serotonergic neuron firing, even when it does not lead to complete cessation of serotonergic neuron firing. The limitation of this experiment is that 5-HT is produced without any rate-limiting step from 5-HTP and that 5-HT may have been produced also by other monoaminergic cells which have AADC. The advantage, however, is that this would override possible genotype-dependent adaptive changes in the activity of Tph2. In sum, the present data show that autoinhibition produced by endogenous 5-HT is preserved in the 5-Htt Het and KO mice, in spite of the decrease in functional response of 5-HT_{1A} receptors. Data from the Tph2 KO mice line also confirmed the presence of autoinhibition at a lower concentration of 5-HTP than WT mice, probably reflecting sensitisation of 5-HT_{1A} receptors.

In behavioural experimental settings, application of 5-HTP is often used to restore endogenously produced 5-HT in Tph2 KO mice (Angoa-Perez et al., 2012; Liu et al., 2011) or

to further increase extracellular 5-HT levels in 5-Htt KO mice (Fox et al., 2008). Furthermore, administration of 5-HTP is used to enhance 5-HT transmission in human subjects (Turner et al., 2006; Maron et al., 2004). Although studies on behavioural and physiological responses to 5-HTP application depending on TPH2 polymorphism in humans or Tph2 genotypes in animal models are still sparse, evidence suggests that responses to 5-HTP are influenced by human 5-HTTLPR or murine 5-Htt genotypes (Fox et al., 2008; Maron et al., 2004). Of course, one cannot deny the possibility that deferential responses to 5-HTP depending on 5-HTTLPR or 5-Htt genotypes are purely due to the different levels of serotonin transporter availability. It can be inferred from the results presented here, however, that one should take into account the fact that serotonergic neurons in Tph2 KO mice and 5-Htt KO mice have higher responsiveness to 5-HTP than in WT controls in terms of autoinhibition of serotonin neuron firing.

4.1.6. 5-HT synthesis and autoinhibition

Desensitisation/downregulation of 5-HT_{1A} receptors observed in 5-Htt KO mice is expected to lead to less autoinhibition of serotonergic neuronal activities. In the serotonin synthesis level, this was actually reported in a past study. Kim et al. (2005) reported the conversion rate of 5-HT into 5-HTP was 30-60% increased in different brain regions of 5-Htt KO mice *in vivo* compared to wildtype controls. Since no alteration in Tph2 mRNA levels or in maximal *in vitro* Tph activity in the brainstem of 5-Htt KO mice compared to wildtype controls was found, Kim and colleagues (2005) concluded that this increase in the conversion rate is rather an outcome of decrease in the efficacy of 5-HT_{1A} receptors to autoinhibit serotonergic neurons than upregulation of Tph2 activity. Indeed, it was demonstrated that activation of 5-HT_{1A} receptors by their agonists such as 8-OH-DPAT and ipsapirone suppressed increase in 5-HTP when AADC was at the same time inhibited in serotonergic neurons and, on the contrary, 5-HT_{1A} receptor antagonists increased accumulation of 5-HTP (Kim et al., 2005). Data in the present study, however, demonstrate that autoinhibition as assessed by decrease in firing rate due to *de novo* synthesised 5-HT is no different in 5-Htt KO mice compared to WT mice. Autoinhibition triggered by newly synthesised and released 5-HT involves multiple factors including the 5-HT synthesis pathway, reuptake, 5-HT_{1A} receptor sensitivity of serotonergic neurons. As evidenced in the present study, it is therefore not possible to discuss the level of autoinhibition from a single factor, e.g. the conversion rate of 5-HTP into 5-HT as

in the study of Kim et al. (2005). Rather, it should be considered as a result of synergistic interactions among factors mediating serotonergic neurotransmission.

4.1.7. Desensitisation or downregulation?

So far, the term sensitisation/desensitisation was used indiscriminately from upregulation/downregulation. What was actually meant was "functional (de)sensitisation", referring to augmentation or diminution of 5-HT_{1A} receptor function. As mentioned earlier, this may be directly linked to upregulation/downregulation of 5-HT_{1A} receptors, respectively, i.e. the increase and decrease in the number of 5-HT_{1A} receptors. However, a series of past studies investigating effects of chronic SSRI treatment on sensitivity of 5-HT_{1A} receptors point to the fact that desensitisation can take place without altering expression levels of 5-HT_{1A} receptors. In human studies, Sargent et al. (2000) reported that binding potential of 5-HT_{1A} receptors to WAY-100635, a 5-HT_{1A} receptor antagonist, in PET imaging was not altered in depressed patients before and after 6-weeks of SSRI treatment. However, other human studies indicated reduction in functional responsiveness of 5-HT_{1A} receptors after repeated SSRI treatment (Sargent et al., 2000 and references therein). The authors also referred to inconsistent findings in animal studies after SSRI administration. Some reported downregulation of 5-HT_{1A} receptor expression in the DRN and others reported no changes, although evidence suggests chronic SSRI treatment may still alter 5-HT_{1A} receptor function (Sargent et al., 2000 and references therein). These series of findings indicate that functional desensitisation of 5-HT_{1A} receptor function may not necessarily be linked to reduction in the number of 5-HT_{1A} receptors. In fact, in Cornelisse's study (Cornelisse et al., 2007), reduced GIRK currents after chronic SSRI treatment were also observed when GIRK currents were induced by direct stimulation of $GABA_B$ receptors by their agonists. Since 5-HT_{1A} receptors and $GABA_B$ receptors share the common G-protein pathway (Gi proteins) to be coupled with GIRK channels (Innis and Aghajanian, 1987; Colmers and Williams, 1988; Innis et al., 1988; Williams et al., 1988), the result indicates that desensitisation took place downstream of the signal transduction pathway not at the surface level of receptor expression. This is in agreement with radioligand binding assays showing that chronic SSRI (fluoxetine) treatment reduced the coupling of 5-HT_{1A} receptors and G-proteins without affecting 5-HT_{1A} receptor levels (Cornelisse et al., 2007 and references therein). Furthermore, in 5-Htt KO mice, which model mice chronically treated with SSRI, similar adaptive changes in 5-HT_{1A} receptor-stimulated G protein binding were found in DRN (Mannoury la Cour et al., 2001, 2004). As

expected from the shared pathway, reduction in G-protein coupling was observed in 5-Htt KO mice when this pathway was stimulated by $GABA_B$ receptors as well as 5-HT_{1A} receptors (Mannoury la Cour et al. 2004). However, Li et al. (1999, 2000) demonstrated reduction of 5-HT_{1A} receptor density in the hypothalamus, dorsal raphe nucleus and amygdala of 5-Htt KO mice without significant reduction in G-protein coupling. They concluded that the functional desensitisation of 5-HT_{1A} receptors is likely to be attributable to reduction in the receptor density but not to alteration in G-protein coupling. Moreover, Fabre et al. (2000) reported decrease in ^{35}S-GTP-γ-S binding evoked by 5-HT_1 receptor agonists in the DRN (~ 66%) and in the substantia nigra (~ 30%) of 5-Htt KO mice, which corresponds to the observed decrease in receptor density of 5-HT_{1A} receptors and 5-HT_{1B} receptors, respectively. Alternatively, desensitisation of negative coupling of 5-HT_{1A} receptor stimulation to the production of cAMP is proposed to be mediated by receptor phosphorylation through PKC as well as through G protein-coupled receptor kinases (Pineyro and Blier, 1999 and references therein). To make matters even more complicated, desensitisation at the level of effectors, namely K^+ channels, which are coupled with 5-HT_{1A} receptors via G-proteins, has been proposed (Pineyro and Blier, 1999). Caution should be taken, however, that experiments to investigate desensitisation mechanisms of 5-HT_{1A} receptors were not necessarily conducted on mammalian serotonergic neurons, i.e. either non-neuronal model cells which artificially express 5-HT_{1A} receptors or neuronal cells from non-mammalian organisms. It is unlikely that results obtained from such experiments may be directly extrapolated to desensitisation mechanisms of 5-HT_{1A} receptors in the mammalian CNS (Pineyro and Blier, 1999).

Given the presence of contradictory findings and uncertainties, it is not possible to comment on the precise mechanism of functional desensitisation of 5-HT_{1A} receptors in 5-Htt KO mice. It seems, however, probable that functional desensitisation can be attributed both to downregulation of 5-HT_{1A} receptors, i.e. the decrease in the number of 5-HT_{1A} receptors and desensitisation of 5-HT_{1A} receptors, most likely reduction in G-protein coupling. Similarly, functional sensitisation of 5-HT_{1A} receptors as seen in Tph2 KO mice may well be traceable both to upregulation of 5-HT_{1A} receptors and sensitisation of 5-HT_{1A} receptors driven by an as-yet-unknown mechanism.

4.1.8. Interaction between 5-HT_{1A} receptors, 5-HTT, and other 5-HT receptors

In general, genetic deletion of 5-Htt in mice is known to induce downregulation of 5-HT_{1A} autoreceptors in the DRN (Fabre et al., 2000; Li et al., 1999, 2000). In a human postmortem

study, Arango et al. (2001) reported that expression levels of both 5-HT_{1A} autoreceptors and 5-HTT in the DRN from depressed patients were decreased compared to controls. They speculated that this reduction in both 5-HT_{1A} autoreceptors and 5-HTT is a homeostatic compensatory mechanism to enhance the action of serotonergic neurons. In line with this, David et al. (2005) reported that carriers of the s-allele of the 5-HTTLPR, which leads to lesser expression of 5-HTT, had lower 5-HT_{1A} receptor binding potential in all brain regions investigated compared to homozygous carriers of the l-allele. On the contrary, in the study by Lee et al. (2005b), it was found that s-carriers had higher 5-HT_{1A} binding than ll-carriers in pregenual and subgenual cingulate regions while in other regions including the DRN, no difference was detected. More recent study by Borg et al. (2009), however, could not detect any differences in 5-HT_{1A} receptor density between carriers and non-carriers of the s-allele of 5-HTTLPR. Instead they found that s-carriers had a superior performance compared to ll-carriers in a cognitive test. They concluded that functional consequences of 5-HTTLPR are not likely to be mediated by differences in 5-HT_{1A} expression levels and that other biomarkers must be taken into account to infer differences at phenotypic levels. Similarly, Bose et al. (2011) failed to detect robust, intra-regional associations between 5-HT_{1A} receptor and 5-HTT densities. Inter-regionally, on the other hand, DRN 5-HT_{1A} receptor levels were shown to be related to cortical and paralimbic, but not to limbic 5-HTT levels. The authors inferred that the latter finding may suggest differences in 5-HT tone between individuals and underline potential substrates more sensitive to variations in DRN 5-HT function. These series of findings testify complexity of interaction between 5-HTT and 5-HT_{1A} receptors. In the present study using 5-Htt KO mice, absence or reduced levels of 5-Htt were correlated with desensitisation of 5-HT_{1A} autoreceptors as measured by R8HD response. As pointed out by Lee et al. (2005b), the differences between results obtained from animal and human studies may stem from the fact that genetically modified mice incur massive manipulations of homeostatic systems while human studies address naturalistic relationships of genetic variation which may result in changes in 5-HTT availability. The results in the present study, however, attest that autoinhibition is not necessarily a potential substrate or phenotype which has a predictive value for 5-HT_{1A} autoreceptor densities/sensitivity in the DRN. Instead, it would seem more reasonable to consider interaction between 5-HTT and 5-HT_{1A} autoreceptor to predict consequences of 5-HT_{1A} receptor-mediated functions such as autoinhibition.

Moreover, a past study indicates that there is a complex interaction between 5-HT_{1A} receptors and other 5-HT receptor subtypes. For example, Fox et al. (2010) reported interesting interactions between presynaptic 5-HT_{1A} receptors and postsynaptic cerebral 5-HT_{2A}

receptors, both of which were previously shown to be desensitised/downregulated in 5-Htt KO mice (Rioux et al., 1999; Fabre et al., 2000; Li et al., 1999, 2000). Cerebral 5-HT_{2A} receptors, when stimulated by (+/−)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), are known to induce head twitches. Although stimulation of 5-HT_{2A} receptors by DOI induced less head twitches in 5-Htt KO mice, as expected from previously reported downregulation of these receptors, indirect activation of these receptors by blockade of 5-HT_{1A} autoreceptors by WAY-100635 induced 5-fold more head twitches in 5-Htt KO mice compared to wildtype controls. The result conveys two important messages related to the present study. First, blockade of 5-HT_{1A} autoreceptors successfully increased 5-HT availability which subsequently stimulated postsynaptic 5-HT_{2A} receptors, implying that autoinhibition was functional as a default despite previously reported functional desensitisation of 5-HT_{1A} receptors *per se*. Second, sensitivity/expression levels of presynaptic/postsynaptic receptors alone cannot reliably predict functional consequences unless the presence of autoinhibition, which influences firing of presynaptic serotonergic neurons and consequently 5-HT availability at the postsynaptic level, is taken into account.

4.1.9. Desensitisation of 5-HT_{1A} autoreceptor is not necessarily associated with behavioural changes but may be secondary to the increased extracellular 5-HT

Autoinhibition of serotonergic neuron firing in the DRN has been repeatedly implicated in mood disorders. Experimental evidences both in animal studies and human subjects suggest that deficient 5-HT_{1A} autoreceptor function leads to behavioural alterations related to anxiety and depression (reviewed in Pineyro and Blier, 1999; Sharp et al., 2007). Moreover desensitisation of autoinhibitory 5-HT_{1A} receptors following chronic SSRI administration has been proposed as a putative mechanism for SSRIs to take their therapeutic effects (see Introduction). However, results from animal studies using SSRI administration indicate that desensitisation of 5-HT_{1A} autoreceptors is rather an adaptive mechanism to counter elevated extracellular 5-HT levels, not a primary factor leading to behavioural alteration. In the study by Cornelisse and colleagues (2007), 5-HT_{1A} receptor function assessed by GIRK current was no different between socially stressed rats and controls, although socially stressed rats exhibited anhedonia, a core symptom of depression. Difference in GIRK current became evident only when rats were treated by SSRI, regardless of whether the rats were stressed or not. Study by Richardson-Jones and coworkers (2010) also indicated dissociation between desensitisation of 5-HT_{1A} autoreceptors and behavioural effects of chronic SSRI treatment. In

their study, genetically modified mice with higher expression of 5-HT_{1A} receptors (1A-High mice) were shown to have elevated baseline behavioural despairs. Using 8-OH-DPAT-induced hypothermia as an assay for 5-HT_{1A} receptor function, the authors demonstrated marked desensitisation of 5-HT_{1A} receptors in 1A-High mice chronically treated with an SSRI, fluoxetine, although behavioural despairs were not ameliorated by chronic fluoxetine treatment. As opposed to canonical hypothesis that decreased autoinhibition due to desensitised 5-HT_{1A} receptors after chronic SSRI treatment mediates antidepressant action, the study by Richardson-Jones (2010) demonstrates that desensitisation of 5-HT_{1A} autoreceptors is not enough for antidepressants to take their effects. Taken together, these series of evidence demonstrate that desensitisation of 5-HT_{1A} autoreceptors is likely to be secondary to altered extracellular 5-HT levels and is not necessarily associated with behavioural changes. Similarly, loose-seal cell-attached recording data obtained from 5-Htt KO mice, which mimic mice chronically treated with SSRIs, indicate that desensitisation of 5-HT_{1A} receptors took place as a response to elevated levels of extracellular 5-HT. In other words, desensitisation occurred not for reducing or abolishing autoinhibition. Instead, together with elevated levels of extracellular 5-HT, desensitisation of 5-HT_{1A} receptors eventually helped the organism normalise the level of autoinhibition, which otherwise is too intense.

4.1.10. 5-HT_{1A} receptor sensitivity and its functional consequences

Despite the importance of 5-HT_{1A} autoreceptors in regulation of serotonergic firing autoinhibition, past studies rarely investigated autoinhibition of serotonergic neuron firing *per se*. Instead, functional levels of autoinhibition in human patients with mood disorders and animal models were indirectly inferred from 5-HT_{1A} receptor agonist responses or from binding affinity to those receptors (reviewed in Drevets et al., 2007 and Savitz et al., 2009; Li et al., 1999, 2000; Bouali et al., 2003). For example, one of the most consistent findings among depressed patients is their blunted hypothermia in response to 5-HT_{1A} receptor agonists (Jacobsen et al., 2012b and references therein). Such responses are usually ascribed to desensitisation of somatic 5-HT_{1A} receptors (reviewed in Jacobsen et al., 2012a). In fact, 5-Htt KO mice were previously shown to have reduced hypothermia in response to 5-HT_{1A} autoreceptor stimulation by R8HD (Li et al., 1999, 2000; Bouali et al., 2003), implying functional desensitisation of 5-HT_{1A} autoreceptors. Desensitisation of 5-HT_{1A} receptors is consistent with the findings from DRC of R8HD application shown in the present study. However, this does not mean that, as demonstrated in other experiments using Trp and 5-HTP

application, autoinhibition of 5-Htt KO mice is impaired. In other words, as indicated in the present study, neither responses of serotonergic neurons to 5-HT_{1A} receptor agonists nor 5-HT_{1A} receptor expression levels are necessarily reliable measures of 5-HT_{1A} autoreceptor-mediated functions such as autoinhibition of serotonergic neuron firing.

On the other hand, unaltered 5-HT_{1A} receptor expression levels or G-protein coupling do not necessarily indicate that 5-HT_{1A} receptor-mediated hypothermia is also unaltered. Mice homozygous for targeted insertion of G1449A, which substitutes Arg^{439} with His^{439}, are known to have 25-40% of the normal 5-HT content compared to WT controls, while their 5-HT_{1A} autoreceptor levels and G-protein coupling remain unchanged (Beaulieu et al., 2008; Jacobsen et al., 2012b). Hypothermia via 5-HT_{1A} autoreceptor stimulation, however, was shown to be blunted. The authors (Jacobsen et al., 2012b) postulated that blunted hypothermia in response to 5-HT_{1A} receptor agonist in the absence of any change in 5-HT_{1A} receptor expression/function could be attributed to decreased baseline extracellular 5-HT levels.

Taken together, functional consequence of 5-HT_{1A} receptor stimulation cannot be inferred simply from receptor expression levels. This is particularly true of autoinhibition of serotonergic neuron firing since its direct assessment in human population was hindered due to experimental limitations. Evidence suggests that hypothermic response to 5-HT_{1A} receptor stimulation does not necessarily reliably predict autoinhibition. Moreover, hypothermic response itself is not directly related to 5-HT_{1A} receptor expression or its G-protein coupling. It is likely that functions of 5-HT_{1A} autoreceptors depend on the baseline serotonergic tone in organisms, which is influenced by several factors including but not limited to the availability of 5-HTT and the activity of TPH2.

4.1.11. Conclusion for Study 1

The present study showed that intrinsic basal firing rates of serotonergic neurons are similar in all the genotypes tested, whose genes encoding important mediators of serotonergic transmission are genetically modified. This means that, despite different levels of life-long availability of 5-HT, there are no major adaptive changes in pacemaking properties of serotonergic neuron firing. Moreover, it was demonstrated that 5-HT_{1A} receptor functional response, as assayed by direct agonist R8HD, changes markedly only in the case of an extreme increase of extracellular 5-HT availability as in 5-Htt KO mice. The general conclusion that could be drawn from the series of experiments where *in vivo* 5-HT synthesis was simulated by *in vitro* application of 5-HT precursors, i.e. Trp or 5-HTP, as presented here,

is that autoinhibition is such an important property of serotonergic neurons that it is homeostatically regulated to function regardless of 5-HT_{1A} receptor sensitivity, even when the sensitivity itself is changed due to alterations in 5-HT synthesis or uptake, which have been incurred by genetic impairment of these mechanisms. In other words, serotonergic neurons buffer the changes in levels of extracellular 5-HT through changes in responsiveness to 5-HT_{1A} receptor stimulation. This was particularly evidenced as the presence of autoinhibition in 5-Htt KO mice despite substantial desensitisation of their 5-HT_{1A} receptors. Desensitisation of 5-HT_{1A} receptors is rather an outcome of adaptive changes in response to excessive levels of extracellular 5-HT due to genetic deletion of 5-Htt. The same logic seems to be applicable when the extracellular 5-HT is elevated by SSRI treatment. Studies investigating molecular mechanisms of functional sensitisation of 5-HT_{1A} receptors in Tph2 KO mice or linking autoinhibition with behavioural changes of Tph2 KO mice are still scarce. However, results from the present study suggest that the overall principle of 5-HT_{1A} receptor desensitisation in 5-Htt KO mice may still apply to Tph2 KO mice, i.e. autoinhibition is maintained regardless of 5-HT_{1A} receptor sensitivity. The result therefore poses precaution in directly inferring the level of autoinhibition from 5-HT_{1A} receptor sensitivity.

The findings presented here have significant clinical relevance since responses to 5-HT_{1A} agonists, e.g. 5-HT_{1A} binding potential or hypothermia tests with R8HD, are commonly used to draw conclusions on functions of the serotonergic system, especially in patients with mood disorders, although responses to 5-HT_{1A} agonists may only reflect adaptive responses of 5-HT_{1A} receptors to keep the serotonergic system functioning at the proper level and are not necessarily predictive of the serotonergic tone *in vivo*. Moreover, therapeutic effects of SSRI were believed to be mediated by reduction in autoinhibition, which was traditionally attributed to desensitisation of 5-HT_{1A} autoreceptors. As the present study suggests, even if 5-HT_{1A} autoreceptors are actually desensitised following SSRI administration, it does not necessarily mean that autoinhibition is also attenuated. The results presented here further imply that depressive and anxious behaviours of organisms are not necessarily mediated by deficit in autoinhibition of serotonergic neuron firing and that other endophenotypes must be taken into account when ascribing behavioural phenotypes to genetic predispositions. Moreover, based on the finding that autoinhibition is maintained in animal models of emotional disorders, it could cautiously be inferred from the present study that human subjects with genetic polymorphisms in genes encoding 5-HTT or TPH2, which have secondary effects on expression levels or sensitivity of 5-HT_{1A} receptors, may still have normal autoinhibitory function of serotonergic neuron firing. In sum, the present study

proposes that Trp and/or 5-HTP challenges should be used instead of direct 5-HT_{1A} agonists to estimate autoinhibition of serotonergic neuron firing as well as the serotonergic tone in organisms.

4.2. WC recording from serotonergic neurons in the DRN of Tph2 KO mice (Study 2)

4.2.1. Membrane resistance was decreased in neurons of KO mice

The whole-cell data obtained from serotonergic neurons in the DRN of Tph2 KO and WT mice showed almost identical electrophysiological properties between these two genotypes. The only parameter which showed statistically significant difference was membrane resistance, with neurons from KO mice having lower resistances (**Fig. 3.14.A**). The conductance of a neuron, defined as the inverse of membrane resistance, therefore became significantly higher in KO mice compared to WT controls (**Fig. 3.15.A**). However, when the cell conductance was normalised by cell capacitance, which correlates positively with cell sizes, the difference in cell conductance became non-significant. Thus, it is possible that higher conductance of neurons from KO mice may have derived from larger cell sizes. To confirm the potential difference in cell sizes between WT and KO mice, direct histological studies are necessary. Other parameters which showed trend of statistical significance were action potential amplitude (**Fig. 3.18.A**) and conductance in response to voltage ramp (**Fig. 3.20**). Action potential amplitude is susceptible to the membrane potential when the action potential was generated. As shown in **Fig. 3.13**, the resting membrane potential at a steady state was not different between WT and KO mice. Moreover, when the action potential was generated by step-wise current injection, the membrane potential before step-wise current injection was adjusted at around -65mV by steady-state current injection, so that the action potential shapes were measured under similar conditions (see Materials and Methods). It is therefore not possible to attribute the difference in action potential amplitude to the difference in membrane potential when the action potentials were generated. Action potential amplitude is a rather fixed parameter determined by internal and external ionic composition and their reversal potentials. This means that action potentials are generated in an all-or-none fashion and its amplitude is normally not graded. Due to the nature of the whole-cell patch clamp recording method, i.e. the washout effect of the intracellular component, the intracellular ionic

composition should be similar across the recorded neurons. It is therefore possible that this trend in difference in action potential amplitude is only transitory and the difference may diminish when more recoding data are obtained.

4.2.2. Neurons from KO mice had larger inward-rectifying current in response to negative voltage ramp

Increased conductance of neurons from KO mice in response to voltage ramp is in accordance with increased conductance at a steady state, calculated as the inverse of membrane resistance. The current observed in response to hyperpolarising voltage-ramp showed inward-rectification which started at around -95 mV. Williams et al. (1988) reported that in the absence of 5-HT_1 receptor agonists, there are two kinds of voltage-activated inwardly rectifying current in the voltage range more negative than the resting membrane potential: one is activated instantaneously (< 5-7 msec) at around the potassium equilibrium potential (E_K), termed inward current (I_{IR}); the other is activated slowly (0.5-1 sec till full activation) when the membrane potential is stepped to potentials more negative than -70 mV, which resembles I_Q or I_H current as has been observed in other neurons such as hippocampal pyramidal neurons and in smooth muscles. Since the inwardly rectifying current shown in **Fig. 3.20.A** was activated in response to slow voltage ramp (over 600 ms range), it is likely to represents I_Q current. Like I_{IR} current, I_Q current is activated at negative membrane potentials and is closed at positive potentials. While I_{IR} current is predominantly composed of K^+ current, I_Q current derives from an ionic flow of both Na^+ and K^+ ions (Edman et al., 1987). It has been suggested that, although the I_Q current contributes to only a small portion of the total membrane conductance of dorsal raphe neurons in normal extracellular K^+ concentration, it is under the influence of neurotransmitters and the opening state of other potassium channels and thus plays a role in determining the activity of dorsal raphe neurons (Williams et al., 1988). Moreover, evidence suggests that kinetics of I_Q current is modulated by the second messenger cAMP, implying functional significance of this current (Hille, 2001; Chapter 5, p.158). The difference in the I_Q current we observed between Tph2 WT and KO mice, although it did not reach statistical significance, may therefore indicate a potential difference in the activity of dorsal raphe serotonergic neurons.

In sum, the whole-cell recording from serotonergic neurons in Tph2 KO mice revealed electrophysiological properties quite similar to those of WT counterparts. The only statistically significant difference found was decreased membrane resistance, i.e. increased

conductance in the neurons of Tph2 KO mice. Together with increased conductance in response to voltage ramp, the results may indicate that a certain type (or types) or ion channels are more active in the neurons of Tph2 KO mice.

4.3. WC recording from hippocampal CA1 pyramidal neurons in 5-Htt KO mice (Study 3)

4.3.1. CA1 pyramidal neurons from 5-Htt KO mice had increased conductance

Whole-cell patch clamp recording from CA1 pyramidal neurons in the hippocampus revealed overall similar electrophysiological properties between 5-Htt KO and WT mice. One consistent difference, however, exists in terms of conductance, i.e. neurons of KO and Het mice showed higher conductance than those of WT mice. This was first observed at the steady state as reduced membrane resistance (**Fig. 3.21**). Moreover, increased conductance was evident in response to voltage changes (**Fig. 3.28**) as well as inward current accompanying action potential generation (**Fig. 3.29**). Although it did not reach statistical significance, the amplitude of EPSC was also larger in 5-Htt KO and Het mice in comparison to WT controls (**Fig. 3.26**). Moreover, the reduced number of action potential generation in response to current injection as observed in neurons of Het and KO mice is a reflection of reduced membrane resistance and increased conductance (**Fig. 3.25**). Namely, the injected current was less effective in raising the membrane potential to the action potential threshold since substantial amount of current went through the neurons without raising the membrane potential because of the decreased membrane resistance. In a biological setting, this may read as decreased excitability in response to a certain synaptic input.

In the following section, potential mediators which endowed neurons in KO and Het mice with higher conductance were discussed. Due to the nature of these mediators, they may have also served as compensatory mechanisms for the lack of 5-Htt to keep the balance of 5-HT in the extracellular space. Overall retention of electrophysiological properties in 5-Htt KO mice may have been attributable to such compensatory mechanisms.

4.3.2. Non-5-HTT mediated serotonin transport may have compensated for the lack of 5-HTT and contributed to the increased conductance in CA1 neurons of 5-Htt KO mice

Past studies suggested the presence of two different types of 5-HT transport; one with a high affinity and low capacity, the other with a low affinity and high capacity, termed uptake1 and uptake2, respectively (Zhou et al., 2007). The uptake1 system solely consists of 5-HTTs, which work in a Na^+/Cl^--dependent manner. On the other hand, the uptake2, whose molecular correlate is also known as the extraneuronal monoamine transport system (EMT; Lazar et al., 2003), may consist of several different types of transporters, including members of the organic cation transporter (OCT) family and the plasma membrane monoamine transporter (PMAT). Here the possibility that these mediators of non-5-HTT serotonin transport system compensated for the lack of 5-HTT is discussed. Moreover, the possibility that these transport system contributed to the increased conductance of CA1 pyramidal neurons is also discussed.

i). OCT3

OCTs transport a wide range of organic cations. They work both as a sodium-dependent and sodium-independent manner (Koepsell, 1998 as in Schmitt et al., 2003). So far, three subtypes of potential-sensitive organic cation transporters, OCT1, OCT2, and OCT3 have been cloned and characterised. Whereas OCT1 and OCT2 are mainly expressed in the kidney and liver, OCT3 were shown to be present in the brain (especially in the cerebellum, the dorsal raphe, hypothalamic nuclei, the cortex, and the hippocampus), as well as intestine, kidney, and placenta. A wide spectrum of substrate specificity has been reported for OCT3 including 5-HT and DA, albeit with a low affinity. (Schmitt et al., 2003 and references therein). Similar to 5-HTT, OCT3 is also a target of several types of psychostimulant drugs, such as MDMA, cocaine, D-amphetamine (Amphoux et al., 2006), implying relevance of OCT3 in emotion regulation. Moreover, it was recently demonstrated that corticosterone, which is released as a stress response mediated by the HPA axis, inhibit 5-HT uptake by OCT3 (Baganz et al., 2010).

Past study revealed that the expression level of OCT3 was upregulated in 5-Htt KO and Het mice, including the pyramidal and granular cells of the hippocampus (Schmitt et al., 2003; Baganz et al., 2008). Study by Schmitt et al. (2003) also confirmed that OCT3 is preferably, if not exclusively, expressed in neurons and not in extraneuronal cells, e.g. astrocytes and oligodendrocytes (in this sense the term EMT may not be appropriate for OCT3). The authors assumed that upregulation of OCT3 and resulting enhanced 5-HT reuptake may be a compensation mechanism to counter increased extracellular 5-HT in 5-Htt KO mice, playing a critical role in maintaining 5-HT-dependent functions of the hippocampus in 5-Htt deficient

mice. In fact, a past study using an OCT3 blocker, 1,1'-diethyl-2,2'-cyanine (decynium-22; D-22), demonstrated that D-22 reduced 5-HT reuptake and exerted antidepressant-like effects only in 5-Htt KO mice but not in WT mice, suggesting greater contribution of OCT3 to 5-HT uptake in 5-Htt KO mice (Baganz et al., 2008).

OCT3-mediated transport is electrogenic, associated with the transfer of positive charges into the cell (Kekuda et al., 1998). Such function of OCT3 is shown to be membrane potential-dependent (Kekuda et al., 1998), having higher conductance at negative membrane potentials. Given that OCT3 was found to be upregulated in 5-Htt deficient mice, it is probable that the larger amount of inward current observed in 5-Htt KO and Het mice in comparison to WT mice at negative membrane potentials might have derived from increased expression of OCT3. Such a hypothesis is also supported by the fact that most serotonin is positively charged at pH 7 (Chattopadhyay et al., 1996). Since 5-Htt KO mice have abundant 5-HT in the synaptic cleft, positively charged 5-HT passing through OCT3s may have contributed to the increased current flow. On the other hand, the increase in current flow was previously reported only when the membrane potential was in the negative potential range (Kekuda et al., 1998). The result in the present study, on the other hand, showed increase in current flow also at the positive membrane potential range in 5-Htt KO and Het mice (**Fig. 3.28**). It is therefore not likely that OCT3 alone accounts for the increased conductance of 5-Htt KO and Het mice. More likely, some other factors also contributed to the enhancement of current flow as was observed in the present experimental data.

ii). PMAT

PMAT is a type of organic cation transporter which shows a broad range of specificity for organic cations and endogenous biogenic amines, working in a Na^+-independent manner. Among the monoamine neurotransmitters, PMAT has the highest affinity for 5-HT and its mRNA is widely expressed in the CNS. PMAT is known to be expressed in neuronal cells but not in astrocytes (Dahlin et al., 2007). Unlike 5-HTT, however, its expression is not limited to serotonergic neurons but various neuronal subtypes including pyramidal neurons and interneurons which receive serotonergic inputs were shown to express PMAT(Dahlin et al., 2007). Although the apparent affinity (K_m) of recombinant PMAT for 5-HT in heterologous expression systems is much lower than that of 5-HTT, PMAT has a much higher transport capacity (V_{max}), yielding grossly comparable uptake efficiencies (V_{max}/K_m) to 5-HTT (Zhou et al., 2007 and references therein). Therefore, 5-HT transport mediated by non-5-HTT transporters may play a non-negligible role in 5-HT clearance in certain brain regions,

especially where 5-HTT expression is low, and/or when extracellular 5-HT concentrations are abnormally elevated due to pharmacological blockade of 5-HTT or genetic deletion of 5-Htt as in 5-Htt KO mice.

Itagaki et al. (2012) performed a series of electrophysiological experiments on PMAT and revealed that PMAT-mediated transport was associated with inward currents, i.e. net transfer of positive charges into the cell, and the magnitude of current flow was increased as the membrane potential became more negative. In the same study, it was shown that histamine, one of the substrates of PMAT-driven transport, became protonated to a singly charged cation and mediated inward current flow into the cell at negative membrane potentials. Although not directly demonstrated, the same mechanism may apply to the transport of other biogenic amines such as 5-HT. It was not tested in the study of Itagaki et al. (2012) whether PMAT mediate augmentation of outward current in positive membrane potentials in a voltage-dependent manner. Moreover it is still unknown whether PMAT is upregulated in 5-Htt KO mice (Daws, 2009) although it is likely given that OCT3 was shown to be upregulated as a compensatory mechanism to the lack of 5-Htt. Since the hippocampus is one of the regions where PMAT is richly expressed, there is a possibility that PMAT-mediated flow of cations may have contributed to the increased conductance of hippocampal neurons in 5-Htt KO and Het mice.

4.3.3. 5-HT_3 receptors may also have contributed to the increased conductance

Unlike other members of the 5-HT receptor family, 5-HT_3 receptors are known to be ionotropic receptors, directly conducting ions in response to the binding of 5-HT. The ion channels of 5-HT_3 receptors are permeable to N^+, K^+, Ca^{2+}, and organic cations. As mentioned earlier (see Introduction), upregulation of 5-HT_3 receptors has been reported in 5-Htt KO mice and there is a possibility that this upregulation contributed to the increased conductance of CA1 pyramidal neurons. However, past studies demonstrated that 5-HT_3 receptors are mainly expressed on hippocampal interneurons, not on pyramidal neurons. Therefore, it is rather unlikely that 5-HT_3 receptors are the direct mediators of increased ion conductance as was observed in 5-Htt KO and Het mice. Indeed past investigations in the pyramidal neurons of the CA1 region aimed at detecting rapid response to 5-HT application, which is supposed to be mediated by ionotoropic 5-HT_3 receptors, produced negative results (Yakel and Jackson, 1988 and references therein). Another more plausible mechanism for increased conductance of CA1 pyramidal neurons is that 5-HT activates 5-HT_3 receptors on interneurons, which then

release GABA onto pyramidal neurons. In principle, released GABA can activate both $GABA_A$ and $GABA_B$ receptors on pyramidal neurons. $GABA_A$ receptors are ionotropic receptors which, when activated, open Cl^- channels. On the other hand $GABA_B$ receptors are metabotropic receptors, coupled to K^+ channels. Given that the resting membrane potential of CA1 pyramidal neurons remained near -60 mV and that the reversal potential of K^+ ion channels is around -90 mV, the contribution of K^+ channels to increased conductance is likely to be small. Contrarily, the reversal potential of Cl^- channel is near the resting membrane potential (-60mV) and changing the ionic composition of pipette solution to artificially change the reversal potential of Cl^- channels to 0 mV (symmetric Cl^- solution) shifted reversal potential of KO neurons more positive than in neurons of WT mice (**Fig. 4.2**). Importantly, Cl^- channels have been implicated in depression and the mechanisms of antidepressant drugs (Goren et al., 2007; Tunnicliff et al., 1999; Tunnicliff and Malatynska, 2003).

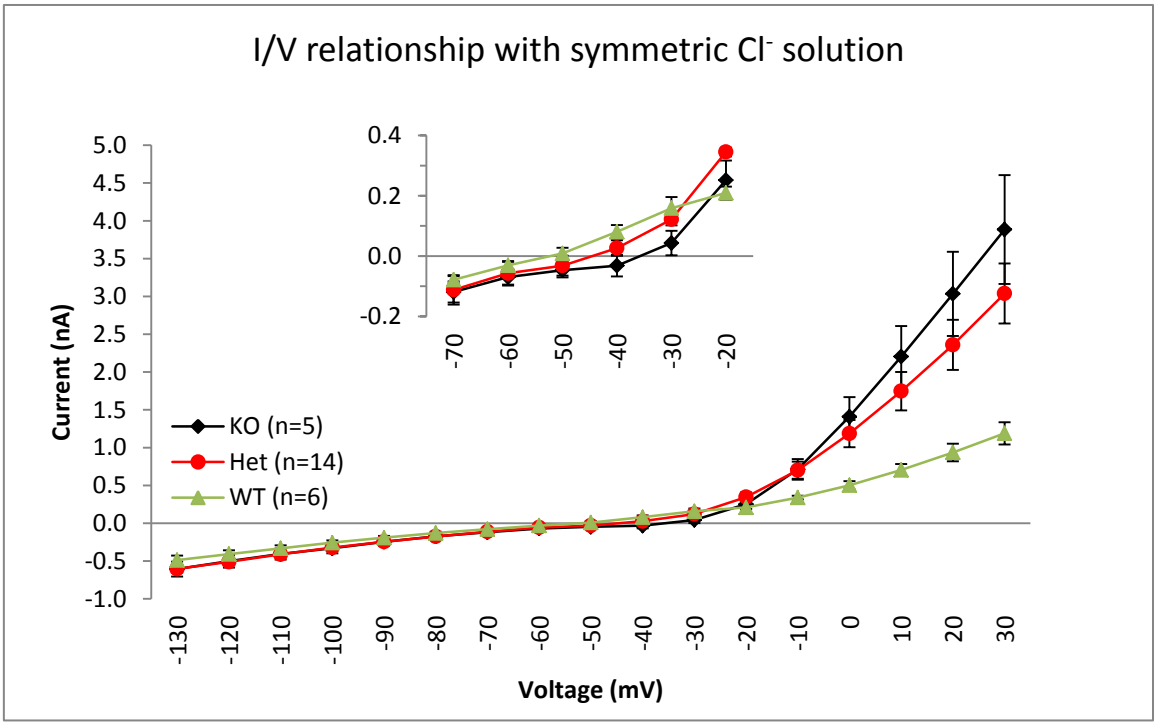

Fig. 4.2. I/V relationship obtained with symmetric Cl^- solution. K-gluconate in the pipette solution was replaced with KCl to make the concentration of Cl^- inside and outside the neuron equal. Therefore E_{cl} becomes 0mV. The point where the I/V plot crosses 0 current axis shifted more positive in KO mice than in WT mice, indicating higher Cl^- conductance in KO mice. The inlet is the magnification at the voltage range from -70 mV to -20 mV.

Potential increase in Cl^- conductance cannot, however, explain increase in inward current associated with action potentials (**Fig. 3.29**). This may suggest that the increased conductance

in 5-Htt Het and KO mice is attributable to several different types of ion channels and transporters, which were discussed in previous sections, resulting in the net increase in the conductance. This is evidenced by the fact that the resting membrane potential remained the same across the genotypes and the reversal potential of the IV plot was near the resting membrane potential of the neuron.

4.3.4. Morphological analysis

Overall, there was no striking difference found in the morphology of CA1 pyramidal neurons among different genotypes of the 5-Htt KO mice line. Although there was statistically significant difference found in the Sholl analysis at the 200-220 μm segment, the result is rather difficult to interpret. First of all, the difference is only evident at this segment and there was no significant difference at other segments, both segments closer to the soma and away from the soma. Moreover, it is unlikely that the difference in this segment is related to electrophysiological differences observed in the present experimental data. This is because the whole-cell patch clamp mostly reflects electrophysiological properties of neuronal material close to the soma. In fact, electric signals significantly attenuates along the somatodendritic axis (for review see Spruston, 2008). The morphological analysis in brain slices is also prone to potential errors considering the fact that dendritic arborisation might have been severed during the slicing procedure. To avoid this problem, it is necessary to record from neurons located relatively deep inside the brain slices. However, staining reaction on thicker brain slices as in the present study (300 μm thickness) entails difficulty in terms of penetration of staining reagents into the tissues, as opposed to standard immunohistochemical studies, and recording from neurons deep inside the tissue makes staining even more difficult. Overall, it is not possible to draw a more reliable conclusion from this study due to the relatively small number of neurons investigated, partly because of the above mentioned difficulty. At least one past histological study done on adult 5-Htt KO mice (Nietzer et al., 2011), however, demonstrated that there was no morphological difference in CA1 pyramidal neurons in the hippocampus. Given this, it is still probable that there are indeed no morphological differences in hippocampal CA1 neuronal morphology of young mice used for the present whole-cell patch clamp recording, although caution should be taken since results obtained from adult mice may not directly be extrapolated to the case of young mice.

4.4. Extracellular field potential recording of LTP (Study 4)

The results obtained from the LTP study showed that intensity of LTP induction was not influenced by the level of brain 5-HT. This was rather unexpected given numerous past findings demonstrating effects of 5-HT on LTP and synaptic plasticity (see section 1.5.5.), although it is at the same time true that 5-Htt deletion has been demonstrated to have less impacts on terminal areas including the hippocampus than on the central serotonergic origins (Fabre et al., 2000; Mannoury la Cour et al., 2001; Li et al., 1999, 2000).

There are several possibilities to explain this lack of differences. One is of biological origin, either by as-yet-unknown compensatory mechanisms to counteract changes in the level of brain 5-HT, whose possibility is discussed in other sections of Discussion, or by interaction with some other neurotransmitter systems. Another possibility is that LTP inducibility is dependent on experimental conditions, such as behavioural paradigm used in combination with LTP induction. Moreover, the result obtained indicates that LTP inducibility at the particular pathway investigated, i.e. Schaffer collateral/commissural pathway, was not necessarily associated with changes in 5-HT availability. These possibilities will be discussed in turn.

4.4.1. LTP was possibly maintained by interaction with other neurotransmitters

One of the plausible explanations for the maintenance of normal LTP inducibility in Tph2 KO mice is interaction with other neurotransmitters. In general, 5-HT works adversely to LTP although facilitation of LTP by 5-HT has also been reported (see section 1.5.5.). On the contrary, evidence suggests that NE promotes protein synthesis-dependent late phase of LTP (L-LTP) in the DG via activation of β-adrenergic receptors, which are positively coupled to adenylyl cyclase and thus leading to increase in cAMP and activation of PKA (Stanton and Sarvey 1985; Andersen et al., 2007). Similar LTP enhancing effects of NE were seen in the CA1 *in vitro* via activation of α_1 receptors (Izumi and Zorumski, 1999). It should be noted that although an *in vivo* study using a SNRI reported suppression of LTP, this was likely due to enhanced release of 5-HT induced by activation of α_1 receptors on serotonergic neurons in the DRN (Tachibana et al., 2004). Moreover, DA is also known to work positively for the induction of L-LTP in CA1 with a similar mechanism to that of NE. Importantly, Gutknecht et al. (2012) reported consistent reduction in NE content across brain regions and hippocampus-specific reduction in DA in Tph2 KO mice. Given these lines of evidence, one possible explanation for the absence of changes in LTP inducibility observed in the present study in the CA1 area of Tph2 KO mice could read as follows; if the depletion of 5-HT is not

accompanied by reduction in NE and DA, it will probably enhance LTP inducibility in Tph2 KO mice. However, since NE and DA, which as a default promote LTP induction, are also reduced along with the depletion of 5-HT in Tph2 KO mice, the overall efficacy of LTP induction is unchanged in these mice. The same surmise seems to be applicable to the case of 5-Htt KO mice. Namely, the adverse effect of excessive levels of 5-HT on LTP induction could have been counteracted by enhancement of NE transmission. Indeed, it was shown that chronic treatment with a SSRI fluoxetine, which is partly comparable to knockout of 5-Htt, led to an increase of tyrosine hydroxylase (TH) gene expression in the LC (Brady et al., 1992 as in Gutknecht et al., 2012).

4.4.2. Changes in LTP inducibility in 5-Htt KO and Tph2 KO mice may become evident in particular behavioural paradigms

In the present study, LTP induction of 5-Htt KO and Tph2 KO mice was investigated without any prior behavioural treatments. It seems that, to investigate influence of 5-HT on LTP in these KO mice, it is important to combine the LTP induction protocol with behavioural paradigms which are most relevant to functions of 5-HT in emotional processing. For example, Dai et al. (2008) demonstrated that without behavioural treatments, there was no difference in LTP inducibility in CA1 between WT and Lmx_{1B} KO mice, another animal model of central 5-HT deficiency. However, after foot-shock stress paradigms, WT mice showed impairment in LTP induction while Lmx_{1B} KO mice were resilient to the stress paradigms and maintained the same LTP inducibility as was without foot shocks. Effects of environmental factors, including stress, on genetic predispositions are called gene-by-environmental interactions and have recently attracted attention due to their significant roles for the development of neuropsychiatric disorders (for review see Canli and Lesch, 2007; Lesch, 2011; Gross and Hen, 2004; Dick, 2011; Caspi and Moffitt, 2006). Although not directly demonstrated in terms of LTP inducibility, environmental stressors were shown to influence learning abilities of Tph2 KO and 5-Htt KO mice at the behavioural level. CMS, such as overnight illumination, tilted cage, food or water deprivation, confinement to a restricted space, was shown to enhance cued and context fear learning of Tph2 KO mice (Gutknecht et al., submitted). Moreover, prenatal stress (PS) impaired object recognition, which belongs to a domain of memory performance, in 5-Htt WT controls, whereas 5-Htt Het mice showed resilience to PS (van den Hove et al., 2011). Since 5-HT has been repeatedly implicated in the modulation of stress response in the hippocampus, it would be plausible that

differences in LTP inducibility between Tph2 KO or 5-Htt KO mice and WT controls become visible only after some behavioural stress paradigms. This would particularly be the case given that the hippocampus has been implicated in contextual fear learning. Moreover, 5-Htt KO mice were shown to have impaired fear extinction recall, whereas Tph2 KO mice have enhanced fear learning (see section 1.6.2.). As mentioned earlier (see section 1.5.5.), ATD is also known to enhance learning abilities of human subjects in response to aversive stimuli. The results in the present study indicate that those behavioural alterations may not derive from LTP inducibility at the baseline but rather LTP inducibility may have been modified after experimental behavioural paradigms.

4.4.3. Alterations in learning abilities of 5-Htt KO mice and Tph2 KO mice are not correlated with changes in Schaffer-collateral- CA1 LTP

As mentioned in Introduction, 5-Htt KO mice are known to exhibit deficits in fear extinction recall (Wellman et al., 2007), while Tph2 KO mice show enhanced fear learning (Gutknecht et al., submitted). The results in the present study indicate that these learning impairment/enhancement are not related to changes in LTP inducibility in the Schaffer collateral/commissural pathway of the hippocampus. There is a possibility that factors other than LTP may have contributed to behavioural changes of 5-Htt KO and Tph2 KO mice, related to learning and memory. Given the multitude of LTP induction protocols proposed so far (Bortolotto et al., 2001; Malenka and Nicoll, 1999), it is also possible that other LTP induction protocols may have teased out potential alterations in LTP inducibility. However, evidence suggests that dissociation of memory functions from LTP is still possible. For example, past studies demonstrated that MDMA enhances LTP in the CA1, although MDMA is reported to cause memory deficits in the behavioural level (Rozas et al., 2012; Morini et al., 2011; Mlinar et al., 2008).

On the other hand, the highly organised and hierarchical structure of the hippocampus suggests that some other pathways or subregions of the hippocampus, either singly or in harmony, led to changes in memory functions of 5-Htt KO and Tph2 KO mice. For instance, the CA1 filed in the hippocampus has a unique function, combining the present sensory information with past memories. As a centre of intensive cognitive computation, the CA1 field of the hippocampus is particularly suited to act as a comparator between present direct sensory information and past associative memory traces. This is because CA1 receives information from the entorhinal cortex (EC) through two distinct pathways that terminate on

segregated regions of the apical dendrites of the CA1 pyramidal neurons. One pathway directly reaches the distal dendrites of the CA1 pyramidal cells in the stratum lacunosum-moleculare (SLM) through the perforant path (pp) and provides sensory information. Another pathway terminates on the proximal dendrites of the CA1 pyramidal neurons in the stratum radiatum (SR) through trisynaptic indirect route; the Schaffer collateral (sc) from CA3 to CA1, the mossy fibre pathway from the DG to CA3, and the pp from EC to DG. This indirect pathway is proposed to provide mnemonic information. Due to the trisynaptic nature, information carried via the indirect pathway reaches CA1 field 10-20 ms after information from the direct pathway (Dudman et al., 2007). On the other hand, Otmakhova et al. (Otmakhova and Lisman, 2000; Otmakhova et al., 2005) demonstrated that the direct pp-CA1 synaptic inputs respond to modulatory effects of monoamine neurotransmitters, such as 5-HT, DA, and NE. It is possible that changes in learning behaviours of 5-Htt KO and Tph2 KO mice do not necessarily require changes in LTP at the Schaffer collateral/commissural pathway on the CA1. Instead, some other pathways or subregions of the hippocampus may have more relevance to learning alterations of 5-Htt and Tph2 KO mice, either by themselves or in combination with functions of the Schaffer collateral/commissural pathway.

4.5. Impacts of changes in the 5-HT level could have been buffered by as-yet-unknown biological compensatory mechanisms

4.5.1. Tph2 KO mice

i). Residual serotonin

It should be noted that, in Tph2 KO mice, a residual amount of 5-HT was detected in the brain. This is probably due to unintended contamination from blood which contains cells with high 5-HT content, such as platelets or mastocytes (Savelieva et al., 2008). However, even in mice with double knockout of Tph1 and Tph2, which should theoretically eliminate 5-HT from the periphery too, a small amount of 5-HT was still detected in the brain by HPLC measurement. This could be due to another neurochemical with similar HPLC mobility or to another enzyme which has tryptophan hydroxylase-like activity, such as phenylalanine hydroxylase (PAH) in the liver (Renson et al., 1962). Another explanation for a trace amount of 5-HT found in the brain of Tph2 KO mice is that 5-HTP produced in the peripheral tissues by Tph1 crossed the blood brain barrier and was converted into 5-HT by 5-HTP decarboxylase and AADC, which is rather ubiquitously expressed (see section 1.2.1.). In fact, the data presented in this study confirmed that serotonergic neurons from Tph2 KO mice are able to produce 5-HT when

supplied with 5-HTP. On the other hand, the possibility is ruled out that 5-HT synthesis in the brain of Tph2 KO mice is mediated by Tph1 as a compensatory mechanism since a previous study showed that Tph1 is not upregulated in Tph2 KO brains (Gutknecht et al., 2008). Moreover, in the case of prenatal mice, the possibility cannot be ruled out that a low amount of 5-HT comes from the placenta of pregnant mothers, which may have contributed to the almost normal development of Tph2 KO mice (see discussion in Gutknecht et al., 2012). It is rather unlikely, however, that the trace amounts of 5-HT from all those possible sources mentioned above alone can compensate for the dramatic reduction of brain 5-HT levels observed in Tph2 KO mice. It is more reasonable to assume that some other as-yet-unknown adaptive changes in Tph2 KO mice also enabled these mice to retain gross morphological and electrophysiological properties of the serotonergic system as seen in control animals.

ii). Upregulation of 5-HT_{1A} receptors

As demonstrated in the present study, 5-HT_{1A} receptors in the DRN of Tph2 KO mice were mildly sensitised, i.e. shift of EC_{50} from c.a. 3 nM in WT to 1.5 nM in KO in response to R8HD. This is consistent with quantitative autoradiography study by Gutknecht et al. (2012), where upregulation of the density and G-protein coupling of 5-HT_{1A} and 5-HT_{1B} receptors were detected across several brain regions of Tph2 male KO mice, reaching 11.7 % increase in the case of DRN 5HT_{1A} receptors. As for the hippocampus, they reported about 20% of 5-HT_{1A} receptor upregulation. The functional extent of 5-HT_{1A} receptor sensitisation in the hippocampus was not electrophysiologically investigated in the present study but it is likely that a similar level of sensitisation will be observed in brain regions outside of the DRN as heteroreceptors as a general compensatory reaction of receptors to the lack of their ligands. It is, however, still questionable whether the functional sensitisation, if at all present, is sufficient to maintain 5-HT mediated function from a minute amount of 5-HT detected in Tph2 KO mice. At least for some brain functions where 5-HT is involved to some extent, such as aggression, this upregulation is not sufficient to maintain normal behaviours from a trace amount of 5-HT. For example, although stimulation of 5-HT_{1A} or 5-HT_{1B} receptors by their agonists is known to reduce aggressive behaviours and 5-HT_{1A} and 5-HT_{1B} receptors are upregulated in Tph2 KO mice, these mice still exhibit high aggression, confirming that uprelation is not sufficient to reinstate normal behaviours from the trace amount of 5-HT in KO mice (Gutknecht et al., submitted). Similarly, it is rather unlikely that the lack of difference in LTP inducibility between Tph2 KO and wildtype counterparts is solely due to the potential functional upregulation of 5-HT_{1A} receptors in the hippocampus unless the

magnitude of upregulation is so intense as to compensate for almost complete absence of brain 5-HT.

iii). Interaction with other neurotransmitters

There is a possibility that impacts of lack of 5-HT are compensated by some other neurotransmitters. This is because some of the serotonergic receptors are known to share common second messenger signalling pathways or ion channels with receptors for other neurotransmitters (reviewed in Ciranna, 2006). For example, activation of GIRK current in the hippocampus was shown to be mediated by multiple receptors. (Sodickson and Bean, 1998 as in Ciranna, 2006). To be precise, it was shown that agonists for serotonin, adenosine, somatostatin, and metabotropic glutamate receptors all induced GIRK current that is the same as the one caused by $GABA_B$ receptor stimulation. In fact, the common downstream signal transduction pathway between 5-HT_{1A} receptors and $GABA_B$ receptors has been previously demonstrated (Mannoury la Cour et al., 2004; see Introduction). The difference, however, exists in terms of half-maximal effective agonist concentration (EC_{50}) and the latency for the activation/inactivation of GIRK current caused by each different receptor type. Importantly, when the concentration of those agonists is at the sub-saturating level, they work synergistically to produce supra-additive GIRK current. Moreover it was demonstrated that both carbachol, a non-hydrolysable cholinergic agonist and 5-HT induce low-threshold transient (LTT) Ca^{2+} in dissociated interneurons of the CA1 lacunosum-moleculare (LM) layer (Fraser and MacVicar, 1991). Prior to this finding, LTT Ca^{2+} currents had been shown to induce theta bursts in other neuronal types and both cholinergic afferents from the septum and serotonergic innervation from the raphe nuclei are believed to play a crucial role in generating rhythmic theta bursting in the hippocampus. Given the importance of theta bursting for learning and memory formation mediated by the hippocampus, this interchangeable role between acetylcholine and 5-HT bears a particular significance, especially when one of these neurotransmitters is abrogated. It should be noted, however, a resent *in vivo* study revealed that 5-HT supresses hippocampal theta rhythm through activation of 5-HT_{2C} receptors (Sorman et al., 2011). The same group also found NE either enhances or inhibits theta oscillations depending on the behavioural states of animals (Sorman et al., 2011 and references therein). As mentioned earlier (see section 1.6.1.), complete absence of brain 5-HT in Tph2 KO mice was found to be accompanied by reduction in NE contents. This may indicate compensatory mechanisms of organisms to keep balance between excitation and inhibition of various biological functions, including theta oscillations of the hippocampus.

In sum, a series of evidence attest that part of brain serotonin function in Tph2 KO mice could have been represented by some other neurotransmitters which all converge on the same effector, e.g. ion channel. Although this possibility is described only for the hippocampus here, it is likely that a similar compensatory mechanism may have taken place in some other brain regions such as in the DRN.

4.5.2. 5-Htt KO mice

As discussed previously, OCT3 and PMAT are potential candidates that ameliorated consequences of 5-Htt deficiency. Additionally, it was demonstrated in past studies that DAT and NET can reuptake 5-HT into catecholaminergic neurons (Schmitt et al., 2003 and references therein). 5-HT transport by DAT is particularly evident when normal 5-HT reuptake by 5-Htt is abrogated. In detail, DAT was shown to reuptake 5-HT in 5-Htt KO mice, but not 5-Htt WT or Het mice, although the expression level of DAT was not changed in 5-Htt KO mice (Fox et al., 2008 and references therein). In addition, Pan et al. (2001) demonstrated that in midbrain-hindbrain cell cultures obtained from 5-Htt KO mice, DATs reuptake 5-HT. Similarly, Ravary et al. (2001) observed low-affinity serotonin uptake in dopaminergic neurons of the substantia nigra *in vivo* in 5-Htt KO mice. In brain regions with high DAT expression, such as in the striatum, contribution of DAT to 5-HT transport is not negligible as was evidenced by enhanced reduction of striatal tissue levels of 5-HT in DAT and 5-Htt double KO mice compared to 5-Htt KO mice (Schmitt et al., 2003 and references therein). Moreover, a blocker to DAT was shown to be effective in reducing or abolishing ectopic 5-HT accumulation in dopaminergic neurons in the substantia nigra and the VTA of 5-Htt KO mice (Zhou et al., 2002). However, it was found that elevated levels of extracellular 5-HT was present even in the striatum where DAT expression is high, indicating that DAT can only partially compensate for the absence of 5-Htt. Moreover, the tissue level of 5-HT in the hippocampus, frontal cortex, brain stem, and hypothalamus was not further decreased by additional knockout of DAT in 5-Htt KO mice. Taken together, in brain regions apart from the striatum, other transporters, such as OCT3 mentioned earlier, are more likely to participate in clearance of elevated extracellular 5-HT in 5-Htt KO mice (Schmitt et al., 2003).

Conclusion

In a series of studies presented here, 5-Htt KO and Tph2 KO mice showed some characteristics which differentiate themselves from WT controls. At the same time, these mice displayed remarkable levels of resilience to genetic manipulation, resulting in almost the same characteristics in some electrophysiological parameters. The main finding from the investigation of autoinhibitory function in the dorsal raphe serotonergic neurons is that autoinhibition is maintained despite the fact that 5-HT_{1A} receptor sensitivity was altered. This may have clinically important implications since the autoinihibitory mechanism has substantial relevance to the mechanism of antidepressant action. Whole-cell patch clamp recordings in the DRN of Tph2 KO mice and CA1 pyramidal neurons of 5-Htt KO mice both displayed electrophysiological characteristics almost similar to those of each respective control animals. The only difference observed was increased conductance in the neurons of KO mice. Further investigation is required to infer its functional significance. In the case of 5-Htt KO mice, findings from past studies hint the upregulation of other cation carriers which may have contributed to increased conductance of neurons from KO mice. Finally, comparison of LTP inducibility in the CA1 area of the ventral hippocampus revealed no difference across all the genotypes tested. This was rather surprising given that the hippocampus and one of its main functions, i.e. learning & memory, have been repeatedly implicated in the aetiology of emotional disorders. It could be that the difference reveals itself only under a certain condition, for example after stress exposure. Alternatively, behavioural alterations in 5-Htt KO and Tph2 KO may not necessarily be reflected in the LTP inducibility. Overall, while some findings from the present study may have improved our understanding of the serotonergic system in relation to emotional disorders, they at the same time presented a few new open questions. The complexity of brain function together with potential biological compensatory mechanisms counteracting genetic manipulation makes it difficult to unmask underlining mechanisms which led to behavioural phenotypes of the KO mice. Further investigation addressed to open questions raised here, with some support from newly developed techniques such as conditional knockout mice, which may circumvent potential compensatory mechanisms concomitant with constitutive knockout, promises to produce further findings which will enhance our understanding of the serotonergic system and the mechanism of emotional disorders.

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Appendices

Appendix A. List of figures

Appendix B. List of frequently used abbreviations

5-HT	5-hydroxy-tryptamine = serotonin
5-HTP	5-hydroxy-L-tryptophan
5-HTT (*5-HTT*)	serotonin transporter of humans and in general (gene)
5-Htt (*5-htt*)	murine serotonin transporter (gene)
5-HTTLPR	5-HTT linked polymorphic region
AADC	aromatic l-amino acid decarboxylase
ACSF	artificial cerebrospinal fluid
AHP	afterhyperpolarisation
AP	action potential
C	capacitance (F)
CA1	Cornu Ammonis 1 of the hippocampus
CA3	Cornu Ammonis 3 of the hippocampus
CNS	central nervous system
DA	dopamine
DRC	dose response curve
DRN	dorsal raphe nucleus
EC_{50}	half-maximum Effective Concentration
EPSC	excitatory postsynaptic current
fEPSP	field excitatory postsynaptic potential
FR	firing rate
G	conductance (S)
GABA	gamma-aminobutyric acid
GIRK	G-protein-coupled inwardly rectifying K^+ channel/current
Het	heterozygous (+/-)
HHW	half-height width
I	current (A)
KO	knockout (-/-)
LTP	long-term potentiation
MDMA	3,4-Methylendioxy-N-methylamphetamin = ecstasy
MRN	medial raphe nucleus
NE	norepinephrine
OCT3	organic cation transporter 3
PMAT	plasma membrane monoamine transporter
R	resistance (Ω)
R8HD	R-8-hydroxy-2-(di-n-propylamino)tetralin (R-8-OH-DPAT)
SSRI	selective serotonin reuptake inhibitor
TB10	theta-burst stimulation consisting of 10 bursts of 5 stimuli
TB5	theta-burst stimulation consisting of 5 bursts of 5 stimuli
TPH2 (*TPH2*)	tryptophan hydroxylase-2 of humans and in general (gene)
Tph2 (*Tph2*)	murine tryptophan hydroxylase-2 (gene)
Trp	L-tryptophan
UD_2I	Up-to-the second Downstroke Interval
UDI	Up-to-Downstroke Interval
V	voltage (V)
WC	whole-cell
WT	wildtype (+/+)

Appendix C. List of publications

Goto H, Watanabe K, **Araragi N**, Kageyama R, Tanaka K, Kuroki Y, Toyoda A, Hattori M, Sakaki Y, Fujiyama A, Fukumaki Y, Shibata H (2009) The identification and functional implications of human-specific "fixed" amino acid substitutions in the glutamate receptor family. BMC Evol Biol. Sep 8; 9:224.

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Gutknecht L, **Araragi N**, Merker S, Waider J, Sommerlandt FM, Mlinar B, Baccini G, Mayer U, Proft F, Hamon M, Schmitt AG, Corradetti R, Lanfumey L, Lesch KP (2012) Impacts of Brain Serotonin Deficiency following Tph2 Inactivation on Development and Raphe Neuron Serotonergic Specification. PLoS One 7:e43157.

Gutknecht L, Waider J, Popp S, **Araragi N**, Sommerlandt FMJ, Weber M, Göppner C, Colaço MBN, Post A, Wischmeyer E, Sommer C, Reif A, Palme R, Lesch KP. Brain serotonin deficiency promotes resilience to chronic stress via sex-specific adaptive mechanisms. (submitted)

Araragi N and Lesch KP. Serotonin (5-HT) in the regulation of depression-related emotionality: insight from 5-HT transpoter and tryptophan hydroxylase-2 knockout mouse models. (submitted to Current Drug Targets)

Araragi N, Gutknecht L, Baccini G, Mlinar B, Lesch KP, Corradetti R. 5-HT_{1A} receptor-mediated autoinhibition of serotonergic neurons is preserved in mice with severely altered serotonin homeostasis. (in preparation)

Appendix D. Curriculum vitae

Aus datenschutzrechtlichen Gründen wurde der Lebenslauf bei der online-Veröffentlichung entfernt.

For protection of private information, the curriculum vitae has been removed from the online publication.

Aus datenschutzrechtlichen Gründen wurde der Lebenslauf bei der online-Veröffentlichung entfernt.

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