“Gene and environment interactions in serotonin transporter knockout mice – how stress influences gene expression and neuronal morphology”

Dissertation zur Erlangung des naturwissenschaftlichen Doktorgrades der Julius-Maximilians-Universität Würzburg

vorgelegt von

Sarah Louise Nietzer

Marktheidenfeld

Würzburg 2010

Eingereicht am: .....

Mitglieder der Promotionskommission:

Vorsitzender: .....

Gutachter : **Prof. Dr. Klaus-Peter Lesch**

Gutachter: **Prof. Dr. Thomas Dandekar**

Tag des Promotionskolloquiums: .....

Doktorurkunde ausgehändigt am: .....

"Gene and environment interactions in serotonin transporter knockout mice – how stress influences gene expression and neuronal morphology"

Abstract

Serotonin (5-HT) is an important modulator of many physiological, behavioural and developmental processes and it plays an important role in stress coping reactions. Anxiety disorders and depression are stress-related disorders and they are associated with a malfunction of the 5-HT system, in which the 5-HT transporter (5-HTT) plays an important role. *5-Htt* knockout (KO) mice represent an artificially hyperserotonergic environment, show an increased anxiety-like behaviour and seem to be a good model to investigate the role of the 5-HT system concerning stress reactions and anxiety disorders. As synaptic proteins (SPs) seem to be involved in stress reactions, the effect of acute immobilization stress on the expression of the three SPs Synaptotagmin (Syt) I, Syt IV and Syntaxin (Stx) 1A was studied in the *5-Htt* KO mouse model as well as the expression of the two immediate early genes (IEGs) FBJ osteosarcoma oncogene (c-Fos) and fos-like antigen 2 (Fra-2). Additionally, the expression of the corticotrophin releasing hormone (CRH) and its two receptors CRHR1 and CRHR2 was investigated as part of the hypothalamic-pituitary-adrenal (HPA) stress system.

Based on gender- and genotype-dependent differences in corticosterone levels, expression differences in the brain were investigated by performing a quantitative real time-PCR study using primer pairs specific for these SPs and for the IEGs c-Fos and Fra-2 in five different brain regions in *5-Htt* KO and *5-Htt* wild-type (WT) mice. Mainly gender-dependent differences could be found and weaker stress effects on the expression of SPs could be demonstrated. Regarding the expression of IEGs, stress-, gender- and genotype-dependent differences were found mainly in the hypothalamus. Also in the hypothalamus, gender effects were found concerning the expression of CRH and its both receptors. Additionally, in a second study, male *5-Htt* WT and male 5-Htt deficient mice were subjected to a resident-intruder-paradigm which stresses the animals through a loser experience. The morphological changes of neurons were subsequently analyzed in Golgi-Cox-stained sections of limbic brain areas in stressed and unstressed animals of both genotypes using the computer-based microscopy system Neurolucida (Microbrightfield, Inc.). While no differences concerning dendritic length, branching patterns and spine density were found in the hippocampus and no differences concerning dendritic length and branching patterns could be shown in the cingulate cortex (CG), pyramidal neurons in the infralimbic cortex (IL) of stressed *5-Htt* WT mice displayed longer dendrites compared to unstressed *5-Htt* WT mice. The results indicate that, although in this model drastic alterations of neuronal morphology are absent, subtle changes can be found in specific brain areas involved in stress- and anxiety-related behaviour which may represent neural substrates underlying behavioural phenomena.

“Gen- und Umweltinteraktionen in Serotonintransporter-Knockout-Mäusen – wie Stress Genexpression und neuronale Morphologie beeinflusst”

Zusammenfassung

Serotonin (5-HT) ist ein wichtiger Modulator vieler physiologischer, verhaltensbiologischer und entwicklungsbiologischer Vorgänge und spielt zudem eine wichtige Rolle bei der Stressbewältigung. Angsterkrankungen und Depression sind stressbedingte Störungen und sie sind mit einer Dysfunktion des serotonergen Systems assoziiert, in dem der Serotonintransporter (5-HTT) eine wichtige Funktion einnimmt. *5-Htt* Knockout (KO)-Mäuse haben reduzierte Serotoninkonzentrationen im Gehirn, zeigen erhöhtes Angst-ähnliches Verhalten und scheinen ein gutes Modell für die Erforschung der Rolle des serotonergen Systems in Bezug auf Stress-Reaktionen und Angsterkrankungen darzustellen. Da synaptische Proteine (SPs) in die Stress-Reaktion involviert zu sein scheinen, wurden die Auswirkungen von akutem Immobilizierungsstress auf die Expression der drei SPs Synaptotagmin (Syt) I, Syt IV und Syntaxin (Stx) 1A in diesem *5-Htt* KO-Maus-Modell untersucht. Ebenso wurde die Expression der zwei „immediate early genes“ (IEGs) FBJ osteosarcoma oncogene (c-Fos) and fos-like antigen 2 (Fra-2) unter die Lupe genommen. Außerdem wurde die Expression des „Corticotrophin Releasing Hormone“ (CRH) sowie seiner beiden Rezeptoren CRHR1 and CRHR2, als Teil des Hypothalamus- Hypophysen-Nebennieren-(HPA)-Systems, analysiert.

Basierend auf Geschlechts- und Genotyp-spezifischen Unterschieden der Kortikosteron-Konzentrationen im Blut der Tiere wurden die Expressionslevel dieser SPs und der beiden IEGs mittels quantitativer Real-Time (qRT)-PCR in fünf verschiedenen Gehirnregionen von *5-Htt* KO- und *5-Htt*-Mäusen mit wildtypischer (WT) Gen-Konstitution untersucht. Dabei konnten vor allem Geschlechts-spezifische Unterschiede in der Genexpression gezeigt werden und es konnte ein im Vergleich dazu schwächerer Einfluss des akuten Immobilisierungs-Stresses auf die Genexpression nachgewiesen werden. Die Expression der IEGs wurde durch Stress, Geschlecht und Genotyp vor allem im Hypothalamus beeinflusst. Ebenfalls im Hypothalamus konnte der Einfluss des Geschlechts auf die Expression des CRH und seiner beiden Rezeptoren gezeigt werden.

In einer zweiten Studie wurden männliche *5-Htt* KO-Mäuse sowie *5-Htt* WT-Mäuse dem „Resident-Intruder-Paradigma“ unterzogen, in welchem die Tiere mittels einer mehrfachen Verlierer-Erfahrung gestresst wurden. Morphologische Veränderungen von Neuronen limbischer Gehirnareale wurden daraufhin an Golgi-Cox-gefärbten Gehirnschnitten dieser gestressten und ungestressten *5-Htt* KO- und *5-Htt* WT-Tiere mittels des Computer-gestützten Mikroskop-Systems Neurolucida (Microbrightflield, Inc.) analysiert. Während keine Unterschiede bezüglich der Länge des dendritischen Materials, des Verzweigungsmusters und der Spinedichte im Hippocampus gefunden werden konnten und keine Unterschiede in Länge des dendritischen Materials und des Verzweigungsmusters in der Area cinguli (CG) gezeigt werden konnten, wiesen Pyramidenzellen im infralimbischen Kortex (IL) von gestressten *5-Htt* WT-Mäusen längere Dendriten auf als die entsprechenden Zellen in den ungestressten Tieren desselben Genotyps. Diese Ergebnisse zeigen, dass, obwohl in diesen Tieren keine drastischen stressbedingten Änderungen der neuronalen Morphologie vorliegen, doch subtile Änderungen der neuronalen Morphologie stress- und angstinvolvierter Gehirnareale gefunden werden können. Diese Änderungen können die neuronale Basis verschiedenster Verhaltens-Phänomene darstellen.

1. Introduction

1.1. Stress related disorders in humans: Depression and anxiety disorders

1.1.1. Genetic as well as environmental factors involved in psychiatric disorders

Depression and anxiety disorders are common and widespread disorders. About 10% of population develop a depression during their lifetime. Depression as well as anxiety disorders are associated with stress as they are to a great extent powered by stress or can be triggered by stressful life events (Sapolsky 1996). In depressed patients overreaction of hypothalamic-pituitary-adrenal (HPA) axis (Chapter 1.4.1.) has been documented (Holsboer 2001). During development and during adulthood the environment as well as the genetic endowment play an important role in this process of developing such stress-related disorders. The question arises as to which proteins and genes are involved in the onset of depression or anxiety disorders. One molecule known to be involved in this relationship is the serotonin transporter (5-HTT). Its corresponding gene (*SLC6A4*) as well as the promoter of the *SLC6A4* which is important for the transcription rate and hence the availability of the 5-HTT are also important for the clinical image of depression and anxiety disorders. There are polymorphisms in the 5' regulatory region of *SLC6A4* as well as within the intron 2 of the *SLC6A4* which are associated with personality traits like anxiety and depression (Lesch, Bengel et al. 1996; Mazzanti, Lappalainen et al. 1998; MacKenzie and Quinn 1999; Greenberg, Li et al. 2000; Stoltenberg and Burmeister 2000). However, there are also studies which deny this influence of the *SLC6A4* on the possibility of developing a depression (Risch, Herrell et al. 2009). But in an impressive study Caspi and collegues could show that humans who exhibit the short allelic variant of the *SLC6A4* promoter responsible for lower transcription rate of the *5-HTT* gene have a higher risk to become depressed after serious life events compared to humans who possess the longer allelic variant of the promoter which leads to a higher amount of 5-HTT in the organism (Caspi, Sugden et al. 2003).

1.1.2. The role of the 5-HTT in the serotonergic system

Serotonin (5-HT) is a biogenic amine which plays a very important role in neurotransmission not only during adulthood but also during development, as it is shown to be a regulator of early embryogenesis and morphogenesis (Buznikov, Lambert et al. 2001). Comparisons of developmental processes in various animal phyla show that 5-HT, together with other neurotransmitters, regulates the basic development of an organism, including cell proliferation, migration, differentiation, and morphogenesis. For that reason serotonergic neurotransmission is involved in a wide range of behaviour including food intake, reproductive activity, sensory processing, motor activity, cognition and emotion. Altered serotonergic function is implicated in the etiology and pathogenesis of a variety of psychiatric disorders such as anxiety disorders, depression and also attention-deficit hyperactivity

disorder (ADHD). 5-HT neurotransmission is basically regulated by clearance of 5-HT from the extracellular space by the NaCl-dependent 5-HT-transporter (Blakely, Berson et al. 1991). Therefore 5-HTT is a major component of the serotonergic neurotransmission as shown in Figure 1.1.

After an action potential and the following cascade of events in the synapse (Chapter 1.3.) serotonin is released into the synaptic cleft. Released 5-HT attaches itself onto its receptors on the surface of the postsynapse as well as onto autoreceptors localized on the presynapse. To finish the signal forwarded by the released 5-HT, the 5-HTT reuptakes the 5-HT from the synaptic cleft and leads it back into the presynapse where it can be metabolized by the monoamine oxidase (MAO type A and MAO type B) or can be stored in synaptic vesicles again.

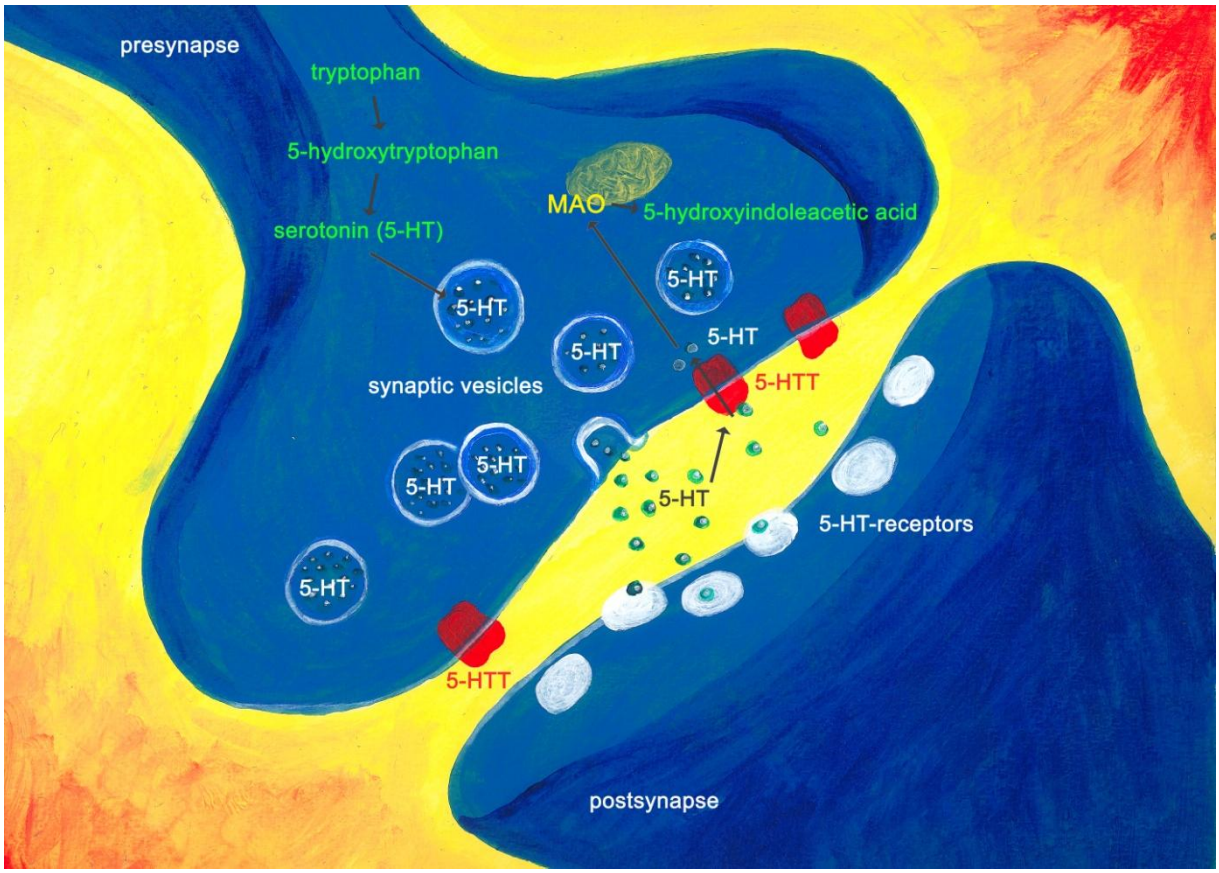

Figure 1.1: The function of the 5-HTT. 5-HT is synthesized in the presynaptic neuron and is stored in synaptic vesicles. After an action potential 5-HT is released into the synaptic cleft and attaches itself onto 5-HT receptors on the postsynapse (white ovals) or onto autoreceptors on the presynapse (not shown). 5-HTT stops the signal when it removes 5-HT from the synaptic cleft back into the presynapse, where it is metabolized by the MAO or stored away again in synaptic vesicles.
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1.1.3. The Monoamine Deficiency Hypothesis

If there is less 5-HTT available because of a lower transcription rate caused by the short variant of the promoter, the signal can't be finished as quickly as normal. This circumstance has some of the consequences described by the monoamine deficiency hypothesis (Heninger, Delgado et al. 1996) which emanates from a deficit of monoamine and serotonergic neurotransmitters reaching the receptors of depressive patients. This hypothesis is supported by former studies which revealed that cerebrospinal fluid (CSF) in depressive patients shows reduced concentrations of 5-hydroxyindoleacetic acid (5-HIAA), the major metabolite of 5-HT (Coppen, Prange et al. 1972) Moreover these patients have lower tryptophane concentrations in plasma (Coppen, Brooksbank et al. 1972; Koyama, Lowy et al. 1987) as well as a decreased number of 5-HTT binding sites for specific antidepressants (Nemeroff 1992) as the 5-HTT is target of many antidepressants such as selective 5-HT reuptake inhibitors like fluoxetine and other antidepressant or anxiolytic drugs (Torres and Caron 2003). Additionally 5-HTT is the working point of drugs like 3,4-Methylendioxy-N-methylamphetamin (MDMA, Ecstasy) and cocaine. This last one affects the dopamine transporter (DAT) too.

In mice it could be shown that loss of 5-Htt leads to adaptation mechanisms which redress the homeostatic balance of the organism's serotonergic system. In these 5-Htt deficient mice (Chapter 1.2.) 5-HT receptors become down regulated and synthesis of 5-HT becomes reduced. During the development of the brain this process is believed to decrease the 5-HT in the whole brain and is caused by some compensation mechanism although concentrations of 5-HT are quite high in the synaptic cleft of serotonergic synapses. These mechanisms are probably similar in humans. Thus the serotonergic system and mainly the 5-HTT have an important role in the onset and treatment of affective disorders like depression and mania as well as anxiety disorders and obsessive-compulsive disorders (Lesch and Beckmann 1990).

Additionally to affecting the 5-HTT there are other ways antidepressants have an impact on serotonergic neurotransmission to increase the levels of 5-HT, for example by influencing synthesis and storage of 5-HT as well as influencing receptor binding and 5-HT metabolism. But as antidepressants are usually effective only after 2-3 weeks (Gelenberg and Chesen 2000) the crucial factor for effective therapy of depression is possibly not the immediate increase of extracellular 5-HT but depends rather on the long term effects of changes in serotonergic neurotransmission and resulting changes in expression of various pre- and postsynaptic proteins (Chapter 1.3.5.).

In the present study these changes in expression of synaptic proteins (SPs) were investigated in wild-type (WT) mice and *5-Htt* knockout (KO) mice. The *5-Htt* KO mouse is seen as a mouse model for anxiety disorders in humans.

1.2. The *5-Htt* KO mouse – a mouse model for anxiety disorders

To investigate the function of the 5-HTT and its role in the serotonergic system, *5-Htt* KO (*5-Htt* -/-) mice were generated by inactivation of the murine *5-Htt* gene using homologous recombination (Bengel, Murphy et al. 1998), as there is no analogue to the human length polymorphism of the human 5-HTT promoter naturally occurring in mice. These *5-Htt* KO mice express no 5-Htt at all and (like *5-Htt* KO rats) show some characteristics as reviewed by Kalueff et al. (Kalueff, Olivier et al. 2009), which make them a practical model for depression-like disorders as well as for anxiety disorders. Heterozygous (Het) animals (*5-Htt* +/-) express 5-Htt by the half compared to wild-type (WT) mice (*5-Htt* +/+) which express the full amount of 5-Htt.

1.2.1. Behavioural phenotype of the 5-Htt deficient mice

5-Htt KO mice show an anxiety-like phenotype in some behavioural tests (Murphy, Wichems et al. 1999; Holmes, Murphy et al. 2002; Holmes, Yang et al. 2002) and are shown to be less aggressive than WT mice (Holmes, Murphy et al. 2002). For example: they don't like to explore brightly lit spaces or elevated open platforms, which can be judged a sign of anxiety. This behavioural profile couldn`t be replicated in all recently performed studies. Jansen et al. for example, demonstrated that this anxiety-like behaviour can't be seen in naive unstressed mice but only in mice which have experienced some kind of stress (Jansen, Heiming et al. 2010). They could show that male mice of a 5-Htt KO breeding line were not more anxious than WT mice before having experienced repeated social stress. But after having experienced becoming a loser in a territorial fight (resident-intruder-paradigm) for three days running the *5-Htt* KO mice were significantly more anxious than WT or Het mice with the same loser experience (Jansen, Heiming et al. 2010). Another distinct trait of these 5-Htt deficient mice is their depression-like behaviour. That means they give up struggling early when put in an uncomfortable situation (Holmes, Yang et al. 2003; Adamec, Burton et al. 2006; Wellman, Izquierdo et al. 2007), i.e. a behaviour associated with depression. Interestingly, in contrast to WT mice, *5-Htt* KO mice show a depression-like tendency to give up (immobility or behavioural despair) only after repeated exposure to stressors which are analogous to repeated or chronic stressful life events in humans (Wellman, Izquierdo et al. 2007). As Wellman and associates could show, this anxious-depressive phenotype in *5-Htt* KO mice is associated with changes in dendritic branching or amount of dendritic spines in the "fear circuit" including the amygdala and infralimbic cortex (Wellman, Izquierdo et al. 2007). In summary, it can reasonably be said that the altered emotion-related behaviour in the 5-Htt KO mouse line is shown to be shaped by stressful life events during different periods of development, as shown in a study performed by Carola and associates. They could show that heterozygous 5-Htt KO mice displayed increased anxiety- and depression-related

behaviour compared to WT controls only when they had received low maternal care (Carola, Frazzetto et al. 2008).

Interestingly, the same anxious phenotype which is shown by the *5-Htt* KO mice can be seen in 5-HT_{1A} receptor KO mice (Zhuang, Gross et al. 1999). On the other hand, mice with transgenically increased 5-Htt expression appear fearless and explore bright-lit and open spaces more daringly than WT animals do (Jennings, Loder et al. 2006). Phenotypes of all these mouse models suggest that serotonergic neurotransmission plays an important role in affective disorders.

1.2.2. Physiological phenotype of the 5-Htt deficient mice

5-Htt KO mice show not only increased anxiety-like behaviour but they have higher extracellular levels of 5-HT in their brain. This could be demonstrated in the striatum (Mathews, Fedele et al. 2004) and the substantia nigra (Fabre, Beaufour et al. 2000). In contrast to the increased levels of extracellular 5-HT, the overall concentration of 5-HT in the whole brain is decreased compared to normal WT mice (Bengel, Murphy et al. 1998) and the number of serotonergic neurons in the dorsal raphe is decreased too (Lira, Zhou et al. 2003). Deficiency of 5-Htt has an influence on the expression of various 5-HT receptors e.g. 5-HT_{1A}, 5-HT_{1B} and 5-HT_3 receptors (Fabre, Beaufour et al. 2000; Mossner, Schmitt et al. 2004). The 5-HT_{1A} receptor density is lowered in *5-Htt* KO mice compared to WT mice whereas female *5-Htt* KO mice exhibit lower receptor density than male *5-Htt* KO mice (Li, Wichems et al. 2000). One compensation mechanism to balance the effects of increased extracellular 5-HT concentrations could be an upregulation of the expression of the polyspecific organic cation transporter (OCT3) (Schmitt, Mossner et al. 2003). The OTC3 lets organic cations pass as well as, to a lower extent, 5-HT. As they have no serotonin transporter at all, *5-Htt* KO mice aren't responsive to ecstasy (Bengel, Murphy et al. 1998), but they are sensitive to cocaine (Sora, Wichems et al. 1998).

1.2.2. Stress reaction in the 5-Htt deficient mice

Concerning the physiology of stress, *5-Htt* KO mice react to stressful events more strongly than WT mice, as 5-HT is an important factor not only in the brain but also in the stress reaction of the body. In the periphery, 5-HT is synthesized by enteric neurons and enterochromaffin cells of the gut and released into the blood (Ormsbee and Fondacaro 1985). The 5-Htt is expressed in many tissues including the adrenal, as it has to take up blood-borne 5-HT (Kent and Coupland 1984; Schroeter, Levey et al. 1997; Lefebvre, Contesse et al. 1998; Delarue, Contesse et al. 2001; Lefebvre, Compagnon et al. 2001). Here it plays an important role in maintaining normal adrenal 5-HT levels, in the increase of TH transcription during acute stress in mice, and in the stress-induced increase in catecholamine release and adrenomedullary AT2 receptor expression (Armando, Tjurmina et al. 2003). In mice, absence of SERT expression leads to abnormally low adrenal 5-HT levels and inability

to increase 5-HT concentrations, TH transcription and AT2 receptor expression during stress (Armando, Tjurmina et al. 2003). Thus 5-Htt deficient mice are impaired in stress response.
In consequence of chronic stress, *5-Htt* KO mice of both genders show increased corticosterone response (Lanfumey, Mannoury La Cour et al. 2000). But there are big discrepancies in the literature regarding these corticosterone concentrations with and without stress exposure in *5-Htt* KO mice. Whereas Lanfumey et al. revealed reduced basal corticosterone levels in *5-Htt* KO mice compared to WT controls, Tjurmina and associates and Jansen and associates couldn`t show any baseline corticosterone concentration differences in female mice of different *5-Htt* genotypes (Tjurmina, Armando et al. 2002; Jansen, Heiming et al. 2010). Concerning adrenocorticotropic hormone (ACTH) response after acute stress, it seems that ACTH is increased in *5-Htt* KO mice after stress in comparison to WT mice (Li, Wichems et al. 1999). After short immobilization female 5-Htt deficient mice exhibit enhanced blood adrenalin concentration (Armando, Tjurmina et al. 2003) and the amount of 5-HT in the adrenal medulla is decreased after stress compared to WT mice. Additionally, there is no increased expression of tyrosin hydroxylase in the adrenal medulla of female *5-Htt* KO mice, which is a sign for disturbed stress-induced synthesis of catecholamines (Armando, Tjurmina et al. 2003). Fifteen minutes' immobilization stress leads to 80% decreased 5-HT concentrations in the adrendal glands of female *5-Htt* KO mice compared to female WT mice. There is also a reduction of 5-HT concentration in the adrenal glands of *5-Htt* KO mice after stress, whereas in *5-Htt* Het and WT mice an increase of 5-HT concentration in the adrenal glands can be measured after stress (Tjurmina, Armando et al. 2002).

The present investigations focus on corticosterone measurements and of acutely stressed mice of both gender and all three *5-Htt* genotypes to clarify these data. Also expression analysis of SPs was done in mice of both genders.

1.3. SPs and their role in synaptic transmission

The release of serotonin and other neurotransmitters results from Ca^{2+} mediated fusion of neurotransmitter-containing vesicles with the presynaptic membrane during the vesicle cycle. At the beginning of the vesicle cycle (Figure 1.2) synaptic vesicles are built up from post Golgi structures and are charged with transmitter molecules. The vesicles are then transported to the plasma membrane of the synapse, where they dock onto special structures (docking process). Afterwards vesicles are transported to the active zone and are primed for transmitter release (priming process). After Ca^{2+} ions have streamed into the cell, the plasma membrane fuses with the vesicles' membranes and transmitter molecules are released into the synaptic cleft. Finally the plasma membrane invaginates and becomes recycled (Cremona and De Camilli 1997).

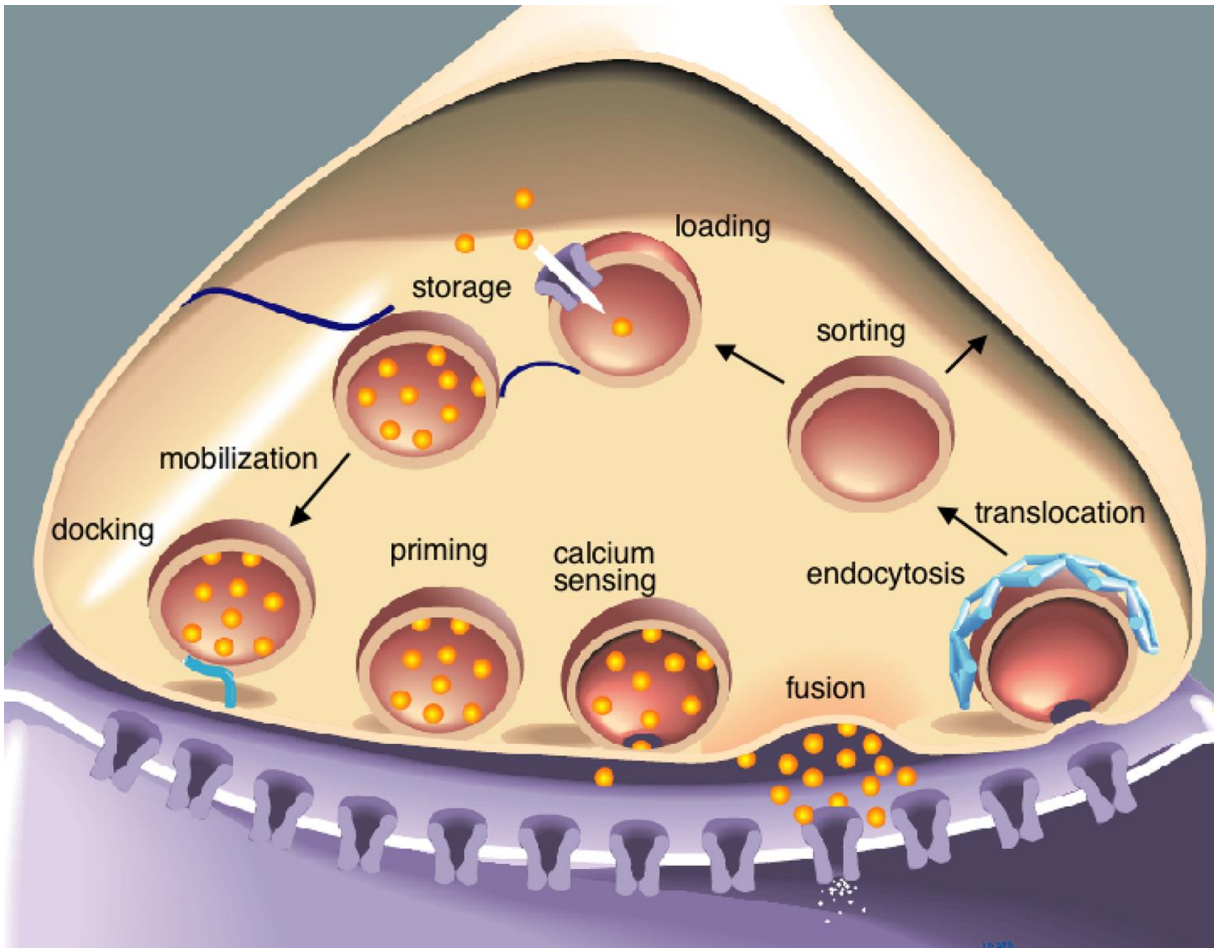


Figure 1.2: The synaptic vesicle cycle. Neurotransmitter molecules are stored in synaptic vesicles after synthesis. The vesicles contain a neurotransmitter transporter which loads vesicles with neurotransmitter molecules. The vesicles are stored until they are mobilized and able to translocate to the plasma membrane of the active zone where they attach themselves to the plasma membrane (docking). During a priming step docked vesicles become fusion-competent (priming). Vesicle fusion is triggered by calcium sensors like synaptotagmin I after a rise in intracellular calcium and then the release of neurotransmitter molecules occurs. Released neurotransmitter can bind and activate receptors on the postsynaptic membrane. The vesicle proteins as well as the membrane are recycled by clathrin-mediated endocytosis.

(from: Richmond, J. Synaptic function (December 7, 2007), WormBook, ed. The C. elegans Research Community, WormBook, doi/10.1895/wormbook.1.69.1, http://www.wormbook.org)

1.3.1. Proteins involved in the synaptic vesicle cycle

Various proteins are responsible for vesicle docking and membrane fusion. Because of their interactions with the „N-ethylmaleimide sensitive fusion protein“ (NSF) and the „soluble N-ethylmaleimide-sensitive factor attachment proteins“ (SNAPs), these proteins are called SNAP receptors (SNAREs). Depending on their localization in the cell they are called v (vesicle) or t (target) SNAREs. Synaptobrevin belongs to the group of v-SNAREs (Sollner, Whiteheart et al. 1993; Sollner and Rothman 1994) and T-SNAREs are e.g. Syntaxin (Stx) and SNAP-25 (synaptosomal attachment protein of 25 kDa). Proteins of the synaptotagmin (Syt) protein family are not directly involved in the formation of the SNARE complex but they exhibit a sensor for Ca^{2+} and probably have an important role in regulating the secretion of neurotransmitters (Chapman 2002; Sudhof 2002). Most synaptotagmins are able to bind the clathrin adaptor (or activator) protein 2 (AP-2) and are therefore presumably engaged in the recycling of membrane proteins. Additionally, SPs are shown to be involved not only in stress reactions but also in antidepressant treatment (Thome, Pesold et al. 2001; Kinnunen, Koenig et al. 2003; Zink, Rapp et al. 2005). Therefore the expression of SPs is of considerable interest from a therapeutic point of view.

In this study I focused on the expression of three SPs, namely syntaxin 1A (Stx 1A), synaptotagmin I (Syt I) and synaptotagmin IV (Syt IV), and their expression after acute stress in mice of all three *5-Htt* genotypes. Mice of both genders were investigated to reveal the influence of gender on the expression of these SPs in addition to environmental factors such as stress and genetic endowment.

1.3.2. Syntaxin 1A

Not only synaptobrevin and SNAP-25 but also syntaxin are components of the SNARE complex. The SNARE complex is composed of four twisted alpha helices. One helix derives from synaptobrevin which is a member of the family of vesicle associated membrane proteins (VAMPs), the second derives from Stx and the third and fourth helix derive from SNAP 25 (Figure 1.3). All proteins involved lie together in a way that the C- and N-termini are parallel (Hanson, Heuser et al. 1997; Hanson, Roth et al. 1997; Lin and Scheller 2000). The synaptobrevin and syntaxin are bound to the plasma membrane of the synapse by their C-terminus and build up a transmembrane complex (Jahn and Sudhof 1999; Lin and Scheller 2000). In the process of building up the SNARE complex, synaptobrevin seems to be a limiting factor as well as a factor triggering membrane fusion (Hu, Carroll et al. 2002). Primarily syntaxin was identified as the retinal antigen HPC-1 (Barnstable, Hofstein et al. 1985). Later the two isoforms Stx 1A and Stx 1B became cloned and characterized (Bennett, Calakos et al. 1992; Leveque, Pupier et al. 1998). Treatment with various antidepressants (desipramine, fluoxetine, tranylcypromine) has been shown to affect the expression of Stx 1A and complexin (Cplx) II, another

SP (Zink, Rapp et al. 2005), whereas the selective serotonin reuptake inhibitor (SSRI) fluoxetine displays stronger effects on Stx 1A expression compared to the effects on the expression of Cplx II. Zink and associates showed in this study that, in contrast to fluorexetine, the tricyclic antidepressant (TCA) desipramine as well as the monoamine oxidase inhibitor (MAOI) tranylcypromine increased the expression of Cplx II and Stx 1A significantly (Zink, Rapp et al. 2005).

Stx 1A is shown to interact with some neurotransmitter transporters, for example with the GABA transporter and also with the 5-HTT (Deken, Beckman et al. 2000; Geerlings, Lopez-Corcuera et al. 2000; Haase, Killian et al. 2001) as well as with the dopamine transporter (Lee, Kim et al. 2004), and it is able to influence the cellular localization of these transporters.

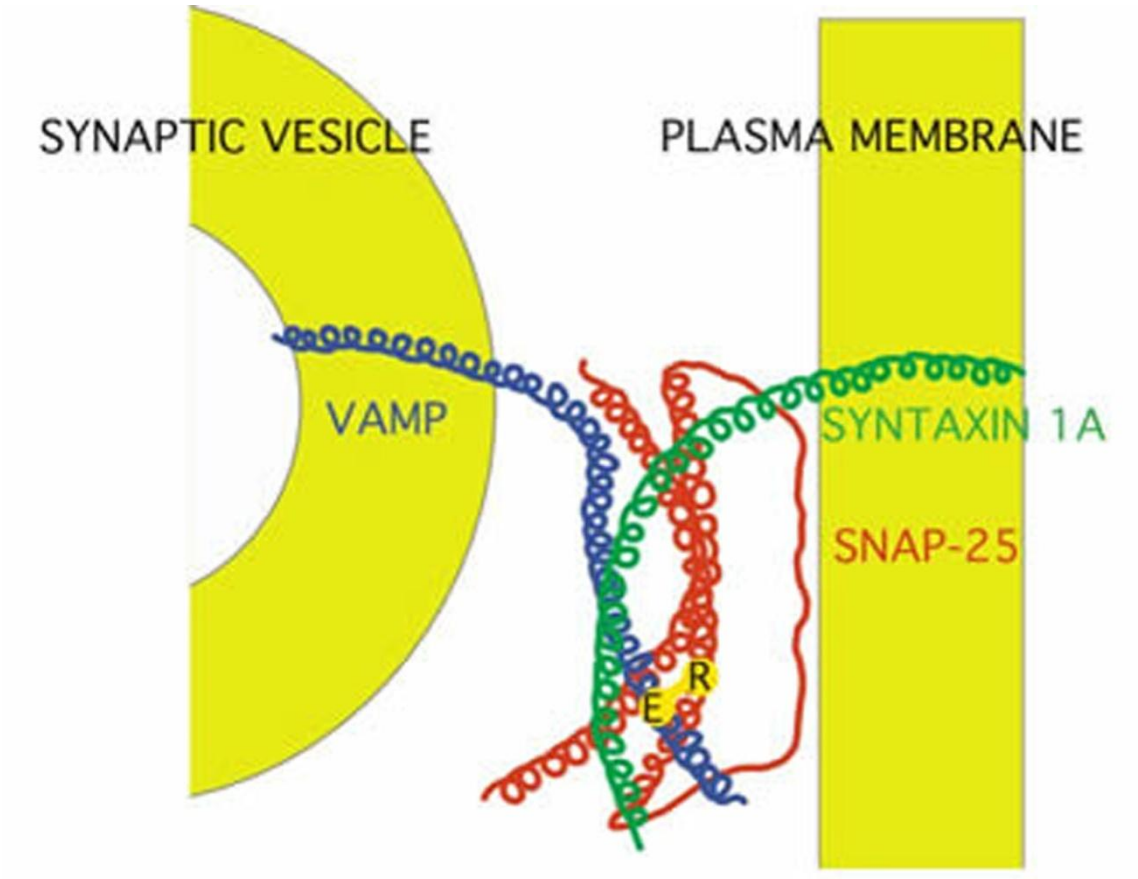


Figure 1.3: The SNARE complex. The SNARE complex is built up by the three proteins synaptobrevin (VAMP), Stx 1A and SNAP-25. Vesicles containing neurotransmitter are brought to the membrane by the complex in a zipper-like manner.

(Image modified from http://dbs.umt.edu/research_labs/haylab/images/fig5.jpg)

1.3.3. The Synaptotagmin gene family

Synaptotagmins belong to a very big family of membrane proteins and are widespread in the brain as well as in other tissue where neurons are present. The first time they were isolated they were only known as proteins on the surface of small synaptic vesicles (SSV). Until now up to 17 synaptotagmins have been identified in mice (Fukuda, Kanno et al. 1999; Fukuda and Mikoshiba 1999; Craxton 2001; Craxton 2007), and they can be classified in different classes (Sudhof 2002). Expression studies showed that there are synaptotagmins which are universally expressed in most brain areas. On the other hand there are isoforms which are specifically expressed in individual cells or in distinct areas of the brain (Mittelsteadt, Seifert et al. 2009). But more protein variants are possible because of the different splicing of RNA (Craxton and Goedert 1999). Genes coding for single synaptotagmins vary in size (< 10 kb to > 60 kb), but the structure of exons and introns is almost equal in each class of synaptotagmins although it varies from class to class. Some of the synaptotagmin genes are composed simply e.g. *Syt IV* which consists of 4 exons, but some of them are quite complex e.g. *Syt VII* which has 14 exons. Only one single exon at one end of the C2 domain is common to each synaptotagmin gene. Each synaptotagmin protein has one single transmembrane region (T) which is important for building up dimers between synaptotagmins. Additionally, each synaptotagmin has 2 C2 domains (C_2A and C_2B), which are able to bind Ca^{2+} and phospholipids (with the exception of Syt IV which can't bind Ca^{2+}). The carboyxterminal domain and especially the amino acid residues W405 and L408 are important for the correct targeting of synaptotagmins to the axon terminations (Krasnov and Enikolopov 2000). Synaptotagmins can either be localized on the surface of the synaptic vesicles (SytI, II and maybe Syt IV) or they can be localized on the plasma membrane of the active zone (SytIII, VI, VII). Highest concentrations of synaptotagmins can be found in synaptic knobs (Matteoli and De Camilli 1991) as well as in the golgi-area (Matteoli, Takei et al. 1991; Hou, Li et al. 1997). In neuronal differentiated phaeochromocytoma 12 (PC12)-cells synaptotagmin-like proteins can also be found where they are located similarly (Elferink, Peterson et al. 1993; Elferink and Scheller 1993; Marxen, Maienschein et al. 1997).

1.3.4. Synaptotagmin I (Syt I) and Synaptotagmin IV (Syt IV)

Syt I is able to bind Ca^{2+} as well as phospholipids. Additionally it is a sensor for Ca^{2+} during the fusion of synaptic vesicles with the synaptic membrane (Geppert, Bolshakov et al. 1994; Davis, Bai et al. 1999). In contrast to this Syt IV cannot bind either Ca^{2+} nor phospholipids but it can bind the activator protein AP-2, a transcription factor which plays a role in early development (Hilger-Eversheim, Moser et al. 2000). It could possibly play a role in the formation of clathrin cages in endocytosis (Ullrich, Li et al. 1994). Syt IV is a protein which is located on vesicles and its expression can be induced by agents

which increase intracellular Ca^{2+} or cAMP concentration (Vician, Lim et al. 1995; Ferguson, Thomas et al. 1999). Moreover it was once characterised to be an IEG and it can be induced by depolarization and by adding forskolin, which elevates cAMP in PC12-cells as well as in cells of the rat's hippocampus (Vician, Lim et al. 1995). Syt IV was (like Syt I) proven to be localized in the membrane of synaptic vesicles (Littleton, Serano et al. 1999; Ferguson, Chen et al. 2000), but there is evidence that Syt IV is not a real synaptic vesicle protein and can also be found in axons and dendrites on vesicles/organelles not yet characterized (Ibata, Hashikawa et al. 2002).

Syt IV doesn't exhibit Ca^{2+}-dependent phospholipid binding sites (Li, Ullrich et al. 1995; von Poser, Ichtchenko et al. 1997; von Poser and Sudhof 2001; Sugita, Shin et al. 2002; Dai, Shin et al. 2004; Bhalla, Tucker et al. 2005) and it's expressed in most areas of the brain but at lower levels compared to Syt XI, which is another synaptotagmin similar to Syt IV (Mittelsteadt, Seifert et al. 2009). *Syt IV* KO mice are viable (Ferguson, Anagnostaras et al. 2000) and in some tests they are less anxious than the control mice. Their hippocampal memory is impaired and they are hyperactive (Ferguson, Anagnostaras et al. 2000; Ferguson, Herschman et al. 2004). Analyses of these *Syt IV* KO mice lead to the hypothesis that Syt IV is a regulator of short-term synaptic plasticity. In contrast to mice deficient in Syt IV, *Syt I* KO mice are nonviable and die immediately after birth (Geppert, Goda et al. 1994). Overexpression of Syt IV leads to minor transmitter release (Littleton, Serano et al. 1999; Wang, Grishanin et al. 2001), assuming that SytIV is a negative regulator of vesicle exocytosis. Syt IV is widely expressed in the whole brain both in rodents (Vician, Lim et al. 1995) as well as in humans (Ferguson, Chen et al. 2000).

1.3.5. Influence of stress on the expression of SPs

Stress can influence the expression of SPs. Chronic immobilization stress leads to decreased immunoreactivity of synaptobrevin in the hippocampus of rats (Xu, He et al. 2004). After acute or chronic stress the mRNA production of synaptophysin is also reduced in the hippocampus as well as in the cortex of rats (Thome, Pesold et al. 2001). In contrast hereto there is an increased Syt III expression shortly after stress in the hippocampus (Thome, Pesold et al. 2001). Fast and differentiated regulation of SPs is connected to morphological and biochemical as well as behavioural changes which are linked to stress-related disorders like depression and posttraumatic stress disorder (PTSD) and to altered synaptic plasticity (Gage 2000; Sapolsky 2000).

1.4. Stress and the stress response

Stress can be defined as the body's non-specific answer to various stress factors which unbalance the organism's homeostasis. During mental stress the discrepancy between perceived circumstances and expected circumstances leads to compensatory stress responses. The interaction of stress reactions and emotional behaviour is coordinated by the limbic system. The limbic system contains the amygdala, the hypothalamus and the thalamus, the Substantia grisea centralis and the septo-hippocampal system as well as sensory cortical areas. In general: the bigger the transmitter's role for the central nervous system (CNS), the smaller its role for stress. 5-HT together with dopamine is responsible for the adequate reaction according to the specific situation, including behaviour pattern, control of stress reaction, intensity and emotion (Chaouloff, Berton et al. 1999). Above all, glucocorticoids released during stress counteract with 5-HT (Chaouloff 2000).

During stress the HPA axis (Figure 1.4) is activated, hence ACTH is released and the release of glucocorticoids increases. This is necessary to get enough energy and attention to cope with the stress (Pich, Koob et al. 1993). Anxiety (Pich, Heinrichs et al. 1993) and depression (Nemeroff 1996; Holsboer 2001) are implicated to be concerned with increased HPA activity. In rats repeated social stress leads to increased corticosterone levels (Keeney, Hogg et al. 2001). Acute stress also leads to higher 5-HT concentration in the extracellular space (Amat, Matus-Amat et al. 1998). In rats the forced swim test leads to a rise of 5-HT in the striatum and frontal cortex as well as to a descent in the lateral septum and the amygdala (Kirby, Allen et al. 1995). These differences lead to the assumption that stress influences different areas of the CNS in different ways and that these differences reflect the changed synthesis, release and turnover of 5-HT during stress (Kirby, Kreiss et al. 1995). Stress also results in a changed expression and binding affinity of 5-HT receptors in the hippocampus (Holmes, French et al. 1995; McKittrick, Blanchard et al. 1995; Flugge, Kramer et al. 1998) and it leads to morphological changes in several brain regions as reviewed by McEwen (McEwen 1999).

Increased HPA activity as well as increased cortisol level in the blood could precede depression. One reason for this could be that the decrease of glucocorticoid receptor sensitivity leads to an increased HPA activity as it causes a diminished negative feedback of corticotropin releasing hormone (CRH) production which in turn results in a superelevated CRH concentration leading to anxiety and depressive behaviour (Holsboer 2001).

Additionally, there is an interaction between the HPA axis and the hypothalamic-pituitary-gonadal (HPG) axis. In principle both axes inhibit each other: stress suppresses sexuality and the other way round sexual activity can inhibit the HPA axis to a certain extent (Rensing, Koch et al. 2006).

In the present study the influence of gender as well as the influence of acute stress on the expression of CRH, its receptors (Chapter 1.4.2. and 1.4.3.) and the expression of IEGs (Chapter 1.5.) was examined to elucidate the connection of the HPA axis and the HPG axis.

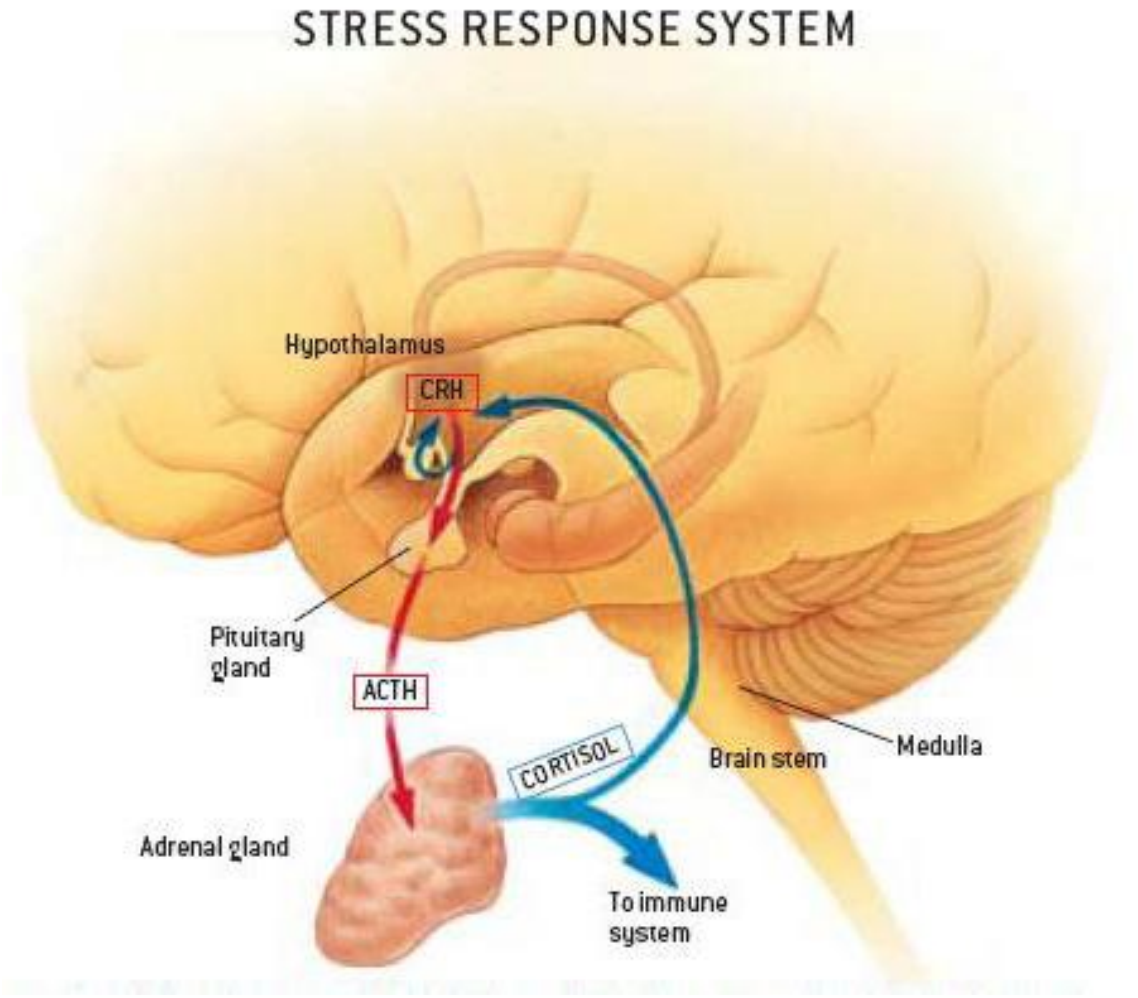


Figure 1.4: The HPA axis forms a feedback loop during stress response. In consequence of stress and impaired homeostasis of the organism CRH is released from the hypothalamus and brings the stress signal via ACTH and the bloodstream to the adrenal gland. ACTH makes sure that cortisol (in mice: corticosterone) is released into the bloodstream. Cortisol is distributed via bloodstream and hence gets back into the brain via the blood brain barrier. Here it acts as a feedback modulator and stops it's own synthesis by stopping CRH release via the glucocorticoid receptors.

(Source: http://speakingoffaith.publicradio.org/programs/2008/stress/images/stressresponse.jpg)

1.4.1. The sympathetic adreno-medullary (SAM) axis and the HPA axis

At first stress results in the quick activation of the SAM axis. After ACTH is released by the hypothalamus it triggers the release of adrenaline, noradrenaline and neuropeptides by the adrenal medulla. There is some delay in the activation of the HPA axis as compared to the activation time of the SAM axis. The most important signal in both axes is the CRH, a peptid consisting of 41 amino acids which is released by CRH positive neurons in the hypothalamus, the limbic system, brainstem and spinal cord (Drolet and Rivest 2001). Hypophysiotropic CRH positive neurons are mostly found in the paraventricular nucleus of the hypothalamus where CRH is synthesized by parvicellular neurons and to a smaller extent also in magnocellular neurons. Axons of these CRH positive cells project to the external zone of the medial eminence and release CRH into the blood which subsequently stimulates the release of ACTH. But there are also abundant CRH-positive fibres in the paraventricular nucleus of the hypothalamus which project into the brainstem. Non-hypophysiotropic CRH neurons can be found primarily in limbic structures, such as the amygdala and locus coeruleus, and they are involved in the processing of sensory information as well as in the regulation of the autonomic nervous system as reviewed by Papadimitriou and Priftis (Papadimitriou and Priftis 2009).

1.4.2. CRH and its receptors

There are two transmembrane receptors CRH can bind to: CRH receptor 1 (CRHR1) and CRH receptor 2 (CRHR2), both encoded by different genes (Chen, Lewis et al. 1993; Lovenberg, Liaw et al. 1995). Stress signals arrive via these receptors at CRH neurons. The signal is transduced to CRH-positive neurons via serotonergic and cholinergic fibers and results in increased expression of CRH and CRHR1. There is a positive feedback loop modulated by CRHR1 by which CRH increases its own synthesis (Drolet and Rivest 2001). But the synthesis of CRH can also be stimulated by angiotensin II, neuropeptid Y, interleukins 1, 2 and 6 as well as the tumor necrosis factor α (TNF- α). Synthesis of CRH is reduced by a negative feedback loop in which cortisol in humans and corticosterone in mice as well as ACTH affect CRH positive neurons and inhibit further expression of CRH. Also opioids act as negative signals.

Both CRH receptors are G-protein coupled receptors and activate adenylate cyclase (Dautzenberg, Kilpatrick et al. 2001). While CRHR1 is mostly found in the CNS, CRHR2 can be found in the periphery for the most part. In the brain CRH influences behaviour as well as emotions. Via CRHR1 it induces anxiety in monkeys (Kellendonk, Gass et al. 2002) as well as despair behaviour, increased attention, alertness and loss of appetite, but it also suppresses libido, slow-wave sleep and social interaction. Depressive patients often show increased CRH level in their cerebrospinal fluid which makes CRH an

interesting target for the development of new antidepressants (Banki, Karmacsi et al. 1992; Geracioti, Loosen et al. 1997). Furthermore CRHR1 seems to prevent stress-induced learning whereas CRHR2 seems to facilitate stress induced learning (Hashimoto, Makino et al. 2001). Compared to CRHR1, CRHR2 also seems to trigger antagonistic effects like inhibition of anxiety.

The CRH system involves not only CRH and its receptors but also three CRH-like peptides: the urocortins I, II and III as well as the CRH-binding protein (CRH-BP). Urocortins (Ucns) are peptides homologous to CRH but encoded by different genes which are expressed in CNS regions involved in the control of the autonomous nervous system. Ucns can activate both CRH receptors, but they bind CRHR1 as well as CRHR2 with greater affinity than CRH does (Vaughan, Donaldson et al. 1995). As urocortins have an 41-fold higher affinity to CRHR2 than CRH, this suggests them to be the main ligands of this receptor. Interestingly, peripheral administration of urocortin (Ucn) decreases food intake but increases energy expenditure (Asakawa, Inui et al. 1999; Hope, Turnbull et al. 2000; Kinney, Scruggs et al. 2001). Hence in the brain Ucns may be responsible for the effects of stress on appetite. In the periphery Ucn II is expressed in the heart while Ucn III is expressed in various organs like the skin, lung, gastrointestinal tract, heart and kidney. Effects of Ucn II and III are peripheral vascular dilatation, augmentation of cardiac output and release of atrial natriuretic peptide. These effects are similar to CRH-mediated actions by the CRHR2.

The role of CRH-BP is not yet fully understood. It was first identified in 1991 (Potter, Behan et al. 1991) and it is able to bind CRH to attenuate its action at CRH receptors. As its expression is stimulated by stress, CRH and glucocorticoids (Cortright, Goosens et al. 1997; McClennen, Cortright et al. 1998) it could be that CRH-BP functions as a feedforward inhibitor in the HPA axis and limits the duration of HPA activation. The expression of CRH-BP is higher in females than in males and it is stimulated by estrogen (Levine 2002).

1.4.3. Gender differences in CRH expression

Gender differences could also be shown regarding CRH expression: female rats show increased CRH synthesis in preoestrus triggered by estrogen. It seems to be that estrogens enhance the activity of the HPA axis (Burgess and Handa 1992; Handa, Burgess et al. 1994; Lund, Munson et al. 2004; Weiser and Handa 2009) and androgens inhibit the activity of the HPA axis (Gaskin and Kitay 1970; Handa, Burgess et al. 1994; Weiser and Handa 2009). But also in humans estrogen triggers the CRH expression (Vamvakopoulos and Chrousos 1993). In contrast to this, testosterone inhibits the synthesis of CRH in male rats (Almeida, Hassan et al. 1992; Bingaman, Magnuson et al. 1994). Furthermore CRH inhibits the gonadotropin releasing hormone (GnRH) and vice versa glucocorticoids

inhibit the synthesis of gonadal hormones (Levine 2002). It is well known that basal and stress-induced hormone secretions in the HPA axis are generally higher in female mice and rats than in males (Gaskin and Kitay 1970; Brett, Chong et al. 1983).

In general it was shown that females exhibit a more robust HPA response than males after stress (Lund, Munson et al. 2004). The exact mechanisms of how gonadal hormones act on HPA axis have not fully been solved yet. The influence of gender on the CRH system is one of the points investigated in the present study.

1.5. Immediate early genes (IEGs)

Genes which are called IEGs belong to a gene family which is transcribed very quickly in response to various cellular stimuli e.g. growth factors (Robertson 1992). Most of the proteins coded by IEGs are transcription factors like cellular FBJ murine osteosarcoma (c-Fos) and Fos-related antigen 2 (Fra-2) which both are rapidly expressed after various cellular simuli e.g. stress. The general structural organization of the c-Fos gene and the Fra-2 gene is very similar (Nishina, Sato et al. 1990; Yoshida, Kawamura et al. 1993; Foletta, Sonobe et al. 1994). However, Fra-2 gene expression patterns and protein function seem to differ from c-Fos. It seems to be that Fra-2 expression may be especially important for long-term neural and endocrine adaptation to repeated stress (Sabban, Hebert et al. 2004; Hebert, Serova et al. 2005), whereas c-Fos is expressed after short stress (Kovacs 1998). While very short Stress (3 min) results in phosphorylation of CREB-1 and Jun, medium (acute) stress (30-120 min) leads to *de novo* synthesis of c-Fos and the early growth response factor 1 (EGR1). Long lasting (chronic) stress causes activation of both EGR1 and Fra-2, (Rensing, Koch et al. 2006).

This study focuses on the expression changes of c-Fos and Fra-2 after acute stress in *5-Htt* KO mice and WT mice of both genders.

1.5.1. The role of c-Fos in gene transcription

As a cellular proto-oncogene, c-Fos has many different effects on gene transcription. Consisting of a leucine-zipper DNA binding domain and a transactivation domain at the C-terminus, it is upregulated in response to various extracellular stimuli e.g. neurotropic factors, neurotransmitters, depolarization and increase of Ca^{2+} influx and elevation of intracellular/intranuclear Ca^{2+} [for review: (Kovacs 1998)]. For this reason, c-Fos expression can be used as a marker for neuronal activity. Neuronal activity as well as stability can be changed by phosphorylation of MAPK, PKA, PKS or CDC2. Together with the transcription factor c-Jun, c-Fos forms the so-called activator protein 1 (AP-1) complex which is a transcription factor and which leads to the transcription of many genes involved in various cellular processes including differentiation, proliferation and apoptosis. Basal expression levels of c-Fos are very low (Hughes, Young et al. 1998) but after stress c-Fos becomes upregulated very quickly (Bullitt

1990). So it is a widely and long used strategy to map neuronal circuits underlying stress-related pathways in the central nervous system by investigating the expression of c-Fos protein (Kovacs 1998).

1.5.2. Stress-dependent expression of c-Fos and Fra-2

It was shown that induction of c-Fos as well as of Fra-2 varies in speed after stress. In the PC01 cells of a cell-culture as well as in several rodent brain regions in vivo c-Fos mRNA is induced within a couple of minutes after an acute challenge, while the expression peaks are formed between half an hour and 1 hour after stress (Kovacs 1998). The highest level of c-Fos protein occurs between 0 and 2 h after the onset of expression before it gradually disappears from the cell nucleus within some hours (Chan, Brown et al. 1993; Imaki, Shibasaki et al. 1993; Ding, Qin et al. 1994; Ikeda, Nakajima et al. 1994). In rats it was shown that the expression of both genes is sensitive to the duration of stress, but that this sensitivity varies with the brain region. It could be shown that the peak of Fra-2 gene induction in the prefrontal cortex and in the lateral septum was delayed (60 min) after a restraint onset, compared to c-Fos (15 min), whereas in the prefrontal cortex, Fra-2 mRNA increased within 15 min of restraint (Weinberg, Girotti et al. 2007).

1.5.3. Gender differences concerning the expression of IEGs

But there are also gender differences as the stress-induced IEG activation in the CNS is mostly suppressed on proestrus (Figueiredo, Dolgas et al. 2002). In rats a unilateral injection of formalin induced bilateral c-Fos expression in the hippocampus, but the number of labeled neurons was twice as high in females as in males. In the septum, an injection of formalin as well as restraint stress increased c-Fos, but this increase tended to be greater in males than females (Aloisi, Zimmermann et al. 1997). In rats, too, it could be shown that, induced by restraint stress, c-Fos expression was increased by female hormones (oestradiol benzoate) in the paraventricular nucleus of the hypothalamus and it was decreased by male hormones (dihydrotestosterone propionate) in the same region (Lund, Munson et al. 2004). Concerning neuronal localization, c-Fos is expressed in neurons of different types e.g. in inhibitory interneurons and also in excitatory pyramidal neurons but not in astrocytes (Staiger, Masanneck et al. 2002).

1.6. Changes in neuronal morphology after stress

Another way of examining the results of stress is to analyse dendritic morphology and the influence of stress upon the shape of neurons such as pyramidal neurons in different brain areas involved in processing of stressful stimuli. This investigation was focused on the morphology of apical dendrites of layer V pyramidal neurons in infralimbic cortex (IL) and cingulated cortex (CG) and on hippocampal cornu ammonis region 1 (CA1) pyramidal neurons. Additionally spines were counted on segments of hippocampal neurons and on total apical dendrites in IL. Experiments were carried out on male *5-Htt* KO mice and WT mice with and without social stress exposure to analyse the influence of genetic endowment and environment on neuronal morphology.

1.6.1. The pyramidal neuron

There are different types of neurons in the brain. One of the most important (and mainly widespread) group of excitatoric glutamatergic neurons are pyramidal neurons (Figure 5) which are abundant in the brain of mammals, birds, fish and reptiles but not amphibians (Nieuwenhuys 1994; Elston 2003). Pyramidal neurons were first investigated by Santiago Ramón y Cajal and can be found in brain structures like the cerebral cortex, hippocampus, amygdala and forebrain structures, which are important for highly developed cognitive functions. Typical pyramidal neurons exhibit a triangular cell body and one single axon, descending from the base of the soma, which branches widely building up many synaptic contacts with other neurons. Important for the function of each single pyramidal neuron is its ability to respond to all synaptic inputs, to produce an action potential and thereupon to excite its postsynaptic targets.

In the majority of cases pyramidal neurons have one single apical dendrite or very rarely two apical dendrites e.g. in CA2 region of the guinea pig's hippocampus (Bartesaghi and Ravasi 1999). The apical dendrite always emanates from the apex of the soma while basal dendrites, which can be much more numerous, descend, like the axon, from the base of the soma (Figure 5). Usually basal dendrites are shorter than apical dendrites. But there are exceptions e.g. in the amygdala where differences in length of basal and apical dendrites are not always so pronounced as compared to cortical regions. The apical dendrite ends in a tuft-like ramification called the apical tuft (Figure 1.5 A). Oblique dendritic branches emanate from the main apical branch at various distances from the soma and at various angles. Concerning synaptic input, it is known that the apical tuft of pyramidal neurons receives excitatory synaptic inputs that have presynaptic origins different from those that form synapses on more proximal apical dendrites or basal dendrites (Spruston 2008) .

Synaptic input of a pyramidal neuron is received at the soma, the axon and the dendrites. While soma, dendritic shafts and axon receive inhibitory GABA (γ-aminobutyric acid)-ergic inputs, apical

and basal dendrites receive excitatory synaptic input via the spines (See Chapter 1.6.2). Proximal dendrites usually receive input from the same area or from adjacent areas while the synapses on the apical tuft, which is located more distally from the soma, receive input from more distant regions. But the way in which these different inputs influence the neuronal activity of the cell and control output of the pyramidal neuron has not yet been fully elucidated.

Pyramidal neuron function is modulated by neurotransmitters such as dopamine, serotonin, noradrenaline and acetylcholine. These and other neuromodulators influence and change various features of pyramidal neuron function by affecting channels which are gated by neurotransmitters, voltage or intracellular messengers and consequently change cellular functions e.g. synaptic strength or firing mode and rates. But neuromodulators can also act on spine development e.g. serotonin is able to regulate the spine density in the hippocampus during development but also during adulthood (Feria-Velasco, del Angel et al. 2002). It is supposed that the 5-HT2A-receptor is involved in this process. This receptor is located especially on the pyramidal neurons (Jones, Srivastava et al. 2009).

1.6.2. Dendritic spines

Dendritic spines are postsynaptic sites for most excitatory glutamatergic synapses and are located on basal and apical dendrites of pyramidal neurons but not on the soma and the axon. In general they look like tiny mushrooms with small sphere-shaped heads on a thin neck. At least one synapse is located on the top of each spine head. The whole number of spines on a neuron (or dendrite) can be used to estimate the minimum number of excitatory synapses on a neuron (or dendrite), as there is mostly one single synapse on one spine. But it is also possible that spines contain more than one synapse. The total number of spines varies according to different brain regions, species and different times. It can vary within a single neuron, e.g. after stress. The exact function of the synaptic spines is not clearly understood, but it seems to be that they increase the dendritic suface area in order to optimize the packing of a large number of synapses onto a given length of dendrite. Alternatively, they might serve as biochemical compartments that restrict the diffusion of important molecules away from the synapse. Spines might also have a role in regulating the electrical properties of the neuron as their thin neck forms an electrical resistance (Spruston 2008). Moreover, spines can be considered as cellular compartmens which create a limited environment for effects of calcium and second messengers to strengthen the signals (Koch and Zador 1993). Thus the narrow spine neck which separates the spine head from the rest of the dendrite is very important. Because the head of the spine is very small, a low calcium influx is sufficient to cause an increased calcium concentration in the head of the spine. As changes in the calcium concentration in spines last quite long, they could help to induce long-term potentiation (LTP) which is a phenomen considered to be one of the major

cellular mechanisms that underlie learning and memory (Bliss and Richter-Levin 1993; Cooke, Wu et al. 2006). The size and shape of a synaptic spine is manifold and varies strongly (Harris, Jensen et al. 1992) not only within the neuron population but also within one same neuron. There are four basic forms of spines classified namely “thin", "stubby", "mushroom", and "branched". But there are many different shapes in between these categories. The spines change significantly in shape, volume, and number within a short space of time and hence play a role in synaptic plasticity. External factors which lead to synaptic activity on spines can induce differences in the number of spines as well as in their shape and size. Shape and size are important for the fine tuning of spines as small calcium stores (Segal, Korkotian et al. 2000). It has been shown that increases in the calcium concentration in a spine leads to its shrinking but that a decrease of calcium concentration within a spine lead to its growth (Segal, Korkotian et al. 2000). Neuromodulators can also change the number and shape of spines, but this role of neuromodulators, in particular of serotonin, in spine remodeling is not well understood yet (Jones, Srivastava et al. 2009).

1.6.3. Neuronal morphology and stress

As stress results in various morphological changes in the brain (McEwen 1999; Izquierdo, Wellman et al. 2006; Shansky, Hamo et al. 2009) these changes in the dendritic composition of neurons can represent adaptive mechanisms to stressful circumstances. In the majority of cases the morphology of pyramidal neurons was analyzed in response to stressful stimuli or after treatment with glucocorticoids. These pyramidal neurons are abundant in the cerebral cortex of mammals, birds, fishes and reptiles where they can be found in forebrain structures, including the cerebral cortex, the hippocampus and the amygdala, but not in the olfactory bulb, the striatum, the midbrain, the hindbrain or the spinal cord (Ramon y Cajal, 1995).

Repeated restraint stress causes retraction of apical dendrites in pyramidal cells of the cortical layer II/III in rats’ medial prefrontal cortex (mPFC), or more precisely, in the anterior CG and IL (Radley, Sisti et al. 2004; Izquierdo, Wellman et al. 2006). Unlike enhanced corticosterone levels, stress is necessary but not sufficient on its own to cause morphological changes, because a chronic administration of corticosterone can by itself produce dendritic atrophy in rats’ hippocampal neurons (Gould, Woolley et al. 1990). Chronic longer-term physical and psychological stress leads to a deficit of hippocampal CA1 and CA3 neurons not only in rats but also in primates (Sapolsky, Krey et al. 1985; Kerr, Campbell et al. 1991; Mizoguchi, Kunishita et al. 1992). Additionally chronic corticosterone treatment for 3 weeks causes a strong reorganization of pyramidal neurons in CG of male rats (Wellman 2001). The anterior cingulate cortex is a part of the medial prefrontal cortex (mPFC), which acts as regulator of HPA activity under stressful conditions (Meaney, Aitken et al. 1985; Diorio, Viau

et al. 1993; Brake, Sullivan et al. 2000). Dysfunction of this brain region is associated with PTSD in humans (Shin, Whalen et al. 2001; Pissiota, Frans et al. 2003; Rauch, Shin et al. 2003; Yamasue, Kasai et al. 2003). In addition, inactivation of the IL induces learned helplessness after uncontrollable stress (Amat, Baratta et al. 2005). In IL morphological changes in pyramidal neurons are found in consequence of brief uncontrollable stress (Izquierdo, Wellman et al. 2006).

Also the *5-Htt* genotype seems to play a role in morphological adaptations of neuronal cells (Wellman, Izquierdo et al. 2007). As in this study, attention in the present investigation was focused on the morphology of pyramidal neurons in prefrontal cortical regions and additionally on hippocampal neurons of the CA1 area and their morphological features with and without the influence of semi-chronic social stress triggered by loser experience acquired by the resident-intruder paradigm on three consecutive days. As the resident-intruder paradigm dosen't work on female mice, morphological studies were only done with male mice.

1.7. Goals of this study

In the present study, changes in the expression of SPs were investigated in WT and in *5-Htt* KO mice. It is supposed that SPs are important targets in the medication of psychiatric disorders such as antidepressants which selectively inhibit 5-HT reuptake. The *5-Htt* KO mouse is seen as a mouse model for anxiety disorders in humans and was therefore analyzed to get an insight into the neurochemistry of the brain when it is changed by various psychiatric disturbances.

This study was focused on the expression of three SPs, namely syntaxin 1A (Stx 1A), synaptotagmin I (Syt I) and synaptotagmin IV (Syt IV) and their expression after acute stress in mice of all three *5-Htt* genotypes. Mice of both genders were investigated to reveal the influence of gender on the expression of these SPs in addition to environmental factors such as stress and genetic endowment. The influence of gender as well as the influence of acute stress on the expression of CRH, its receptors and the expression of IEGs was examined to elucidate the connection between the HPA axis and the HPG axis. In the present investigation, attention was focused on the morphology of pyramidal neurons in prefrontal cortical regions of male mice and additionally on hippocampal neurons of the CA1 area and their morphological features with and without the influence of semi-chronic social stress triggered by loser experience due to the resident-intruder paradigm on three consecutive days. The goal of this study was to investigate environmental influences (such as stress) on the expression of the genes involved in stress-coping, stress-response and also in gender-dependent interference with stress. Moreover, neuronal morphology and its stress- and genotype-dependent changes were analyzed to get a picture of stress- and genotype-dependent adaptations of the murine WT brain and the brain of *5-Htt* KO mice.

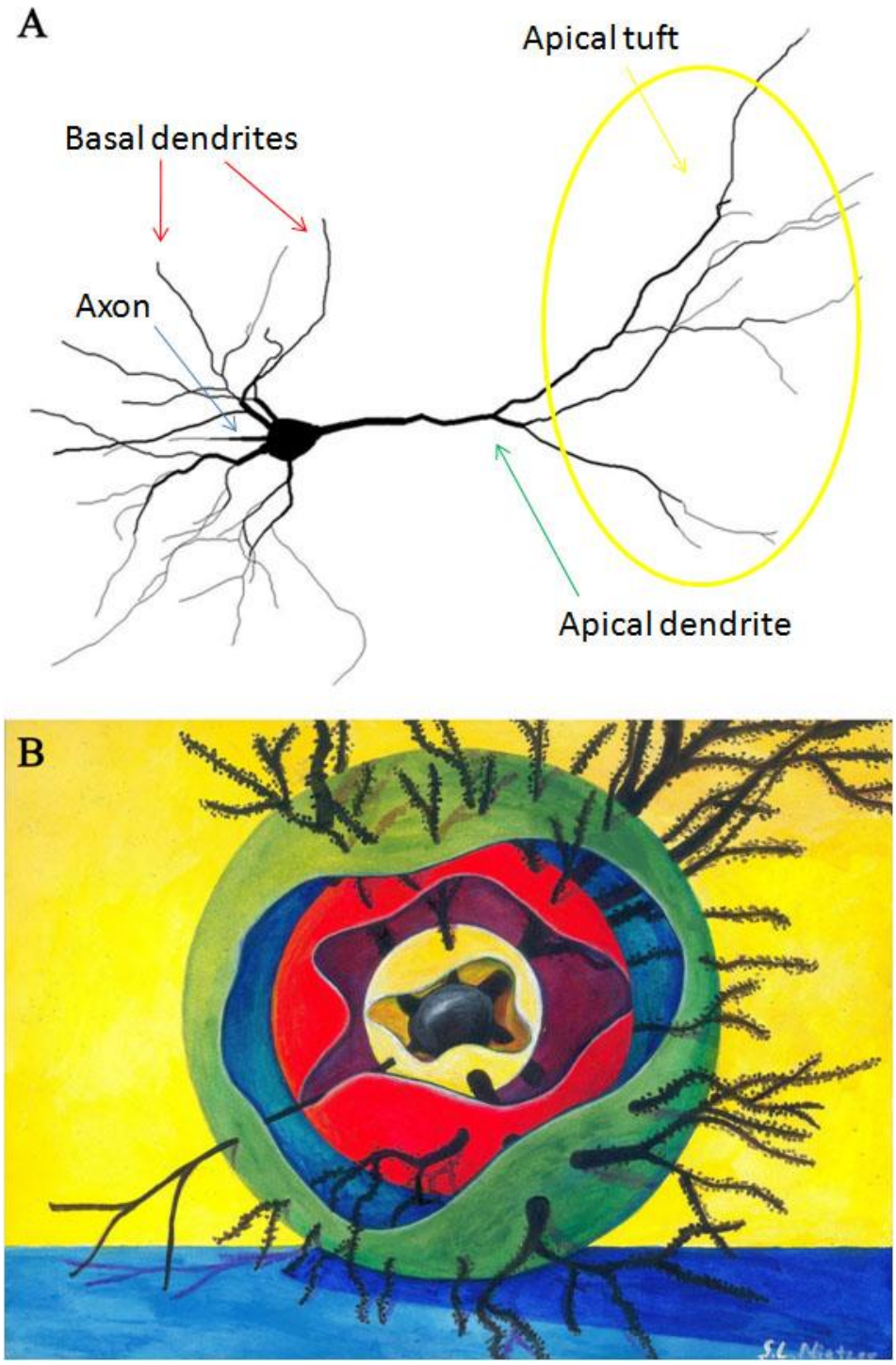


Figure 1.5: Three-dimensional Sholl Analysis of pyramidal neurons. Pyramidal neurons have one big apical dendrite (green arrow in A) and many basal dendrites (red arrows in A). The single axon emanates from the bottom of the soma (blue arrow in A) in opposite direction to the apical dendrite. The apical dendrite ends in a tuft-like ramification called the apical tuft (yellow arrow and yellow ellipse in A). Dendrites are covered with spines where synapses are localized (neuron in B). Three-dimensional Sholl analysis (Sholl 1953) was carried out by putting spheres with increasing radii around the center of the soma (in B schematically shown: smallest sphere in yellow, middle sphere in red, next bigger sphere in green). Sectors between neighbouring spheres were analyzed separately. (© Sarah L. Nietzer)

2. Material and Methods

2.1. Animals

2.1.1. For the gene expression study

Experiments were carried out on male and female *5-Htt* KO (-/-) mice (Bengel, Murphy et al. 1998) with a C57BL/6J genetic background and their *5-Htt* WT (+/+) and heterozygous (HET for the *5-Htt* genotype, +/-) littermates. Animals were housed in a colony room under controlled temperature and humidity conditions, with lights on between 6:00 a.m. and 6:00 p.m., with food and water freely available. All animal protocols have been reviewed and approved of the review boards of the University of Würzburg and of the Government of Unterfranken (Bavaria, Germany), and were in accordance with international guiding principles of the care and use of animals.

2.1.1.1. For *In-situ* hybridization (ISH)

Adult C57BL/6J WT mice were decapitated in isoflurane anesthesia. Brains were dissected and immediately frozen in dry ice-cooled isopentane (AppliChem, Darmstadt, Germany) and tissue was stored at -80°C until being cut in 16 µm thick slices using a cryostat (Microm, HM 500 O).

2.1.1.2. For quantitative real-time PCR and corticosterone measurement

Between 5 and 10 animals (3 to 8 months old) for each gender and genotype were immobilized for 1 hour as illustrated in Figure 2.1 and just as many mice were in each unstressed control group:

Treatment group	n_{mice}
Male WT unstressed	8
Male WT stressed	8
Male HET unstressed	5
Male HET stressed	7
Male KO unstressed	10
Male KO stressed	9
Female WT unstressed	7
Female WT stressed	8
Female HET unstressed	7
Female HET stressed	6
Female KO unstressed	9
Female KO stressed	9

Control animals remained living in their home cage without being exposed to any stressors before being sacrificed. Blood was removed in isoflurane anesthesia (See Chapter 2.5) and brains were removed and frozen in dry ice-cooled isopentane. Brain tissue was stored at -80°C then. For mRNA extraction brain regions were dissected by an experimenter blind to treatment and genotype. The dissected regions were cortical regions, hippocampus, hypothalamus, amygdala and the raphe nuclei containing area.

2.1.1.3. For Nissl staining

Brains of adult mice of *5-Htt* WT genotype were used. Animals were deeply anesthetized with isoflurane and were fixed by perfusion for 10 min using a 4% formaldehyde fixative in 0.1 M phosphate buffer pH 7.4 freshly prepared from paraformaldehyde (PFA; Merck, Darmstadt, Germany). After perfusion brains were dissected and postfixed for 2–3 h in the same fixative at room temperature (RT). Afterwards brains were washed in 0.01 M phosphate buffered saline (PBS; pH 7.4; Cambrex, Wiesbaden, Germany), and either successively infiltrated with 10 and 20% sucrose in PBS, frozen in dry ice-cooled isopentane and stored at -80 °C. Serial 16 µm frontal cryostat (Microm HM 500 O) sections were prepared from the frozen tissue. One series of sections was immediately mounted and Nissl stained for cytoarchitectonic studies.

2.1.3. For the morphological study

Experiments were carried out on adult male mice of a *5-Htt* KO mice breeding line (Bengel, Murphy et al. 1998) backcrossed into a C57BL/6J background for > 10 generations. Mice were bred in pairs of heterozygous KO animals and were raised in the Department of Behavioural Biology (University of Münster, Badestrasse 13, 48149 Münster, Germany). Resulting offspring was of three different genotypes namely wildtype (*5-Htt* +/+), heterozygous KO (*5-Htt* +/-) and homozygous KO (*5-Htt* -/-) mice. After weaning at day 21 ± 1 of age, animals were housed in groups of two to five littermates of the same sex in standard cages and from day 90 ± 15 of age, before beginning of confontations, all animals were housed singly in standard polycarbonate cages (Macrolon cages type III, 38 cm x 22 cm x 15 cm) with *ad libitum* access to food (Altromin 1324, Altromin GmbH, Lage, Germany), water and sawdust as bedding material (Allspan Höveler GmbH & Co.KG, Langenfeld, Germany) as described by (Jansen, Heiming et al. 2010).

Opponents for the generation of loser experiences were males of the strain NMRI, obtained from Harlan Winkelmann GmbH (Borchen, Germany) at an age of 21 days. These mice had to be housed singly from day 40 as they show a high level of intermale aggression (Navarro et al. 1997).

Animals were maintained in a humidity-controlled and temperature-controlled room under a 12 h light/dark cycle (lights on at 7.00 A.M.). All procedures related to maintenance and experimentation were carried out by Jansen et al. and were in accordance with current regulations covering animal experimentation in Germany and the EU (European Communities Council Directive 86/609/EEC) and all experiments were announced to the local authority and were approved of the 'Animal Welfare Officer' of the University of Muenster (reference number: 9.93.2.10.55.07.005).

2.2. Stress treatments

2.2.1. Immobilization stress (acute restraint stress)

This treatment involved simply placing the mice into a 50 ml Falcon Tubes (Greiner BioOne, Frickenhausen, Germany) for 1 h (Figure 2.1), which had one hole for breathing and an additional hole for the tail. Half the respective mice were exposed to this strong type of restraint stress and were subsequently sacrificed. The others were not immobilized and were treated as control group. Male and female mice were stressed at different times. As the keeping of the animals as well as the experimental setup were standardized and as all mice were of the same age at the date of the stress trial, both groups were taken as comparable irrespective of the season.

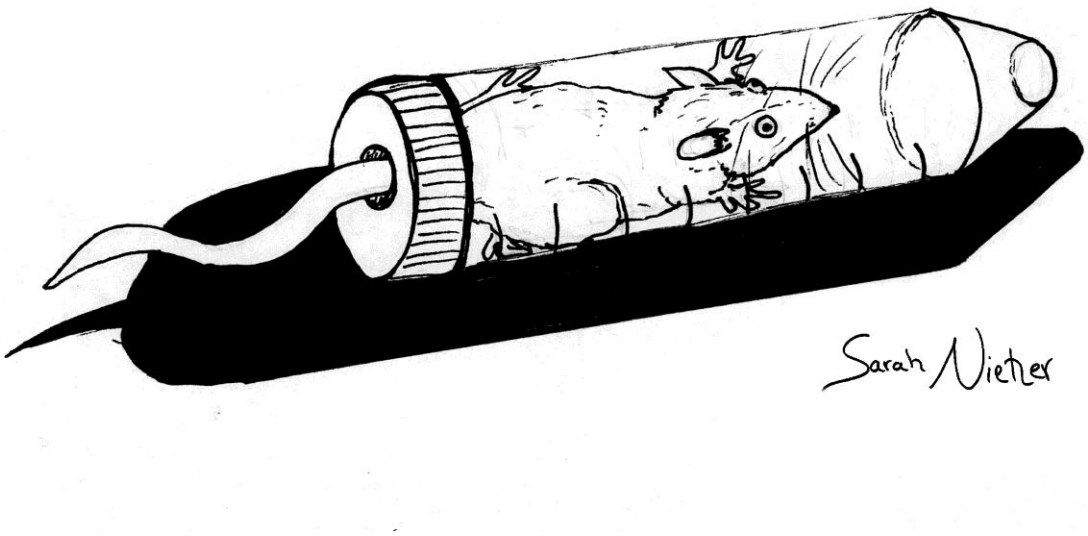


Figure 2.1: Acute immobilization stress procedure. Mice were put into a 50 ml Falcon-Tube with a hole for breathing at the front end of the tube and a hole for the tail in the lid of the tube for 1 hour. Sharp edges were filed until they were blunt so that the mice were not injured. (© Sarah L. Nietzer)

2.2.2. Generation of experimental groups for morphological analysis

Morphological analysis was carried out on animals which were stressed and behaviourally analyzed by Jansen et al. (Jansen, Heiming et al. 2010). We focused our interest on 4 treatment groups: unstressed WT (*5-Htt* +/+) mice (n = 6) as well as unstressed *5-Htt* KO (*5-Htt* -/-) mice (n = 6) served

as control groups. Other WT mice (n = 6) and *5-Htt* KO mice (n = 6) went through the resident-intruder paradigm at days 89 ± 9 to 91 ± 9 (Jansen, Heiming et al. 2010) leading to social stress experience as they had acquired loser experience. In the resident-intruder paradigm the loser mouse was always the intruder who was set into the cage of a very aggressive male NMRI mouse by the experimenter blind to genotype. As male mice defend their territory against the intruder male they start to fight. To prevent mice from severe injury the confrontation was stopped when fighting was too escalated. The test lasted 10 min at the most. Losers had to show at least five loser behavioural patterns in each of the three confrontations (Jansen, Heiming et al. 2010). As the NMRI-mice were very aggressive, the intruder mouse certainly acquired loser experience. Ten (± 1) days after the confrontation these mice were decapitated at the age of 99 ± 9 days and their brains were used for morphological investigations.

2.3. *In-situ* hybridisation (ISH)

2.3.1. Generation of cRNA probes for non-radioactive ISH

Digoxigenin (DIG) labeled cRNA probes were generated by *in vitro*-Transcription from a pCR®II dual promotor vector (Invitrogen™, LIFE TECHNOLOGIES, USA) containing cDNA fragments specific for the respective genes with an average length of 500 bps. Primer sequences are shown in Table 2.1. For transcription, pCR®II-mSyt1 was linearized with XhoI (antisense) or HindIII (sense), pCR®II-mSyt4 was linearized with HindIII (antisense) or XhoI (sense), pCR®II-mStx1A was linearized with HindIII (antisense) or XhoI (sense) and pCR®II-mTph2 was linearized with XhoI (antisense) or SpeI (sense). Phenol-chloroform purified and linearized plamids were ethanol precipitated, and DIG-labeled cRNA was synthesized using the appropriate RNA polymerases (Roche, Mannheim, Germany): SP6 or T7, depending of the orientation of the cDNA insert in the pCR®II-vector. DIG probe synthesis was performed as recommended by the DIG Application Manual for non-radioactive ISH (Roche, Mannheim, Germany). Probes were purified by Phenol-chloroform extraction and precipitated in 0.1 volumes 3 M sodium acetate (pH 5.2) and absolute ethanol (2.5 volumes) before being analyzed on agarose gels (1%).

Name (size in bp)	Sequence 5´ > 3´	Complemementary to nucleotides	GenBank accession No.
mSyt1 (503)	Forward: TGA CCT GCT GCT TCT GTG TC Reverse: TGA TGT CGT GCT TGG AGA AG	443-462 946-927	NM_009306
mSyt4 (600)	Forward: TTA TCC CCA CAT CCA AGA GC Reverse: GCC AGG AAG CTA ACC ATC AC	935-954 1535-1516	NM_009308
mStx1a (697)	Forward: GAG TCC TTT TCC CTC CCA TC Reverse: GAC ACC TGG CTT TCA GAG CAT	980-999 1677-1657	NM_016801

Table 2.1: Sequences of nucleotide primer pairs used for generation of ISH probes.

2.3.2. ISH using non radioacitvely-labeled cRNA probes

All ISH procedures were performed as previously described (Schmitt and Kugler 1999; Schmitt, Asan et al. 2002) and will be described here only in brief. Hybridization was carried out on 16 µm cryostat sections of mouse brains. After fixation for 5 min in freshly prepared cold 4% PFA in 0.1 M PBS and subsequent storage in 100% ethanol for several hours, they were transferred to 2x SSC (sodium saline citrate; Sigma-Aldrich, Munich, Germany) in a graded series of ethanol (100%, 95%, 80%, 70% ethanol) and treated with 0.02 N HCl for 5 min. After washing with 2x SSC, the sections were incubated with freshly prepared 0,25% acetic anhydride in 0.1 M triethanolamine for at least 20 min, washed again with 2x SSC and covered with the prehybridization solution for 1 h at 58°C. The prehybridization solution contained 50% deionized formamide (AppliChem, Darmstadt, Germany), 4x SSC, 1x Denhardt's solution (Sigma-Aldrich, Munich, Germany), 10% dextransulfat and 250 µg/ml denatured Salmon sperm DNA (Sigma-Aldrich, Munich, Germany). Then, sections were incubated with the hybridization solution including prehybridization buffer and DIG-labeled antisense (sense) cRNA (final concentration 10-15 ng/µl) for 16-18 h at 60°C.

Posthybridization washes were done stepwise with 2x SSC (at room temperature, RT), 50 % formamide in 2x SSC (at 60°C), and then again with 2x SSC (RT). To remove unhybridized single stranded RNAs, the sections were treated with 40 µg/ml ribonuclease (RNase) A (50 Kunitz-units/mg, Boehringer) in a solution containing 500 mM NaCl, 10 mM Tris-HCl (pH 8.0), and with 1 mM EDTA at

37°C for 30 min. After several washes with the same buffer without RNase A at RT, the sections were incubated with in this buffer at 60°C for 30 min and rinsed in 1x Tris buffered saline (TBS). cRNA probes were visualized with a sheep anti-DIG alkaline phosphatase (aP) conjugated antibody (1.5 U/ml) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 4-nitro-blue-tetracolium-chloride (NBT) as the substrate of the aP provided by the Nucleic Acid Detection Kit (Roche, Mannheim, Germany). Substitution of the antisense cRNA probes by an equivalent amount of labeled sense cRNA or RNase A treatment before hybridization led to a lack of staining. From these findings it was concluded that the antisense probes were specific and DIG detections did not create labeling artefacts.

2.4. Nissl staining

Nissl staining is used to stain DNA as well as RNA (mainly extranuclear RNA) in tissue slices. As DNA and RNA are acid structures, a basic thiazin pigment like Cresyl violet can be used to stain these structures in blue. These nucleic acids which are stained are mostly to be found in the form of free ribosomes or ribosomes bound to the endoplasmatic reticulum. There is no endoplasmatic reticulum in the plasma of axons. Therefore only the soma and the dendrites of a neuron are stained by Nissl staining. Tissue slices on glass slides had to be dried for 30 min before they were put into a solution of Cresyl violet (Merck, Darmstadt, Germany) for 5 min. Afterwards the tissue slices were washed in doubly distilled (dd) H2O for 2 min. Excessive staining was removed by incubation of tissue slices for 2 min in 96% ethanol to which some drops of acetic acid had been added. Cell bodies achromatize more slowly than fiber bundles and differ clearly from background tissue. In a final step the tissue slices were washed with 100% ethanol for 2 min and afterwards they were washed twice for 5 min in Xylol (Merck, Darmstadt, Germany) before being covered using the embedding medium Vitro-Clud (R. Langenbrinck, Emmendingen, Germany).

2.5. Plasma corticosterone measurement

For the measurement of plasma corticosterone levels, a total of 200 µl heart blood was collected from each stressed and each unstressed mouse immediately after being sacrificed by CO2 intoxication. The blood was collected in pre-chilled tubes containing EDTA di-potassium salt (KABE Labortechnik, Nürnberg-Eisenroth, Germany). Subsequently, the blood samples were centrifuged at 3500 rpm for 10 min (4°C), and plasma aliquots were taken and stored at -80°C until assayed. Plasma corticosterone concentrations were all (at the same time) determined by RIA in duplicates using 125J corticosterone kits (ICN Biomedicals Inc., CA, USA). Results were given as ng/ml.

2.6. Quantitative real-time (qRT) polymerase chain reaction (PCR)

2.6.1. RNA extraction and cDNA synthesis

The total RNA from different murine brain regions was isolated using an optimized guanidinium isothiocyanate phenol extraction method (Chomczynski and Sacchi 1987) combined with the PeqGOLD RNAPureTM system (PeqLab Biotechnology GmbH, Erlangen, Germany) and went through a further cleansing procedure using the RNeasy Mini Kit with DNase-I treatment on a column (Qiagen Inc. Valencia, CA, USA) to ensure that no contaminating genomic DNA was present. First strand cDNA was then synthesized from 500 ng total RNA with a random hexamer & oligo-dT primer mix using the iScript™ cDNA Synthesis Kit (Bio-Rad, Munich, Germany) and was subsequently diluted 1:5 with water free of RNase and DNase.

2.6.2. qRT-PCR

QRT-PCR was performed using the iCycler iQ™ Real-time PCR Detection System CFX384 (Bio-Rad, Munich, Germany) with the gene specific pre-designed primer assays QuantiTectTM Primer Assay from Qiagen (Valencia, CA, USA) in the presence of SYBR Green. As recommended by the manufacturer, each PCR reaction was performed in duplicate with a final volume of 10 µl and contained either 1µl cDNA template or 1µl of differently diluted cDNA amplicons (for the standard curve), 12,5 µl 2x QuantiTectTM SYBR Green PCR Master Mix from the QuantiTectTM SYBR® Green PCR Kit (Qiagen Inc., Valencia, CA, USA) and 2,5 µl QuantiTectTM Primer Assay including forward and reverse primers (Qiagen Inc., Valencia, CA, USA). We used different QuantiTectTM Primer Assays for the gene expression study of three genes of interest (See Table 2.2).

After an initial denaturation for 15 min at 95°C to activate HotStarTaq DNA Polymerase (included in 2x QuantiTectTM SYBR Green PCR Master Mix), 40 cycles of denaturation at 94°C for 15 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec were performed. To confirm amplification specificity, the resulting PCR products were subjected to a melting curve analysis by increasing the temperature in 0.5°C steps (15 sec each) from 70 °C to 95 °C. In addition, the identity of the amplified PCR products was verified by cloning PCR products into vectors and following DNA sequencing. Standard curves were generated from six repeated ten-fold serial dilutions of cDNA amplicons.

Gene name	**Symbol**	**GenBank accession No.**	**QuantiTect primer assay**	**Product size (bp)**
18s Ribosomal	*Rn18S*	X00686	QT01036875	149
β-Actin	*ACTB*	NM_007393	QT01136772	149
β 2-Microglobulin	*B2m*	NM_009735	QT01149547	143
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	NM_01001303	QT01658692	100
Ubiquitin C	*Ubc*	NM_019639	QT00245189	75
acidic ribosomal phosphoprotein P0	*Arbp/Rplp0*	NM_007475	QT00249375	125
Synaptotagmin-1	SytI	NM_009306	QT00167300	86
Synaptotagmin-4	SytIV	NM_009308	QT00147154	84
Syntaxin 1a	Stx1a	NM_016801	QT00101514	109
FBJ osteosarcoma oncogene	cFOS	NM_010234	QT00147308	81
Corticotropin releasing hormone	CRH	NM_295769	QT01055789	183
Corticotropin releasing hormone receptor 1	CRHR1	NM_007762	QT00106232	117
Corticotropin releasing hormone receptor 2	CRHR2	NM_009953	QT00151543	75

Table 2.2: Quantitative real-time RT-PCR primer assays (Qiagen Inc., Valencia, CA, USA). All products were detected by FastSYBR. *Cursive* genes were used as housekeeping genes.

2.6.3. Data preparation and normalization

Following the RT-PCR runs, the threshold cycle (Ct) values for each of the genes given by the iCycler iQ™ Real-time PCR Detection System (BioRad, Munich, Germany) were converted into relative quantities using the algorithm described by Vandesompele et al. (Vandesompele, De Preter et al. 2002). The Q-Value of each Ct-value was obtained as follows:

$$\text{Q-Value} = (\text{PCR-Efficiency})^{([\text{Minimum(Ct-values)}] - \text{Ct-value})}$$

In doing so a PCR efficiency of 100% was calculated as the value 2. To correct for differences in both RNA quality and quantity between cDNA samples, normalization of obtained expression data was performed. For this, relative expression quantities of four to five different reference genes were estimated and the gene expression stability (M) value for each gene was calculated (Vandesompele, De Preter et al. 2002). Normalization factors based on the three best-performing reference genes were calculated using GeNorm algorithm as described by Vandesompele et al. (Vandesompele, De Preter et al. 2002) and were included in estimating the relative expression levels of the genes of interest by dividing each Q-value by the correlative normalization factor.

2.7 Data analysis of the acute stress study

A statistical evaluation was carried out by using the Kruskal-Wallis-test over all groups (**For corticosterone measurements:** *5-Htt* WT unstressed, *5-Htt* WT stressed, *5-Htt* HET unstressed, *5-Htt* HET stressed, *5-Htt* KO unstressed, *5-Htt* KO stressed; **For evaluation of qRT-PCR:** *5-Htt* WT unstressed, *5-Htt* WT stressed, *5-Htt* KO unstressed, *5-Htt* KO stressed) and by using post-hoc Wilcoxon rank sum tests (unpaired) for the evaluation of differences between two groups in case the p-value of Kruskal-Wallis test was lower than 0.1 (which demonstrates a statistically significant group effect) . As many tests were performed with these data (12 tests for qRT-PCR), the level of significance of post-hoc Wilcoxon rank sum tests was adjusted using Bonferroni correction ($p < 0.0021$ for corticosterone measurements and $p < 0.0042$ for qRT-PCR). Statistical analysis was done using R (http://cran.r-project.org/) (version 2.10.0).

2.8. Morphological analysis

2.8.1. Golgi-Cox staining

2.8.1.1. Tissue preparation

Neurons were stained using a modified Golgi-Cox impregnation method (Glaser and Van der Loos 1981) by which approximately 5% of all cells in the brain were stained in black by chance. Whole mouse brains were enveloped in gauze bandage immediately after preparation and completely immersed in Golgi solution (Glaser and Van der Loos 1981). This solution was prepared from 3 stock solutions A, B and C: A is a 5% solution of potassium dichromate ($K_2Cr_2O_7$, Merck, Darmstadt, Germany) in distilled water; B, a 5% solution of mercuric chloride (sublimate, $HgCl_2$, Roth, Karlsruhe, Germany) distilled water; C, a 5% solution of potassium chromate (K_2CrO_4, J. T. Baker, Griesheim, Germany) in distilled water. (i) 5 volume parts of A are mixed with 5 idem of B; (ii) then 4 parts of C were diluted in 10 idem of distilled water; (iii) the AB mixture was poured slowly into the C dilution under continuous stirring with a glass rod. The solution had to be stored in a glass stoppered bottle. Within 5 days a dark reddish precipitate was formed, part of which was on the bottom of the bottle, part of which floated on the surface of the solution. Only fluid free of precipitate was used (Glaser and Van der Loos 1981). This solution was exchanged after 48 hours. The tissue had to be protected from light, temperatures < 10°C as well as from the contact of metal of any kind during impregnation. After 30 days of impregnation the brains were washed 3 x with distilled water (overall washing time: 10 min) and subsequently dehydrated in a graded series of ethanol: 50% for 1,5 h, 70% for 5 hours, 96% for 4 hours, 96% over night, 100% for 2 hours and again 100% for 2-2.5 hours. Dehydration was done at 4°C avoiding light. Afterwards, the brains were put into a 1:2 mixture of ether:ethanol (4 hours) and transferred to 2% celloidin (for 1 day), 4% celloidin (for 3 days) and 8% celloidin (for 4 days). In a final step they were dried in an exsiccator, cut into 150 µm thick slices using a sliding microtome (Reichert-Jung Hn 40) and mounted on glass microscopic slides (R. Langenbrinck, Emmendingen, Germany) using Vitro-Clud (R. Langenbrinck, Emmendingen, Germany).

2.8.1.2. Neuron reconstruction

In cortical areas (IL, CG) only pyramidal neurons in an inner layer with a soma lying between 360 µm and 500 µm below the surface (measured vertically up from the soma along the apical dendrite to the surface of the brain) were chosen for reconstruction. There was no significant correlation between the depth of the neuron and the treatment group. All brain regions were identified according to the mouse brain atlas of Franklin and Paxinos (Franklin and Paxinos, 1997).

In the hippocampus pyramidal neurons of the CA1 area were reconstructed which were defined by their pyramid-shaped soma and their single apical dendrite.

For morphological quantification in each investigated brain region, only complete-looking neurons whose dendrites had as few as possible crossings with dendrites from other neurons were chosen. Only neurons located in the middle part of the sections (z-axis) were reconstructed because of truncated dendrites near the surface of the slice.

In CG at least 5 pyramidal neurons were drawn per animal while in IL only 2 to 5 pyramidal neurons per animal could be reconstructed. As this region is very small, the amount of well-constructable neurons was very low. In the hippocampus 4 CA1 pyramidal neurons were reconstructed per animal. As stained neurons often lie tightly together in columns only a few stained neurons which were situated solitarily could be used for reconstruction.

Neurons were drawn at 100 x 1600 magnification using the Neurolucida microscopy system (Microbrightfield, Inc., Willsiton, VT, USA) with an Olympus BX51 microscope (Olympus, Hamburg, Germany) and an experimenter blind to genotype and stress condition and were evaluated with the Neurolucida Explorer (Microbrightfield, Inc., Willsiton, VT, USA). Additionally, spines were counted on the total apical dendrites of IL pyramidal neurons as well as on single segments of apical dendrites of CA1 pyramidal neurons of the hippocampus. Spines were defined by their mushroom-like head and their thin neck according to Peters and Kaiserman-Abramof (1970). Only protrusions perpendicular to the dendritic shaft and possessing a clear neck and bulbous head were counted. At first, the following parameters were evaluated: total length of apical dendrites and mean length of basal dendrites, total number of branch points, total branch packing (total number of branch points/total dendritic length), total spine number (= quantity of visible spines) of IL apical dendrites as well as total spine densities (= quantity of visible spines/whole dendritic length) of IL apical dendrites.

Additionally, for hippocampal CA1 pyramidal neurons the mean spine density of 3 segments per neuron was calculated. Moreover, to analyse different sectors of dendrites separatly, we used a three dimensional version of Sholl analysis (Sholl 1953) with radii of concentric spheres increased by 20 µm per sphere around the center of the soma. We looked at the number of points where dendrites crossed the surfaces of the spheres around the soma. We also looked at the length of dendritic material between the surfaces of spheres with neighbouring radii and, for IL, the number of spines between the surfaces of these spheres was counted and spine densities in these spaces were calculated (Figure 2.2).

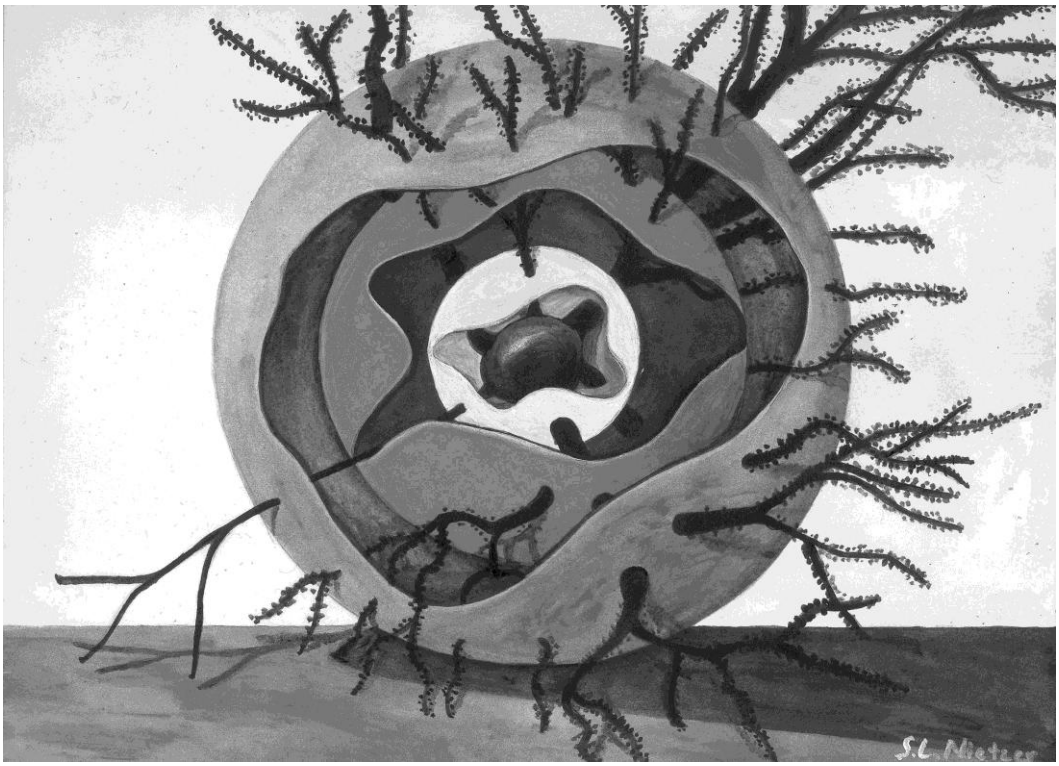


Figure 2.2: A three-dimensional version of the Sholl analysis was carried out on pyramidal neurons. Spheres of evenly distributed radii were put around the middle of the soma. Sectors between neighbouring spheres form equispaced compartments over the whole neuron. (© Sarah L. Nietzer)

2.8.2. The evaluation of morphological data

2.8.2.1. Generation of final values

For each animal the arithmetic mean was calculated for each investigated neuron parameter. Mean values were then analyzed with non-parametric statistical evaluation methods as described in Chapter 2.8.

2.8.2.2. Dendrogram analysis of apical dendrites in CG

Each dendritic tree can be represented by a two-dimensional single rooted tree (Figure 2.3). This tree can be divided in parts of 100 µm length. The longest continuous branch was simply defined as the main branch. Other branches emanating from the main branch were counted within each part of 100 µm length as described in Figure 2.3.

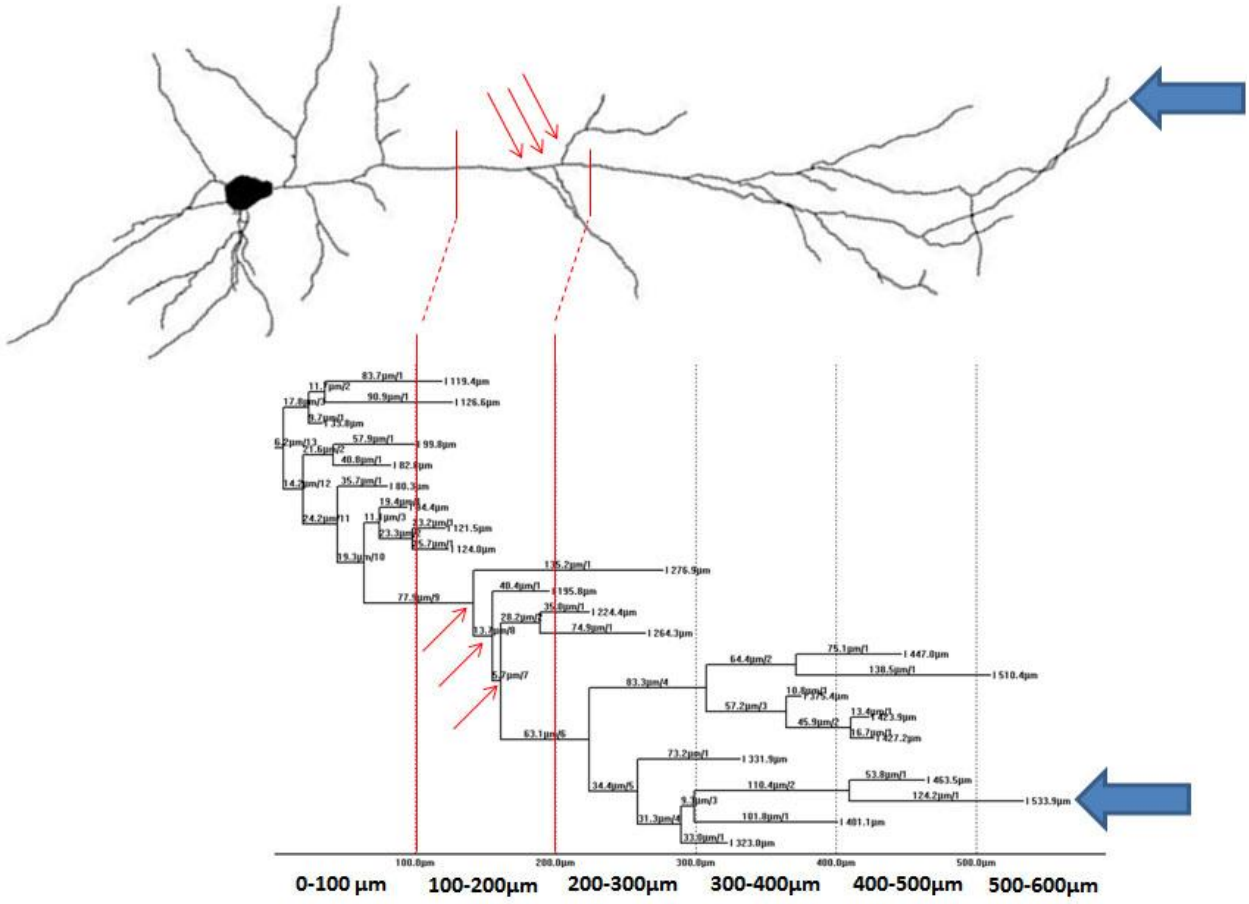


Figure 2.3: Dendrogram analysis of apical dendrites of pyramidal neurons. In order to find out the number of dendritic branches directly emanating from the main dendrite, the total apical dendrite was described as a two-dimensional rooted tree with clearly defined segments in regular distances. The dendrites branching from the main branch were counted (red arrows as an example for the segment in 100 – 200 µm distance from the soma). The longest continuous branch of the two-dimensional tree was defined as the main dendritic branch (blue arrows).

2.8.2.3. Analysis of spine density of dendritic segments in the hippocampus

In the hippocampus, the spine density was evaluated by counting all visible spines on single segments of the apical dendrite. Three segments of each apical dendrite were reconstructed. Most of the segments were terminal branches in the middle part of the apical dendrite. Afterwards, spines were counted on these segments and the spine density was calculated by dividing the total number of spines by the total length of the segment. The mean values of spine densities of all the segments per neuron were used as final values.

2.8.2.4. Sholl Analysis

Concentric spheres with 20 µm increasing radii were put around the centre of the soma of each single neuron (Figure 2.2 and Figure 2.3 A). For each sector (that is the space between the surfaces of spheres with neighbouring radii) the total spine number (black dots) as well as the total length of dendritic material were calculated for the respective neuron e.g. value 1 of spine number results from sector 80 µm -100 µm of one measured neuron of mouse X (Figure 2.4 A) and value 2 of spine number results from sector 80 µm -100 µm of another measured neuron of mouse X (Figure 2.4 B). The same measurement is done for all i reconstructed neurons per animal. The arithmetic mean of (1,2,…,i) was calculated for each animal and was used as final value for further statistical evaluation.

For the evaluation of the spine density of each neuron, the ratio of the number of spines per dendritic length was calculated for each sector between neighbouring radii (Figure 2.4). These quotients were used to calculate the arithmetic mean of all these quotients per animal at each sector. These mean values were then used for further statistical evaluation.

2.8.2.5. Analysis of branch orders

Branch orders can be numbered serially as shown in Figure 2.3 B and branches of the same branching factor can be pooled to get a mean value of each branch order for each investigated parameter. This analysis is valid for neurons which are more spherical (Figure 2.3 B) but not for drawn-out neurons like in the cortex or in the hippocampus (See Chapter 4.2.1). This analysis was used to evaluate the spine density depending on branch order in pyramidal neurons, nut in the end Sholl analysis was preferred.

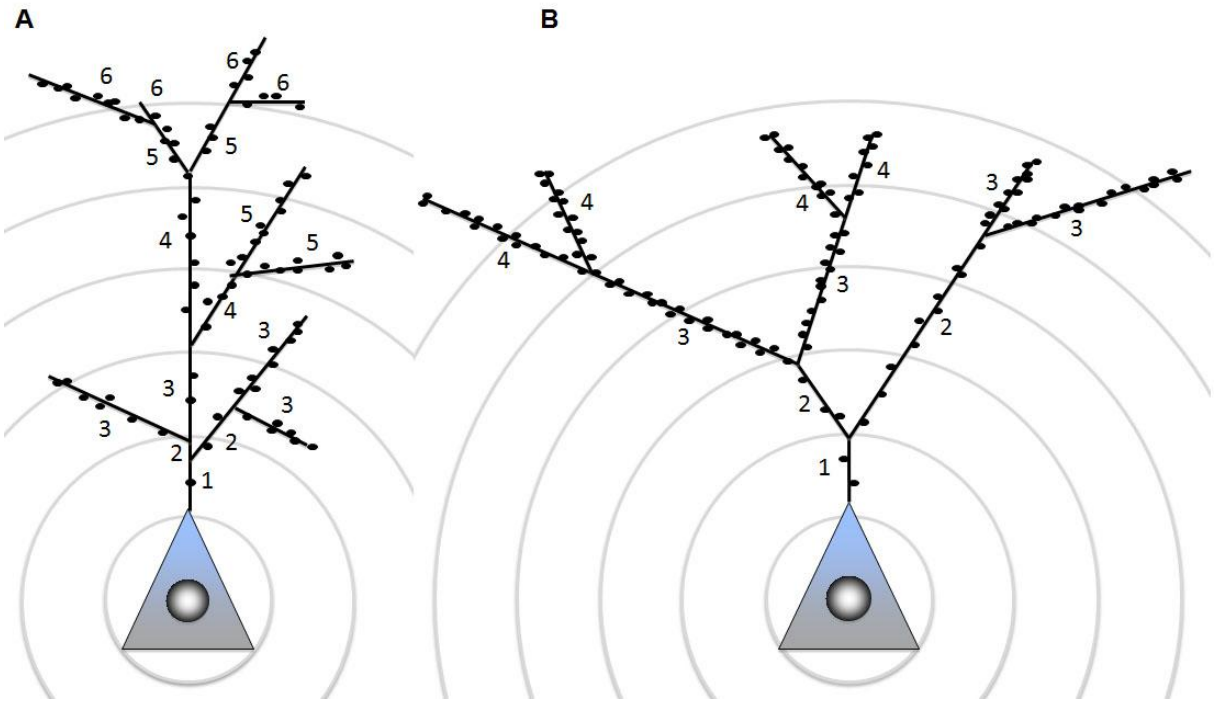


Figure 2.3: Different methods can be used to quantify morphological properties of different neuron types. For cortical neurons with one main apical dendrite and many side branches emanating over the whole length of this dendrite, Sholl analysis (A) is the best for analysing dendritic segments within specific ranges from the neuronal soma. Branch order analysis (B) can be performed on dendrites which are evenly spread over the length of a dendrite, like for example in the case of spherical amygdala pyramidal cells (B) but not in long drawn-out cortical neurons (A) where branching factors are very different in each neuron (See Chapter 4.2.1). In the amygdala as well as in IL (Nietzer, Bonn et al. in preparation), the spine density on apical and basal dendrites increases with rising branch order. (Figure adapted from Nietzer, Bonn et al. in preparation)

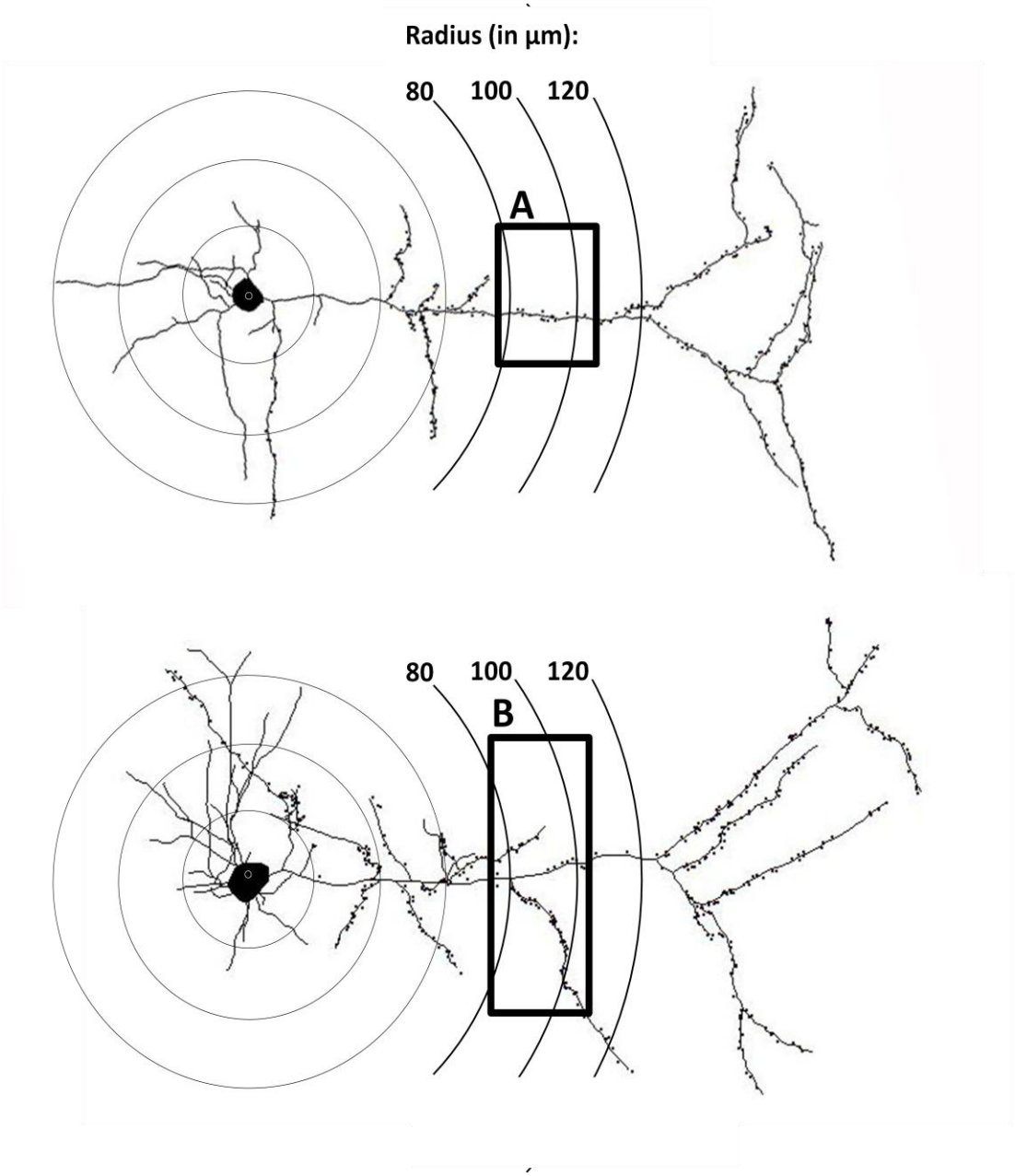


Figure 2.4: Statistical evaluation of Sholl Analysis. For each animal, the arithmetic mean of the investigated neuron parameters were calculated for each single sector. A sector is bordered by two surfaces of spheres with neighbouring radii and contains dendritic material as well as spines (if counted), as seen in A and B for two single neurons of the same animal. For Sholl analysis of spine density the quotients of spine number/spine length were calculated separately for each single sector (quotient of A and quotient of B) for each reconstructed neuron. Afterwards the arithmetic mean (mean of quotient A and quotient B) was calculated separately for each sector from the individual quotients of all the reconstructed neurons of one animal. Mean values were then analyzed with non-parametric statistical evaluation methods (See Chapter 2.9).

2.9 Statistical evaluation

We used the Kruskal-Wallis test over all groups and post-hoc Wilcoxon rank sum tests if the p-value of the Kruskal-Wallis test was < 0.1. The level of significance was defined at $p < 0.01$ as 5 out of 6 possible tests were performed (WT control versus WT loser, *5-Htt* KO control versus *5-Htt* KO loser, WT control versus *5-Htt* KO control, WT loser versus *5-Htt* KO loser, WT control versus *5-Htt* KO loser). $0.01 < p < 0.05$ was defined as a tendency. P-values of $0.1 > p > 0.05$ were mentioned as possible tendencies. Analysis was done using the disposable statistics program R (version 2.10.0) as described in the following chapters.

2.9.1 R-Code for statistical evaluations

The open source program R (http://cran.r-project.org/) was used for a statistical evaluation of the data. The data were prepared in Microsoft Excel the following way and saved as a csv-file:

Group	Spine_Density	Spine_Number	length_apical_dendrite
1	0,147	250	1542,85
1	0,244	356	1465,77
1	0,169	241	1399,18
1	0,14	247	1647,70
1	0,211	442	1928,90
2	0,112	182	1631,07
2	0,127	188	1539,27
2	0,108	254	1815,05
2	0,165	289	1594,73
2	0,198	352	1993,00
2	0,195	327	1580,70
3	0,13	217	1529,26
3	0,164	189	1055,20
3	0,112	219	1699,80
3	0,127	227	1604,08
3	0,166	225	1321,58
3	0,146	194	1244,80
4	0,13	209	1460,83
4	0,178	343	1897,18
4	0,27	454	1694,10
4	0,182	407	2158,63
4	0,166	167	1027,13
4	0,139	243	1744,97

Attention had to be paid to the encoding of animals: same numbers in column “group” stand for animals of the same treatment group. The data were always imported into a variable called “sholl”:

```
sholl <- read.csv2(“C:/… path …/name_of_file.csv”)
```

To perform the same test on each variable (column), the following basic script was used for different testing combinations by changing ***lines*** depending of tested group numbers.

To perform the Kruskal-Wallis test over all groups, wilcox.test was replaced by kruskal.test and ***lines*** was replaced by replacement character (test <- kruskal.test(Fakt.Daten[,i]~Gruppe, data = sholl)). "Name_of_function" was always renamed as necessary.

```
Name_of_function <- function(x){
Fakt.Daten <- subset(x,delect = -1)
dim_Matrix <- dim(Fakt.Daten)
dim_n <- dim_Matrix[c(2)]
for(i in 1:dim_n){
p <- ncol(sholl)
print(variable.names(sholl[ ,i:p]))
test <- wilcox.test(Fakt.Daten[lines,i]~sholl[lines,1], data = sholl[lines, ])
print(test)}}
```

If e.g. group 1 and 3 had to be tested for differences, ***lines*** was replaced by the used lines of the matrix, in this case lines 1 to 5 and lines 12 to 17, which finally looks as follows: c(1:5,12:17).

2.9.2 Problems of statistical analysis of morphological data

2.9.2.1 Pseudoreplication

This is one problem of morphological analysis. The term pseudoreplication means that replicates which are not independent are treated as independent replicates (Hurlbert 1984). All measured neurons of one mouse brain are not independent of each other as they belong to the same organism and are exposed to the same homeostatic environment e.g. hormone state, development, age or inter-neuronal connection. For this reason not all the reconstructed neurons of all the investigated brains were simply taken together as sample size n. Instead only the arithmetic mean of all neurons measured in one mouse was taken to estimate the properties of a "mainstream neuron" in the analyzed region of this individual organism. Hence the sample size n is equal to the number of mice whose brains were analyzed. The arithmetic mean was preferred to the median as in doing so each selected neuron has the same influence on the final value of each investigated mouse brain. We didn't consider the side of the hemisphere as it was not always clear which way the tissue slices had been flipped when they were put onto glass slides.

2.9.2.2 Missing values

Another problem of morphological analysis, especially of Sholl analysis, is dealing with "missing values". As some reconstructed neurons are "shorter" (see Figure 2.5) there are no data available at higher radii. So radii over 400 µm were not taken into account because of too many missing values. But radii < 400 µm were used for statistical evaluation. To avoid overrepresentation of single "longer" neurons, "shorter" neurons were taken into account by replacing missing values by zero. This is a controversial solution but by comparing arithmetic means with and without zero-filling we can see that these missing values distort the representation of reconstructed neurons as single "longer" neurons are overrepresented at higher radii (Table 2.3).

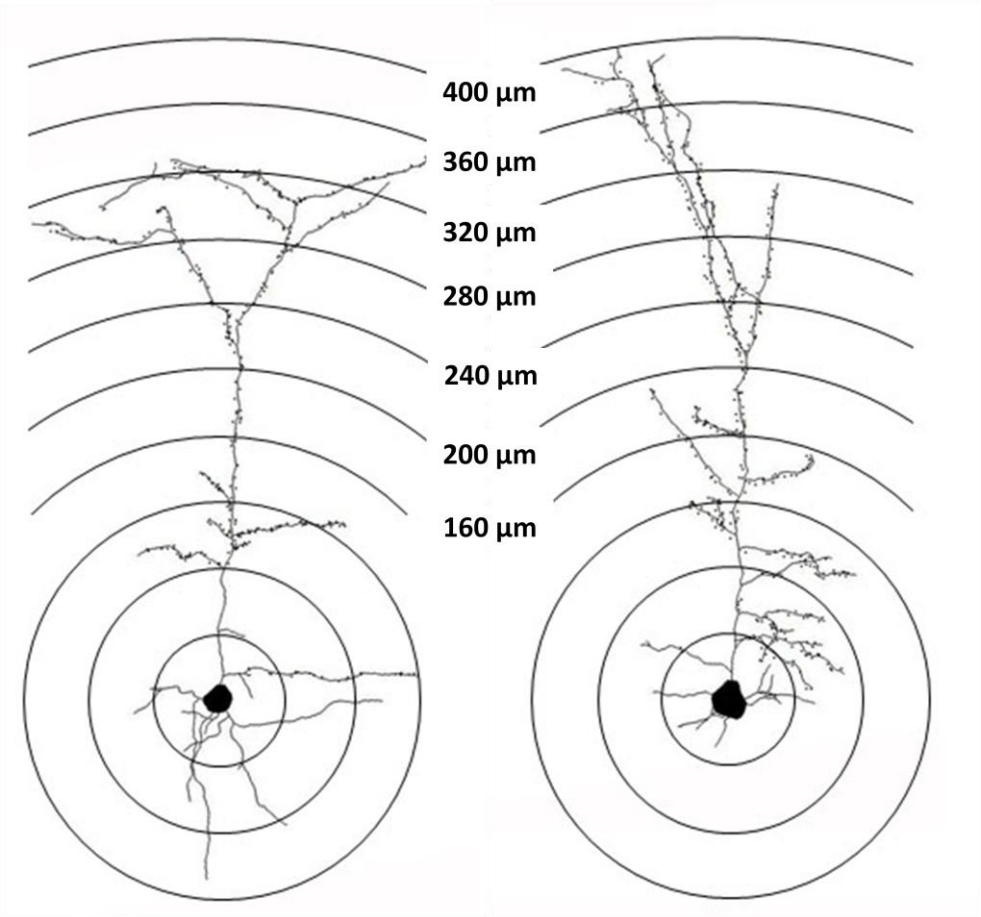


Figure 2.5: Missing values in Sholl Analysis of reconstructed three-dimensional neurons. Concentric spheres with consistently increasing radii and a common centre, (0|0|0) the point of a three-dimensional cartesic coordinate system, lying in the middle of the soma are used to define sectors on the respective neuron. One sector is defined by the surfaces of spheres with neighbouring radii e.g. 360 µm is the inner border of the sector and 400 µm is the outer border of the same sector. In our study the distance between neighbouring radii was set at 20 µm. In this Figure, the neuron on the left ends in the sector between 320 µm to 360 µm but the neuron on the right has dendrites in an additional sector (360 µm to 400 µm) where the neuron on the left doesn't have any dendrites. It has "missing values" in this area.

Radius:	20 µm	40 µm	…	340 µm	360 µm	380 µm	400 µm
Neuron 1	7,7	108,2	…	26,7	31,6	2	**0**
Neuron 2	9,6	108,8	…	20,2	8,2	**0**	**0**
Neuron 3	8,7	49,2	…	35,8	24,3	36,9	29,4
Neuron 4	5,2	43,3	…	146,5	164	178,5	94,7
			Arithmetic mean without zeros:			**72,5**	**62,1**
			Arithmetic mean with zeros:			**54,4**	**31,0**

Table 2.3: Sholl Analysis: length of dendritic material between neighbouring radii. To build up a "mainstream" neuron based on all reconstructed neurons per investigated region, the arithmetic mean of all measured neurons was used for each parameter of interest. At higher radii (black box) there were missing values as some neurons had no dendrites in these sectors (See Figure 2.5, neuron on the left). If these neurons are not taken into account in these outer sectors, the "mainstream neuron" would have greater final values in these sectors (Arithmetic mean without zero-filling) and would not take into account the other neurons in these sectors as done by using zero as a measured value (arithmetic mean with zero-filling). So zero-filling was chosen for final evaluation.

3. Results

3.1. The effect of acute immobilization stress and *5-Htt* genotype on plasma corticosterone levels in male and female mice

Mice of each genotype and gender (Table 3.1.1) were immobilized for 1 hour (stressed) or stayed in their home cage as controls (unstressed). As 24 tests were done with the data, the level of significance of post-hoc Wilcoxon rank sum tests was adjusted using Bonferroni correction and differences between groups which lead to a p-value < 0.0021 were defined as significant differences (**). Differences between groups with 0.05 < p-value > 0.0021 were defined as a tendency (*) and differences which lead to 0.05 < p-value < 0.1 were mentioned, too (#). We used mice of 12 groups with n between 5 and 10 mice per group for corticosterone measurements as well as gene expression analysis by qRT-PCR:

Treatment group	n_{mice}	Corticosterone measurements	qRT-PCR
Male *5-Htt* WT unstressed	8	X	X
Male *5-Htt* WT stressed	8	X	X
Male *5-Htt* HET unstressed	5	X	-
Male *5-Htt* HET stressed	7	X	-
Male *5-Htt* KO unstressed	10	X	X
Male *5-Htt* KO stressed	9	X	X
Female *5-Htt* WT unstressed	7	X	X
Female *5-Htt* WT stressed	8	X	X
Female *5-Htt* HET unstressed	7	X	-
Female *5-Htt* HET stressed	6	X	-
Female *5-Htt* KO unstressed	9	X	X
Female *5-Htt* KO stressed	9	X	X

Table 3.1.1: n_{mice} for corticosterone measurements and gene expression studies. QRT-PCR was carried out only with *5-Htt* WT mice and *5-Htt* KO mice. n_{mice}: number of mice in each treatment group: X: treatment groups which were used for the respective analyses.

3.1.1. The effect of acute immobilization stress and *5-Htt* genotype on corticosterone release

After 1 hour immobilization stress, the animals were immediately sacrificed and blood corticosterone levels were analyzed (Figure 3.1.1 and Figure 3.1.2). The same was done with the unstressed control mice. We hypothesized a) that the stress increases the level of corticosterone in the blood and b) that the *5-Htt* genotype has an influence on the corticosterone levels – with and without stress – but the trend of these changes was not specified in our hypothesis. Additionally, we hypothesized c) that female mice display higher corticosterone levels than male mice (See Chapter 3.1.2).

The level of corticosterone was significantly increased after 1 h immobilization stress in mice of each genotype and gender ($p < \mathbf{0.0021}$) and therefore our hypothesis a) was corroborated. An effect of

genotype on corticosterone concentration was detected only in stressed male mice (Figure 3.1.1 A) but not in stressed female mice (Figure 3.1.1 B). In the group of stressed males, *5-Htt* KO mice showed a tendency to lower plasma corticosterone levels that *5-Htt* WT mice (p = 0.01476) and *5-Htt* HET (p = 0.002176) (Figure 3.1.1 A). This effect of the *5-Htt* genotype on the plasma corticosterone level could not be found in the group of unstressed mice, neither in males nor in females. The difference between corticosterone levels of stressed male *5-Htt* WT mice and of stressed male *5-Htt* HET mice was neither significant nor tendential (p = 0.0973). No genotype effects could be shown in female mice of each stress condition (Figure 3.1.1 B).

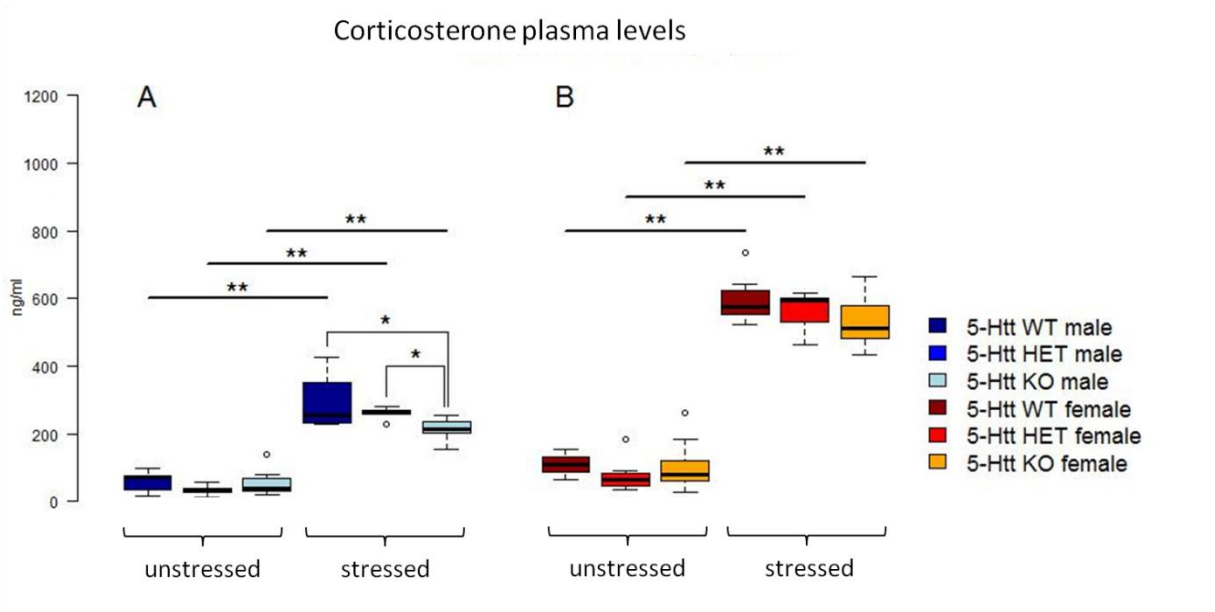


Figure 3.1.1: Corticosterone concentrations in male (A) and female (B) acutely stressed and unstressed *5-Htt* WT mice, *5-Htt* HET mice and *5-Htt* KO mice. Mice of both genders, different *5-Htt* genotypes and of different stress conditions (1 h stressed by immobilization in a 50 ml Falcon tube versus being left unstressed in the respective home cage in a different room) were compared to each other. Significant differences were found within each *5-Htt* genotype between unstressed and stressed mice (thick lines). Genotype effects were detected only in stressed male mice (thin lines in A) but not in female mice (B). Data were analyzed with R (version 2.10.0).
**: $p < 0.0021$, significant with Bonferroni correction; *: $0.05 > p > 0.0021$, tendency.

3.1.2. Gender differences in corticosterone levels of acutely stressed mice

In our study we could reveal basic gender differences: plasma corticosterone concentrations were always higher in female mice than in male mice of the respective treatment group (stressed and unstressed, irrespective of the *5-Htt* genotype) as shown in Figure 3.1.2. This effect was more pronounced in stressed mice of each genotype (p = **0.0003** in WT mice, p = **0.0012** in *5-Htt* Het and p = **0.0001** in *5-Htt* KO mice) than in unstressed mice (p = **0.0140** in WT mice, p = 0.0614 in *5-Htt* Het and p = 0.077 in *5-Htt* KO mice).

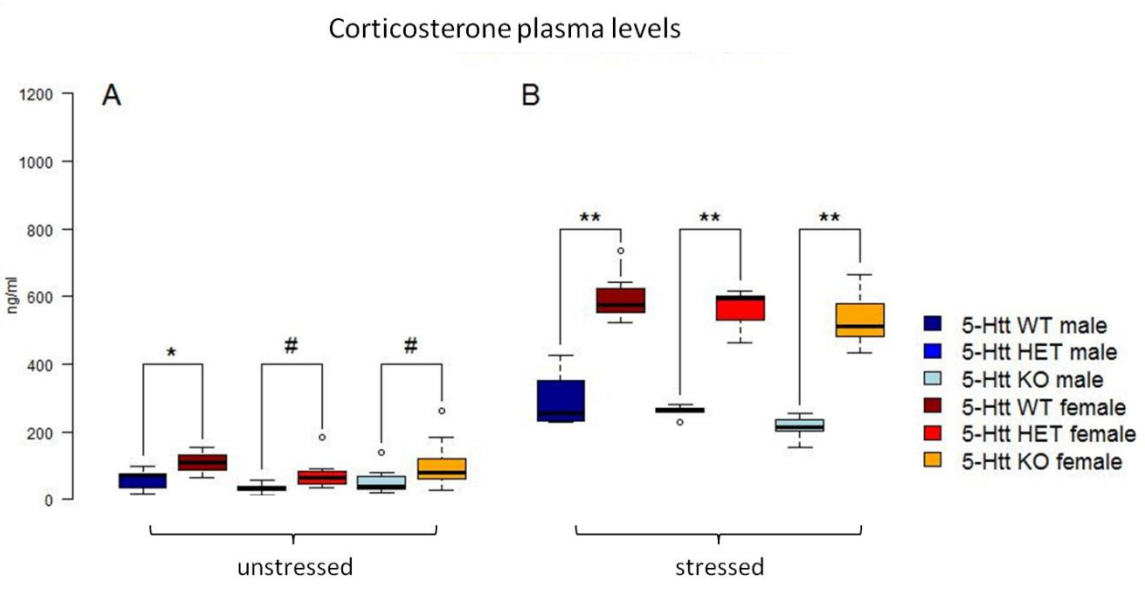


Figure 3.1.2: Corticosterone concentrations in acutely stressed and unstressed mice of both gender. Significant differences between the two genders were found in mice of each genotype (*5-Htt* WT, *5-Htt* HET *5-Htt* KO). In unstressed mice (A) the differences were not as clear as in stressed mice (B). Data were analyzed with R (version 2.10.0).
**: $p < 0.0021$, significant with Bonferroni correction; *: $0.05 > p > 0.0021$, tendency; #: $0.1 > p > 0.005$, possible tendency.

Treatment group	N_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	8	14.62	39.51	**70.00**	59.23	75.80	99.18
Male WT stressed	8	227.7	233.5	**254.8**	291.3	313.6	427.0
Male Het unstressed	5	12.59	25.63	**33.63**	33.66	38.84	57.59
Male Het stressed	7	227.4	256.7	**263.5**	260.7	270.1	280.4
Male KO unstressed	9	19.75	30.79	**38.96**	53.52	66.46	138.20
Male KO stressed	8	153.2	203.1	**213.9**	214.2	233.7	254.4
Female WT unstressed	7	64.70	88.06	**108.50**	109.20	130.30	154.30
Female WT stressed	7	521.2	552.0	**573.4**	597.2	623.8	734.3
Female Het unstressed	6	33.63	44.65	**64.43**	77.11	84.23	184.00
Female Het stressed	6	461.6	544.2	**592.3**	565.3	598.9	616.2
Female KO unstressed	9	25.38	61.52	**79.61**	106.50	121.60	260.40
Female KO stressed	9	432.6	481.3	**510.7**	530.5	576.9	665.5

Table 3.1.2: Mean values of corticosterone concentration in blood in ng/ml. Rounded values were used for box-plots in Figure 3.1.1 and Figure 3.1.2. $N_{values:}$ number of values used for statistical evaluation; Min.: minimum value of all values in each single group; 1st Q.: first quartile value; Median: median of all values in each group; Mean: arithmetic mean of all values in each group; 3rd Q.: Third quartile value; Max.: maximum value of all values in each single group.

3.2. The effect of restraint stress and life-long 5-HT reuptake deficiency on the expression of two different IEGs, three different SPs and on CRHR and its both receptors in both genders - a qRT-PCR study

To monitor the effect of acute immobilization stress of 1 h on neuronal activity in the brain of *5-Htt* KO and *5-Htt* WT mice we performed a qRT-PCR study and compared the expression levels of the two different IEGs c-Fos and Fra-2, which are markers for neuronal activity, in various brain regions of unstressed and stressed *5-Htt* KO and *5-Htt* WT mice. Moreover, the expression of the SPs Syt I, Syt IV and Stx 1A was surveyed in order to find out if there is an effect of the *5-Htt* KO and acute stress exposure on the expression – each condition either by itself or in combination with the other – on synaptic plasticity. Additionally, the effect of stress as well as the effect of the *5-Htt* genotype and gender on the expression of CRH and its both receptors CRHR1 and CRHR2 was investigated, too.
Our hypotheses were that stress and/or life-long 5-HT reuptake deficiency and gender have an influence on the expression of a) the two IEGS, b) the three SPs and c) on the expression of CRH and its receptors in different regions of the brain. We expected that stress-induced changes in IEG expression would result in a higher expression of IEGs in most brain regions of stressed mice compared to unstressed mice. The trends of all other expression changes which we expected in this study were not determined.

The study was done using qRT-PCR. As during qRT-PCR sometimes single PCR reactions don't furnish any usable values, n_{values} of each treatment group can be different from n_{mice} (See Table 3.1.1). A p-value < 0.004 was defined as significant, by using the Bonferroni correction for 12 tests. A p-value of $0.05 < p < 0.004$, that means it was significant without Bonferroni correction, was defined as a tendency.

3.2.1. Strong impact of acute immobilization stress and gender but less pronounced impact of the 5-Htt deficiency on the expression of the two IEGs c-Fos and Fra-2

Using qRT-PCR, we were able to show that acute stress results in increased expression levels of both IEGs, c-Fos and Fra-2, in almost all investigated brain regions (Figures 3.2.1 to 3.2.5). While the strongest stress effects were demonstrated in the hypothalamus of mice of each genotype and of both genders, only subtle effects of stress exposure in IEG expression were found in the cortex. Besides, the hippocampus, the amygdala as well as the raphe showed an intermediate stress-induced increase of IEG expression. In addition, c-Fos and Fra-2 expression showed different spatial induction patterns as a result of acute restraint stress. In the hippocampus, for instance, Fra-2 expression was significantly changed in stressed compared to unstressed mice in almost all experimental groups, whereas only few c-Fos expression changes were detected in this brain region. Interestingly, the gender differences displayed by different basal as well as stress-induced plasma corticosterone levels

in males and females independent of their 5-Htt genotype (Chapter 3.1.2.) could be reproduced by IEG expression levels. In almost all brain regions, the expression levels of c-Fos as well as of Fra-2 were shown to be higher in females than in males (Figure 3.2.1 to 3.2.5). While the strongest gender differences were demonstrated for both IEGs in the hypothalamus and the amgydala, the impact of gender on expression levels of c-Fos and Fra-2 varies between the respective IEG and the different brain region investigated. Moreover, our qRT-PCR study revealed some *5-Htt* genotype effects on IEG expression in the hippocampus and in the raphe region: Whereas acute stress resulted in a more pronounced increase of Fra-2 (and to a lesser extend also of c-Fos) expression in *5-Htt* WT mice compared to *5-Htt* KO mice in the raphe, the opposite could be shown for the hippocampus (Figure 3.2.2). In the hippocampus, the stress-induced increase of Fra-2 expression was stronger in *5-Htt* KO mice compared to *5-Htt* WT mice. Detailed qRT-PCR results are given in the following (Please note that p-values < 0.0042 were classified as significant and p-values between 0.05 and 0.0042 were considered as a tendency, as Bonferroni correction for 12 tests had to be applied):

3.2.1.1. IEG expression in the cortex

In the cortex (Figure 3.2.1), the detected c-Fos expression levels didn`t reveal any influence of *5-Htt* genotype, stress and gender (p-value of Kruskal Wallis test > 0,1). Thus, this brain region appears to be rather inert (Figure 3.2.1 A).

In contrast to c-Fos, we were able to detect at least small effects of stress, *5-Htt* genotype and gender on the expression of Fra-2 in this brain region (Figure 3.2.1 B). Stress led to increased Fra-2 expression levels only in male *5-Htt* WT (p = **0.0023**) but not in male *5-Htt* KO mice, and was completely absent in the cortex of females. As a result of 5-Htt deficiency, we found decreased Fra-2 mRNA levels in stressed male *5-Htt* KO mice compared to stressed male *5-Htt* WT mice (p = **0.0006**). In addition some gender differences were shown regarding the expression of Fra-2, as in unstressed (p = **0.0004**) as well as in stressed (p = **0.0003**) female *5-Htt* KO mice Fra-2 mRNA levels were significantly higher than in male mice.

3.2.1.2. IEG expression in the hippocampus

In the hippocampus (Figure 3.2.2), the expression of c-Fos as well as Fra-2 was changed as a result of restraint stress, *5-Htt* genotype and gender, whereas stress mostly affected Fra-2 mRNA levels and displayed only minor effects on c-Fos expression levels as shown in Figure 3.2.2 A and B. As a result of stress exposure, c-Fos concentrations (Figure 3.2.2 A) were shown to be slightly, but neither significantly nor tendentially increased in male *5-Htt* WT mice (p = 0.0939), but showed a tendency to higher expression levels in male *5-Htt* KO mice (p = 0.0268). Additionally, stressed male *5-Htt* KO mice showed slightly stronger c-Fos expression levels than stressed male *5-Htt* WT mice (p = 0.0499). Therefore, stress reactivity (as indicated by these c-Fos expression changes) seems to be stronger in

5-Htt KO than in *5-Htt* WT mice. Interestingly, these stress effects on c-Fos expression were less pronounced in females and did not reach statistical significance in mice of this gender (p = 0.0939). Additional gender effects could be shown for unstressed *5-Htt* WT (p = 0.0082) and unstressed *5-Htt* KO mice (p = 0.0244), with females displayed higher c-Fos mRNA concentrations than males.

Expression analyses using Fra-2-specific qRT-PCR primer pairs revealed similar expression differences between the various experimental groups as with c-Fos-specific primer pairs (Figure 3.2.2 B). Similar to our results with c-Fos, stress seems to affect Fra-2 expression somewhat stronger in the hippocampus of 5-Htt KO than in the hippocampus of 5-Htt WT mice: Fra-2 mRNA concentrations were significantly increased in stressed male *5-Htt* KO compared to unstressed 5-Htt KO mice (p = **0.0012**), whereas this stress effect was shown to be completely absent between stressed and unstressed male 5-Htt WT mice. However, in contrast to the missing stress effects on c-Fos expression in females, immobilization of 1 h affected Fra-2 expression also in females and resulted in tendentially elevated Fra-2 mRNA concentrations in stressed compared to unstressed female *5-Htt* WT mice (p = 0.0350) as well as in significant expression differences in female *5-Htt* KO mice (p = **0.0018).** Moreover, we showed additional consequences of the 5-Htt genotype in males with increased Fra-2 mRNA levels in stressed *5-Htt* KO compared to stressed *5-Htt* WT mice (p = 0.0077). This genotype effect was less pronounced in females and did not reach statistical significance (p = 0.0823). Gender effects were shown in stressed *5-Htt* WT mice (p = 0.0140) and unstressed *5-Htt* KO mice (p = 0.0134). In both cases the expression was stronger in female mice than in male mice.

3.2.1.3. IEG expression in the hypothalamus

In the hypothalamus (Figure 3.2.3), c-Fos and Fra-2 expression analyses revealed the highest stress reactivity in females as well as in males compared to the other examined brain regions. The expression of c-Fos (Figure 3.2.3 A) was clearly increased in stressed compared to unstressed male *5-Htt* WT mice (p = **0.0012**), *5-Htt* KO mice (p = **0.0021**), unstressed female *5-Htt* WT mice (p = **0.0007**) and female *5-Htt* KO mice (p = 0.0079). No 5-Htt genotype-dependent effects could be detected. But almost each female treatment group showed significantly increased c-Fos expression compared to the respective male treatment group (unstressed *5-Htt* WT mice with p = 0.014; stressed *5-Htt* WT mice with p = **0.0002**; unstressed *5-Htt* KO mice with p = **0.0015**, stressed *5-Htt* KO mice with p = **0.001**).

Expression analyses using Fra-2-specific qRT-PCR primer pairs revealed similar expression patterns between the various experimental groups as with c-Fos-specific primer pairs. The expression of Fra-2 (Figure 3.2.3 B) was significantly increased in stressed compared to unstressed male *5-Htt* WT mice (p = **0.0012**), male *5-Htt* KO mice (p = **0.0004**), female *5-Htt* WT mice (p = **0.0007**) and female *5-Htt* KO mice (p = 0.0111). In females, one 5-Htt genotype-dependent effect could be detected with increased Fra-2 mRNA concentrations in unstressed *5-Htt* KO mice compared to unstressed *5-Htt* WT

mice (p = 0.0256). Significant gender differences with higher expression levels of Fra-2 in females than in males were detected in the group of stressed *5-Htt* WT mice (p = **0.0002**), unstressed *5-Htt* KO mice (p = **0.0000**) and stressed *5-Htt* KO mice (p = **0.0037**), but not in the group of unstressed WT mice.

3.2.1.4. IEG expression in the amygdala

In the amygdale (Figure 3.2.4), stress affects the expression of c-Fos as well as Fra-2, but only in males of each treatment group and not in females. The expression of c-Fos (Figure 3.2.4 A) was significantly increased in stressed compared to unstressed male *5-Htt* WT mice (p = 0.0175) and in stressed compared to unstressed male *Htt* KO mice (p = **0.0004**). The expression of Fra-2 was significantly increased in stressed compared to unstressed male *5-Htt* WT mice (p = 0.041) and in stressed compared to unstressed male *Htt* KO mice (p = **0.0012**). In addition to this missing stress reactivity of c-Fos and Fra-2 expression in females, additional gender effects were shown. Expression levels of the IEG c-Fos were shown to be increased in female *5-Htt* WT mice (p = 0.035), female stressed *5-Htt* WT mice (p = 0.0289) and female unstressed *5-Htt* KO mice (p = **0.0002**) compared to males of the same treatment group. The expression of Fra-2 (Figure 3.2.4.B) was significantly increased in female unstressed *5-Htt* KO mice compared to the respective males of the same treatment group (p = **0.0029**).

3.2.1.5. IEG expression in raphe

In the raphe region (Figure 3.2.5), stress exposure affects the expression of c-Fos (Figure 3.2.5 A) as well as Fra-2 (Figure 3.2.5 B), but predominantly in males. Additionally, in *5-Htt* WT mice stress results in more pronounced elevated c-Fos as well as Fra-2 expression levels than in *5-Htt* KO mice (whereas this effect seems to be more distinct in females). Therefore, in the raphe, stress seems to affect IEG expression somewhat stronger in *5-Htt* WT mice than in mice with a life-long 5-Htt deficiency. In detail, c-Fos mRNA levels were significantly increased in stressed compared to unstressed male *5-Htt* WT mice (p = **0.0003**), male *5-Htt* KO mice (p = **0.0015**), female *5-Htt* WT mice (p = 0.013), but displayed only a tendency to increased mRNA concentrations in stressed compared to unstressed female *5-Htt* KO mice (p = 0.0541). Regarding c-Fos expression, one 5-Htt genotype-dependent effect could be detected with reduced mRNA concentrations in stressed male *5-Htt* KO mice in comparison to stressed male *5-Htt* WT mice (p = 0.0093). Significant gender differences with higher expression levels of c-Fos in females than in males were detected only in the group of unstressed *5-Htt* KO mice (p = 0.0057).

Fra-2 mRNA levels were significantly increased in stressed compared to unstressed male *5-Htt* WT mice (p = **0.0006**), stressed compared to unstressed male *5-Htt* KO mice (p = **0.0029**) and stressed compared to unstressed female *5-Htt* WT mice (p = **0.0013**), but displayed only a tendency to

increased mRNA concentrations in stressed compared to unstressed female *5-Htt* KO mice (p = 0.0663). A genotype-dependency could be demonstrated in stressed males with reduced Fra-2 expression levels in *5-Htt* KO mice than in *5-Htt* WT mice (p = **0.0033**). Gender differences could be shown in stressed *5-Htt* WT mice (p = 0.0499), unstressed *5-Htt* KO mice (p = **0.0015**) and stressed *5-Htt* KO mice (p = 0.0484). The expression of Fra-2 was stronger in female mice than in male mice. Significant gender differences with higher expression levels of Fra-2 in females than in males were detected in the group of stressed *5-Htt* WT mice (p = 0.0499), unstressed *5-Htt* KO mice (p = **0.0015**) and stressed *5-Htt* KO mice (p = 0.0484), but not in the group of unstressed WT mice.

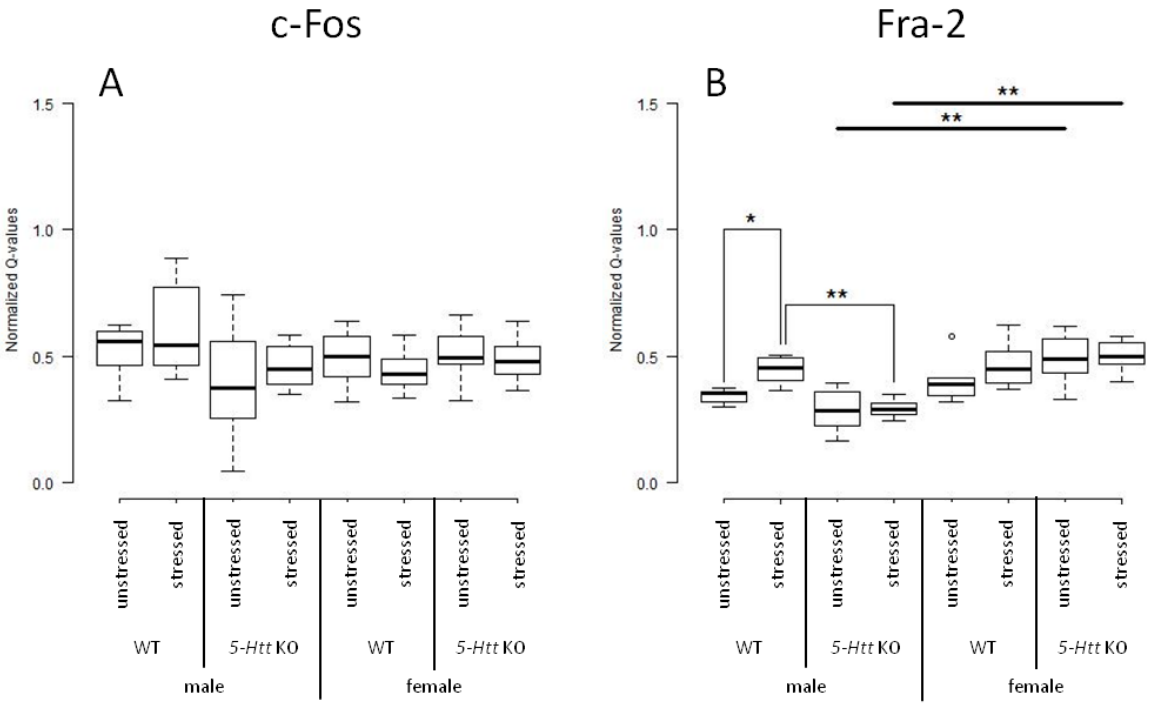


Figure 3.2.1: Expression of c-Fos (A) and Fra-2 (B) in the cortex of male and female acutely stressed and unstressed *5-Htt* WT and *5-Htt* KO mice. No differences in c-Fos expression could be detected in the cortex (A). Concerning the expression of Fra-2 (B) a tendential stress effect could be found in male *5-Htt* WT mice and a significant genotype effect in stressed male mice could be shown. Gender differences were detected in *5-Htt* KO mice but not in *5-Htt* WT mice.
Kruskal-Wallis test cFos: p = 0.3278, Kruskal-Wallis test Fra-2: p = 0.000001; **: p < 0.004 (significant with Bonferroni correction). Thin lines: stress effects and genotype effects; thick lines: gender effects; normalized Q-values of the qRT-PCR were presented as box-plots.

Normalized Q-values of c-Fos in cortex:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0.3232	0.4615	**0.5601**	0.5178	0.5970	0.6244
Male WT stressed	7	0.4100	0.4614	**0.5410**	0.6156	0.7741	0.8871
Male KO unstressed	10	0.0474	0.2713	**0.3758**	0.3943	0.5468	0.7425
Male KO stressed	7	0.3480	0.3866	**0.4508**	0.4612	0.5375	0.5812
Female WT unstressed	6	0.3187	0.4218	**0.4987**	0.4917	0.5749	0.6375
Female WT stressed	8	0.3346	0.3971	**0.4298**	0.4408	0.4723	0.5812
Female KO unstressed	9	0.3224	0.4667	**0.4910**	0.5111	0.5759	0.6646
Female KO stressed	8	0.3619	0.4403	**0.4783**	0.4860	0.5348	0.6360

Normalized Q-values of Fra-2 in cortex:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0.2974	0.3199	**0.3537**	0.3404	0.3590	0.3738
Male WT stressed	7	0.3628	0.4049	**0.4529**	0.4448	0.4927	0.5025
Male KO unstressed	10	0.1665	0.2298	**0.2856**	0.2850	0.3430	0.3952
Male KO stressed	7	0.2466	0.2694	**0.2873**	0.2921	0.3121	0.3480
Female WT unstressed	6	0.3202	0.3554	**0.3911**	0.4063	0.4099	0.5759
Female WT stressed	8	0.3713	0.3952	**0.4460**	0.4634	0.5114	0.6201
Female KO unstressed	9	0.3270	0.4354	**0.4876**	0.4879	0.5679	0.6186
Female KO stressed	8	0.3970	0.4802	**0.4990**	0.5020	0.5446	0.5785

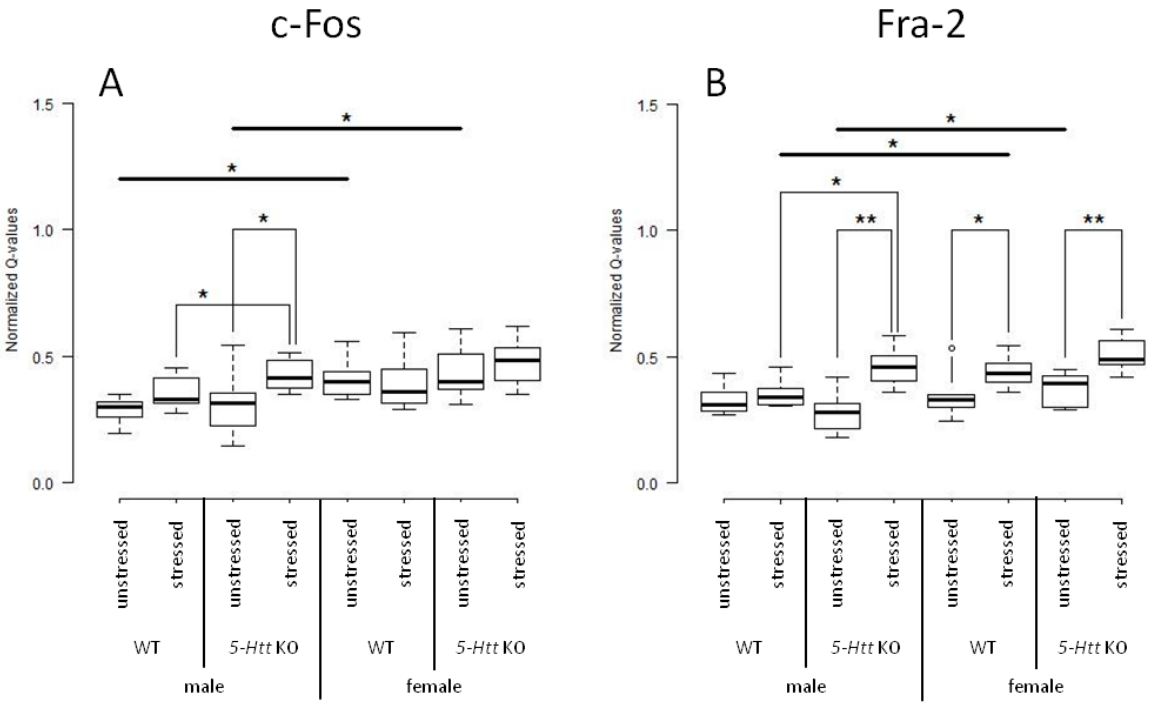


Figure 3.2.2: Expression of c-Fos (A) and Fra-2 (B) in the hippocampus of male and female acutely stressed and unstressed *5-Htt* WT and *5-Htt* KO mice. C-Fos expression was increased after stress in male *5-Htt* WT mice (p = 0.0939, not shown in the diagram) and in male *5-Htt* KO mice. There was a genotype effect in male stressed mice and the same trend could be seen in female mice (p = 0.0939, not shown in the diagram). Fra-2 expression was increased after stress in each group (B), only in male WT mice this effect was very low. Gender effects could be detected in stressed *5-Htt* WT mice and in unstressed *5-Htt* KO mice. Kruskal-Wallis test cFos: p = 0.0015, Kruskal-Wallis test Fra-2: p = 0.00002; **: p < 0.004 (significant with Bonferroni correction), *: 0.05 < p < 0.004 (tendency). Thin lines: stress effects and genotype effects; thick lines: gender effects; normalized Q-values of the qRT-PCR were presented as box-plots.

Normalized Q-values of c-Fos in hippocampus:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0.1950	0.2594	**0.2969**	0.2862	0.3209	0.3507
Male WT stressed	8	0.2751	0.3165	**0.3299**	0.3557	0.4109	0.4522
Male KO unstressed	9	0.1428	0.2256	**0.3153**	0.3065	0.3548	0.5427
Male KO stressed	8	0.3499	0.3840	**0.4153**	0.4257	0.4726	0.5111
Female WT unstressed	6	0.3295	0.3610	**0.4005**	0.4120	0.4281	0.5567
Female WT stressed	7	0.2895	0.3143	**0.3614**	0.3962	0.4509	0.5925
Female KO unstressed	9	0.3081	0.3681	**0.4010**	0.4418	0.5064	0.6092
Female KO stressed	8	0.3491	0.4197	**0.4813**	0.4754	0.5241	0.6191

Normalized Q-values of Fra-2 in hippocampus:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0.2695	0.2859	**0.3081**	0.3281	0.3567	0.4338
Male WT stressed	8	0.3067	0.3080	**0.3385**	0.3501	0.3665	0.4585
Male KO unstressed	9	0.1782	0.2164	**0.2803**	0.2757	0.3132	0.4170
Male KO stressed	7	0.3614	0.4013	**0.4595**	0.4588	0.5017	0.5844
Female WT unstressed	6	0.2429	0.3020	**0.3315**	0.3482	0.3505	0.5340
Female WT stressed	7	0.3572	0.3970	**0.4318**	0.4399	0.4758	0.5452
Female KO unstressed	9	0.2875	0.3004	**0.3918**	0.3696	0.4248	0.4470
Female KO stressed	8	0.4209	0.4703	**0.4863**	0.5073	0.5407	0.6092

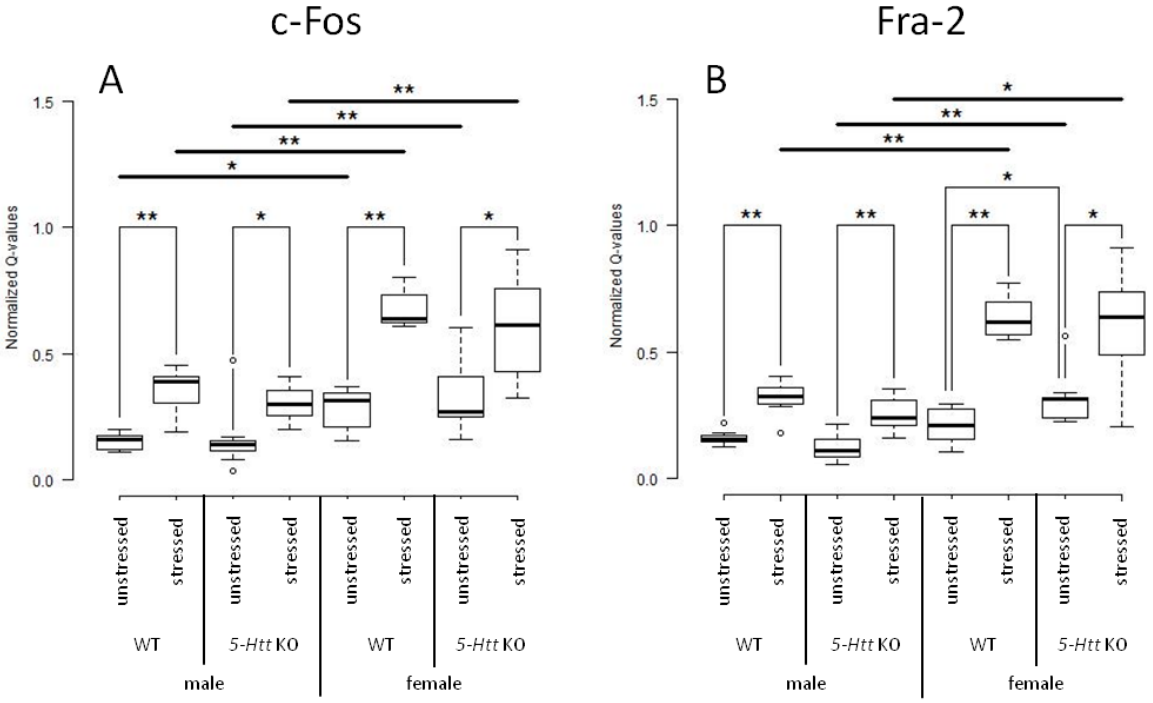


Figure 3.2.3: Expression of c-Fos (A) and Fra-2 (B) in the hypothalamus of male and female acutely stressed and unstressed *5-Htt* WT and *5-Htt* KO mice. Stress increased the expression of c-Fos in hypothalamus in both genders and in each genotype (A). Gender differences could be detected in each treatment group. The expression of Fra-2 was increased after stress in each gender, too (B). There was a genotype effect in female unstressed mice. Gender differences could be detected in each group except in male *5-Htt* WT mice. Kruskal-Wallis test cFos: p = 0.0000000009, Kruskal-Wallis test Fra-2: p = 0.00000002; **: p < 0.004 (significant with Bonferroni correction), *: 0.05 < p < 0.004 (tendency). Thin lines: stress effects and genotype effects; thick lines: gender effects; normalized Q-values of the qRT-PCR were presented as box-plots.

Normalized Q-values of c-Fos in hypothalamus:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,1116	0,1196	**0,1581**	0,1507	0,1742	0,1979
Male WT stressed	8	0,1889	0,3109	**0,3885**	0,3549	0,3985	0,4515
Male KO unstressed	10	0,0376	0,1181	**0,1394**	0,1588	0,1549	0,4717
Male KO stressed	9	0,1979	0,2563	**0,2992**	0,3055	0,3518	0,4088
Female WT unstressed	6	0,1545	0,2313	**0,3127**	0,2844	0,3434	0,3684
Female WT stressed	8	0,6096	0,6243	**0,6355**	0,6736	0,7128	0,8007
Female KO unstressed	9	0,1585	0,2493	**0,2703**	0,3339	0,4069	0,6012
Female KO stressed	8	0,3244	0,4389	**0,6116**	0,6033	0,7054	0,9092

Normalized Q-values of Fra-2 in hypothalamus:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0.1247	0.1453	**0.1571**	0.1623	0.1712	0.2211
Male WT stressed	8	0.1775	0.3025	**0.3241**	0.3167	0.3546	0.4013
Male KO unstressed	10	0.0576	0.0853	**0.1116**	0.1212	0.1455	0.2126
Male KO stressed	9	0.1622	0.2101	**0.2419**	0.2590	0.3084	0.3550
Female WT unstressed	6	0.1034	0.1642	**0.2090**	0.2076	0.2632	0.2938
Female WT stressed	8	0.5456	0.5741	**0.6156**	0.6351	0.6835	0.7699
Female KO unstressed	9	0.2237	0.2414	**0.3156**	0.3160	0.3200	0.5649
Female KO stressed	8	0.2025	0.5318	**0.6366**	0.6049	0.7198	0.9092

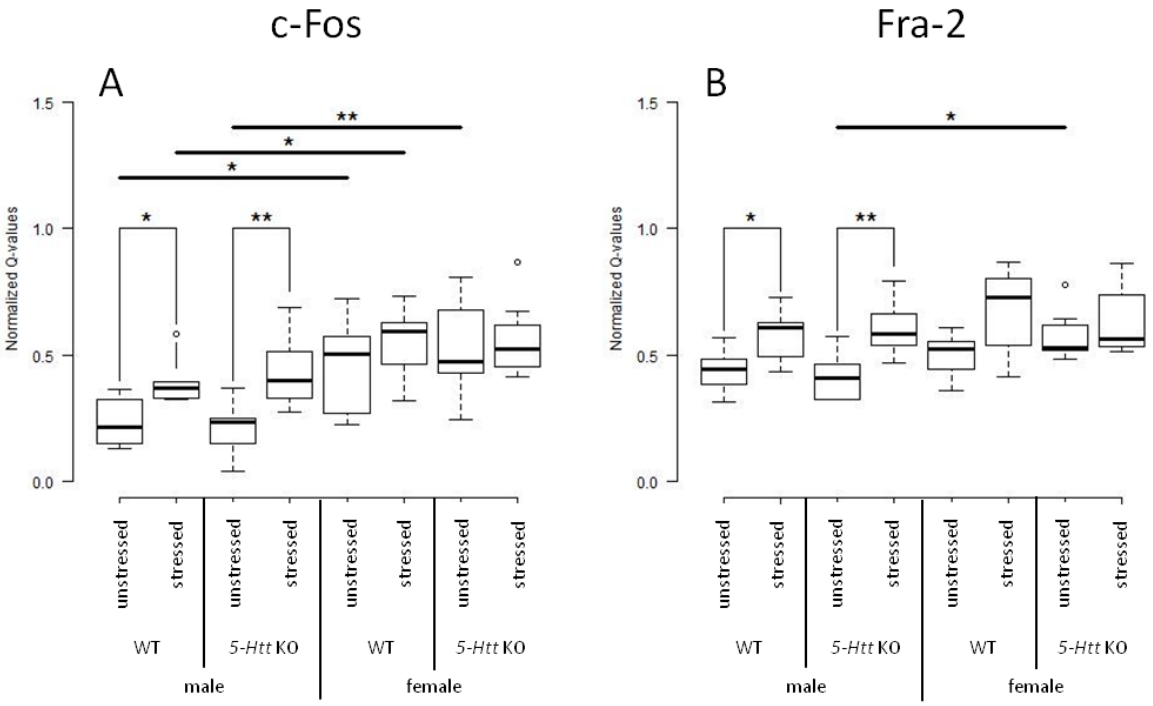


Figure 3.2.4: Expression of c-Fos (A) and Fra-2 (B) in the amygdala of male and female acutely stressed and unstressed *5-Htt* WT and *5-Htt* KO mice. The expression of c-Fos was increased after stress in male mice and to a lower extent in female mice (A). Female mice expressed c-Fos stronger than male mice. Fra-2 expression was increased after stress, too (B). This effect was stronger in male mice than in female mice. Gender differences in Fra-2 expression were not as distinct as in c-Fos expression.
Kruskal-Wallis test cFos: p = 0.00001, Kruskal-Wallis test Fra-2: p = 0.0003; **: p < 0.004 (significant with Bonferroni correction), *: 0.05 < p < 0.004 (tendency). Thin lines: stress effects and genotype effects; thick lines: gender effects; normalized Q-values of the qRT-PCR were presented as box-plots.

Normalized Q-values of c-Fos in the amygdala:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0.1300	0.1481	**0.2141**	0.2366	0.3249	0.3660
Male WT stressed	7	0.3223	0.3272	**0.3702**	0.3875	0.3922	0.5809
Male KO unstressed	10	0.0402	0.1649	**0.2322**	0.2122	0.2482	0.3694
Male KO stressed	9	0.2735	0.3275	**0.3995**	0.4275	0.5128	0.6892
Female WT unstressed	6	0.2253	0.3255	**0.5035**	0.4655	0.5568	0.7202
Female WT stressed	8	0.3193	0.4885	**0.5931**	0.5528	0.6231	0.7302
Female KO unstressed	9	0.2465	0.4292	**0.4740**	0.5357	0.6751	0.8084
Female KO stressed	8	0.4155	0.4692	**0.5242**	0.5598	0.5912	0.8664

Normalized Q-values of Fra-2 in the amygdala:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0.3135	0.3844	**0.4423**	0.4367	0.4830	0.5663
Male WT stressed	7	0.4322	0.4913	**0.6056**	0.5708	0.6251	0.7252
Male KO unstressed	10	0.3238	0.3325	**0.4076**	0.4111	0.4551	0.5729
Male KO stressed	9	0.4664	0.5383	**0.5823**	0.6056	0.6627	0.7917
Female WT unstressed	6	0.3584	0.4620	**0.5230**	0.5018	0.5468	0.6098
Female WT stressed	8	0.4127	0.5747	**0.7294**	0.6771	0.7859	0.8644
Female KO unstressed	9	0.4818	0.5163	**0.5260**	0.5704	0.6197	0.7790
Female KO stressed	8	0.5152	0.5365	**0.5606**	0.6296	0.7166	0.8604

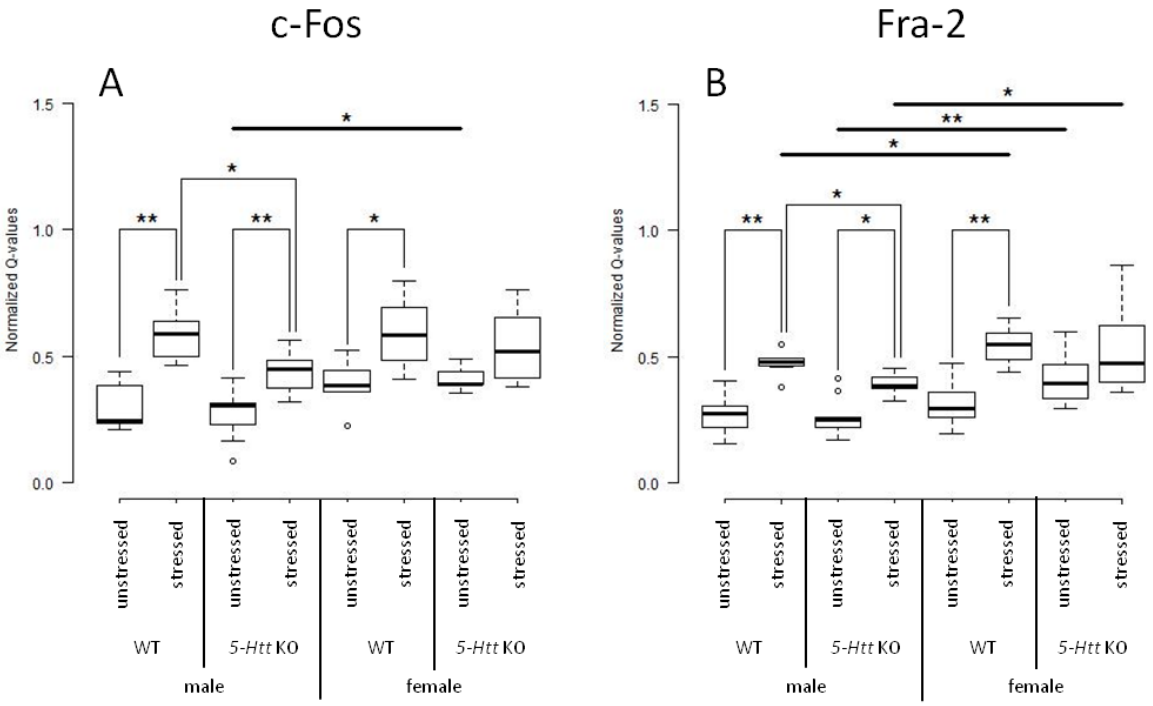


Figure 3.2.5: Expression of c-Fos (A) and Fra-2 (B) in raphe of male and female acutely stressed and unstressed *5-Htt* WT and *5-Htt* KO mice. C-Fos expression in raphe was increased after stress in each group (A). Also in female *5-Htt* KO mice expression of c-Fos was nearly tendential increased after stress (p = 0.0541, not shown in the diagram). Gender effects could be detected only in unstressed *5-Htt* KO mice, but it seems to be that the expression of c-Fos was stronger in female mice than in male mice. Fra-2 was, like c-Fos, increased after stress (B). The expression of Fra-2 in raphe was higher in females than in males. Kruskal-Wallis test cFos: p = 0.000002, Kruskal-Wallis test Fra-2: p = 0.0000005; **: p < 0.004 (significant with Bonferroni correction), *: 0.05 < p < 0.004 (tendency). Thin lines: stress effects and genotype effects; thick lines: gender effects.

Normalized Q-values of c-Fos in raphe:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,2081	0,2342	**0,2435**	0,3038	0,3844	0,4379
Male WT stressed	8	0,4651	0,5025	**0,5873**	0,5844	0,6338	0,7625
Male KO unstressed	10	0,0877	0,2431	**0,3023**	0,2818	0,3135	0,4143
Male KO stressed	9	0,3184	0,3725	**0,4461**	0,4318	0,4837	0,5608
Female WT unstressed	6	0,2231	0,3631	**0,3835**	0,3859	0,4305	0,5232
Female WT stressed	8	0,4105	0,4886	**0,5829**	0,5901	0,6788	0,7986
Female KO unstressed	9	0,3524	0,3893	**0,3911**	0,4116	0,4410	0,4893
Female KO stressed	8	0,3795	0,4189	**0,5165**	0,5384	0,6453	0,7607

Normalized Q-values of Fra-2 in raphe:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0.1538	0.2217	**0.2721**	0.2686	0.3031	0.4049
Male WT stressed	8	0.3804	0.4686	**0.4782**	0.4747	0.4913	0.5454
Male KO unstressed	10	0.1710	0.2232	**0.2478**	0.2580	0.2582	0.4143
Male KO stressed	9	0.3243	0.3734	**0.3839**	0.3924	0.4162	0.4544
Female WT unstressed	6	0.1969	0.2642	**0.2930**	0.3122	0.3466	0.4716
Female WT stressed	8	0.4379	0.4907	**0.5475**	0.5439	0.5832	0.6532
Female KO unstressed	9	0.2964	0.3327	**0.3956**	0.4110	0.4683	0.5983
Female KO stressed	8	0.3574	0.4056	**0.4710**	0.5246	0.5866	0.8618

3.2.2. Minor stress and 5-Htt knockout, but major gender effects on the expression of the SPs Syt I, Syt IV and Stx 1A

The here performed qRT-PCR study revealed only subtle and sporadic effects of acute immobilization stress and/or life-long 5-Htt deficiency on the expression of Syt I, Syt IV and Stx 1A mRNA. The most substantial stress effects were shown for Syt IV while Stx 1A showed an intermediate number of stress-induced expression changes and only few stress effects could be revealed for the expression of Syt I. Interestingly, in contrast to Syt I and Stx 1A, which normally exhibited increased expression levels in consequence of stress exposure, Sty IV expression was mostly shown to be reduced/decreased in stressed compared to unstressed mice.

But, it was possible to disclose significant gender influences on the expression of Syt I, Syt IV and Stx 1A in almost all investigated brain regions. Interestingly, gender often inversely affects the expression of Syt I and Syt IV: Whereas the expression Syt I was shown to be higher in females than in males in the cortex, the hippocampus and the amygdala, the expression of Syt IV levels were higher in male than in female mice. To explore possible reasons for these differential expression patterns which are most likely regulated by gender and stress, an *in situ*-hybridization (ISH) study for visualization of Syt I, Syt IV and Stx 1A mRNA in the murine CNS was performed (Fig. 3.2.6).

In Chapter 3.2.2.2 to 3.2.2.6, all qRT-PCR results are described in detail (Please note that p-values < 0.0042 are classified as significant and p-values between 0.05 and 0.0042 were only considered as a tendency, as Bonferroni correction for 12 tests had to be applied).

3.2.2.1. Differential expression of SPs in the murine brain - an *in situ*-hybridization study

Using ISH, unique regional and cellular expression patterns for each of the investigated SPs could be shown in *5-Htt* WT C57/Bl6 mice (Figure 3.2.6). In the cortex, Syt I displayed higher expression levels in all cortical layers compared to Syt IV which is mainly expressed in layer II to V but is only slightly expressed in layer VI (Figure 3.2.6 B, C). Stx 1A expression could be primarily demonstrated in cortical layer IV (Figure 3.2.6 D). In the hippocampus, the expression pattern could be described as follows: Syt I and Syt IV are expressed in a similar pattern in the Cornu ammonis sector 1 (CA) 1, CA2 and CA3 as well as in the dentate gyrus (DG) (Figure 3.2.6 F, G). In this region, the expression of Syt I and Syt IV was strongest in CA2/3, was less intense in CA1 and only slightly pronounced in DG. Stx 1A was expressed for the most part in CA3/4 pyramidal neurons, whereas in the CA1 region, the expression of Stx 1A was not as strong as the expression of Syt I and Syt IV (Figure 3.2.6 H). In the amygdala Syt I and SytIV were expressed in the lateral amygdaloid nucleus, ventrolateral (LaVL), lateral amygdaloid nucleus, dorsolateral (LaDL) and basolateral amygdaloid nucleus, anterior (BLA) (Figure 3.2.6 J, K), whereas Stx 1A expression could be detected primarily in LaDL (Figure 3.2.6 L). In the hypothalamus, the expression pattern of each investigated SP was different: Syt I was particularly expressed in anterior hypothalamic area (AHA) (Figure 3.2.6 N). In contrast to Syt I, Syt IV was expressed in

paraventicular hypothalamic anterior parvicellular nucleus (PaAP) (Figure 3.2.6 O) and Stx 1A was mainly expressed in lateroanterior hypothalamic area (LA) (Figure 3.2.6 P). All of the investigated SPs were expressed in the raphe region (Figure 3.2.6 R to T). Syt I and SytIV were expressed in a similar pattern in the dorsal raphe in the dorsal part as well as in the inferior part. Stx 1A was expressed to a moderate extent in the whole dorsal raphe (Figure 3.2.6 T) and Stx 1A expressing neurons were sparsely distributed in the brainstem whereas the expression pattern of Syt I and Syt IV expressing neurons was more densely arranged in this region.

3.2.2.2. Gene expression of Syt I, Syt IV and Stx 1A in cortex

In the cortex, only very few expression differences could be detected as a consequence of stress exposure and *5-Htt* knockout. Only one *5-Htt* genotype effect on Syt I expression was detected in male mice with stronger expression levels in stressed *5-Htt* KO than in stressed *5-Htt* WT mice ($p = 0.0379$). Stress exposure was also shown to reduce the expression of Syt IV in *5-Htt* WT mice ($p = 0.0237$). Additionally, gender was shown to influence the expression of all three investigated SPs and has a strong influence on Syt IV as well as on Stx 1A expression but displayed only a marginal influence on the expression of Syt I. With regard to Syt I, unstressed female *5-Htt* KO mice displayed higher Syt I mRNA concentrations than males of the same experimental group ($p = 0.0435$). In contrast to Syt I, the expression levels of Syt IV and Stx 1A were higher in male compared to female mice. Each male treatment group showed significantly increased Syt IV expression compared to the respective female treatment group (unstressed *5-Htt* WT mice with p = **0.0012**; stressed *5-Htt* WT mice with p = **0.0015**; unstressed *5-Htt* KO mice with p = **0.0001;** stressed *5-Htt* KO mice with p = **0.0003**). Similar results were demonstrated for Stx 1A (unstressed *5-Htt* WT mice with p = **0.0012**; stressed *5-Htt* WT mice with p = **0.0003**; unstressed *5-Htt* KO mice with p = **0.0022;** stressed *5-Htt* KO mice with p = **0.0006**).

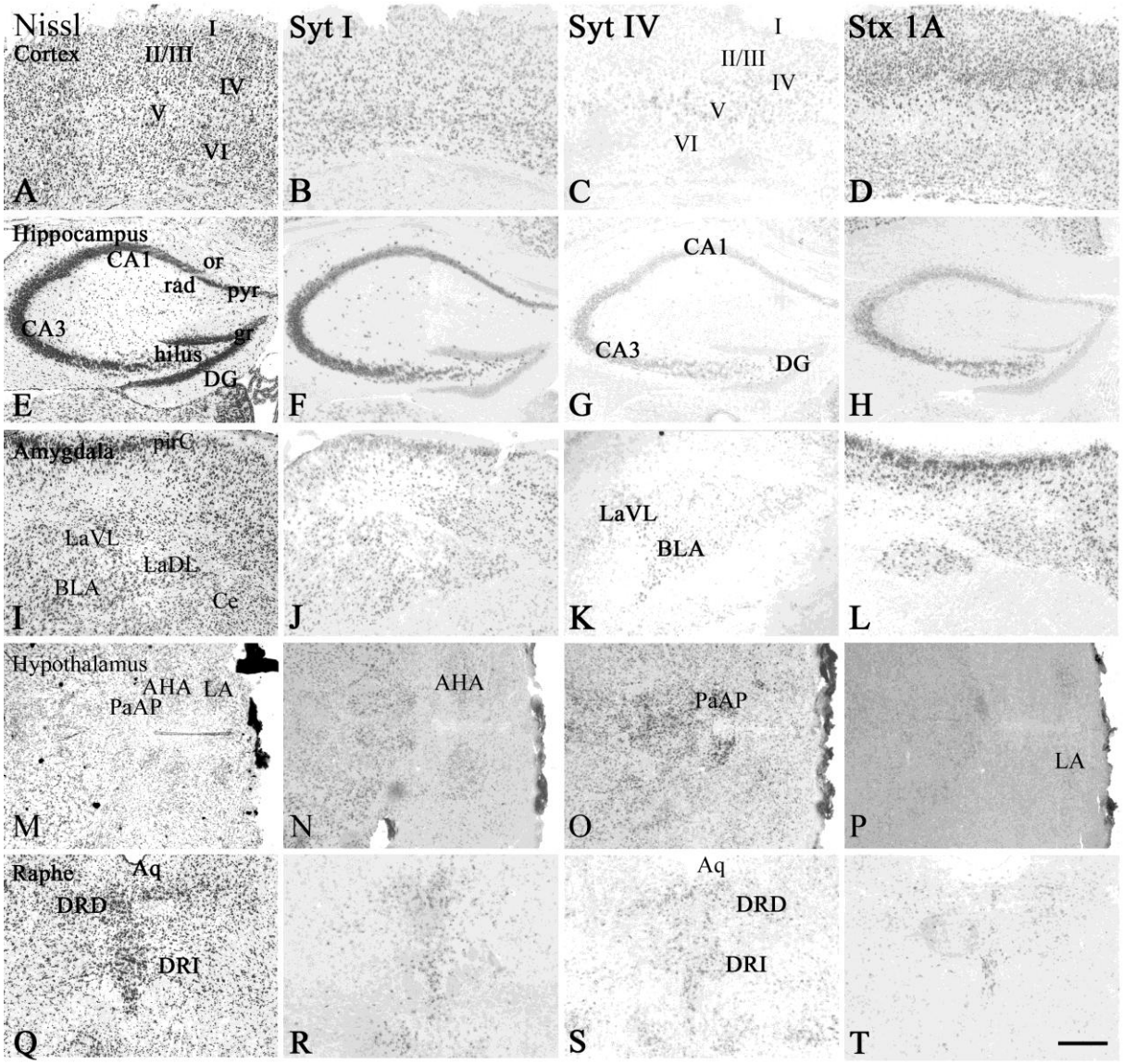


Figure 3.2.6: *In situ*-hybridization study using non-radioactive cRNA probes for Syt I (B,F,J,N,R), Syt IV (C,G,K,O,S) and Stx 1A (D,H,L,P,T) in cortex (A-D), hippocampus (E-H), amygdala (I-L), hypothalamus (M-P) and raphe (Q-R) in the brain of *5-Htt* WT mice. A, E, I, M, Q: Nissl staining, AHA, anterior hypothalamic area; Aq, aqueduct; BLA, basolateral amygdaloid nucleus, anterior; DG, dentate gyrus; CA, cornu ammonis; Ce, central nucleus; DRD, dorsal raphe, dorsal part; DRI, dorsal raphe, inferior part; gr, granule cell layer; LA, lateroanterior hypothalamic area; LaDL, lateral amygdaloid nucleus, dorsolateral; LaVL, lateral amygdaloid nucleus, ventrolateral; mol, molecular layer; or, Stratum oriens; PaAP, paraventicular hypothalamic anterior parvicellular nucleus; pyr, Stratum pyramidale; rad, Stratum radiatum. Scale bar in T: 200 µm for each picture.

3.2.2.3. Gene expression of Syt I, Syt IV and Stx 1A in the hippocampus

In the hippocampus, the largest number of stress- as well as *5-Htt* genotype-dependent effects on the expression of Syt I, Syt IV and Stx 1A (compared to the other brain regions investigated) were demonstrated in addition to some gender effects. As a result of stress exposure, Syt I mRNA concentrations were shown to be significantly increased in stressed female *5-Htt* WT mice compared to unstressed mice of same gender and *5-Htt* genotype (p = **0.0012**), whereas Stx 1A only showed a tendency to increased expression in the same experimental groups (p = 0.0047). Syt IV, however, showed a tendency to having increased expression levels in stressed compared to unstressed male *5-Htt* KO mice (p = 0.0188). Moreover, three *5-Htt* genotype-dependent gene expression differences with higher expression levels in *5-Htt* KO mice compared to *5-Htt* WT mice in each case were revealed: first of all, Syt I mRNA concentrations were significantly increased in female unstressed *5-Htt* KO mice compared to female unstressed *5-Htt* WT mice (p = **0.0027**), secondly Stx1A tended to have higher expression levels in female unstressed *5-Htt* KO mice than in female unstressed *5-Htt* WT mice (p = 0.036) and thirdly, Stx 1A also showed a tendency to higher mRNA concentrations in stressed male *5-Htt* KO versus *5-Htt* WT mice of the particular experimental group (p = 0.0081). In addition to these stress- and genotype-dependent effects, additional gender effects were revealed by using qRT-PCR. Whereas Syt I expression was shown to be higher in females than in males (stressed female versus male *5-Htt* WT mice with p = **0.0003**; unstressed female versus male *5-Htt* KO mice with p = 0.0356; stressed female versus male *5-Htt* KO mice with p = 0.0123), Syt IV expression levels were higher in male in comparison to female mice (unstressed male versus female *5-Htt* KO mice with p = 0.0078; stressed male versus female *5-Htt* KO mice with p = **0.001**). The only gender effects concerning the expression of Stx 1A are seen in the group of stressed *5-Htt* WTs with higher expression levels in females compared to males (p = **0.0015**)

3.2.2.4. Gene expression of Syt I, Syt IV and Stx 1A in the hypothalamus

In the hypothalamus, expression differences due to stress exposure and *5-Htt* knockout were exclusively shown by using Stx 1A-specific qRT-PCR primer pairs, since Syt I as well as Syt IV showed only a few gender-dependent expression differences. As shown for the cortex, the hippocampus, and the amygdala, gender also inversely affects Syt I and Syt IV expression in the hypothalamus. In this brain region, Syt I exhibited higher expression levels in males than in females (unstressed male versus female *5-Htt* WT mice with p = 0.0082) and Syt IV expression levels were higher in female compared to male mice (unstressed female versus male *5-Htt* KO mice with p = **0.0022**). Gender-dependent effects on Stx 1A expression were revealed in stressed *5-Htt* WT mice (p = **0.0002**) and in unstressed *5-Htt* KO mice (p = 0.0133) with higher expression levels detected in females than in males in both cases. Tendential Stx 1A expression differences resulting from stress exposure were detected in male *5-Htt* KO mice (p = 0.0411) and female *5-Htt* WT mice (p = 0.0127) with increased expression levels in

stressed compared to unstressed mice. Additionally, the *5-Htt* KO appears to affect Stx 1A expression as we revealed a tendency to higher Stx 1A mRNA concentrations in male stressed *5-Htt* KO mice compared with *5-Htt* WT mice (p = 0.0484).

3.2.2.5. Gene expression of Syt I, Syt IV and Stx 1A in the amygdala

In the amygdala, Stx 1A expression was neither influenced by *5-Htt* genotype nor by stress and gender (p-value of Kruskal Wallis test > 0,1). In contrast to Stx 1A at least small effects of gender and stress on the expression on Syt I and Syt IV were detected in this brain region. Gender was shown to affect the expression of Syt I in stressed *5-Htt* WT mice with higher expression levels detected in females compared to males (p = **0.0012**). In contrast to Syt I, Syt IV mRNA concentrations were higher in male unstressed *5-Htt* KO mice compared to females of the respective experimental group (p = 0.0086). Restraint stress only affected the expression levels of Syt I in male *5-Htt* WT mice which tended to show reduced mRNA concentrations in stressed compared to unstressed mice (p = 0.0474).

3.2.2.6. Gene expression of Syt I, Syt IV and Stx 1A in the raphe

In the raphe, Stx 1A expression was neither influenced by *5-Htt* genotype nor by stress and gender (p-value of Kruskal Wallis test > 0,1), just like in the amygdala. But we detected gender-dependent effects on the expression of Syt I and at least one small effect of stress on the expression of Syt IV. Syt I mRNA concentrations were shown to be higher in female stressed *5-Htt* WT mice (p = **0.003**), female unstressed *5-Htt* KO mice (p = 0.022) and female stressed *5-Htt* KO mice (p = **0.0015**) compared to males of the corresponding experimental group. Syt IV was shown to tend to decreased mRNA concentrations in stressed male *5-Htt* KO mice compared to unstressed *5-Htt* KO mice (p = 0.0373).

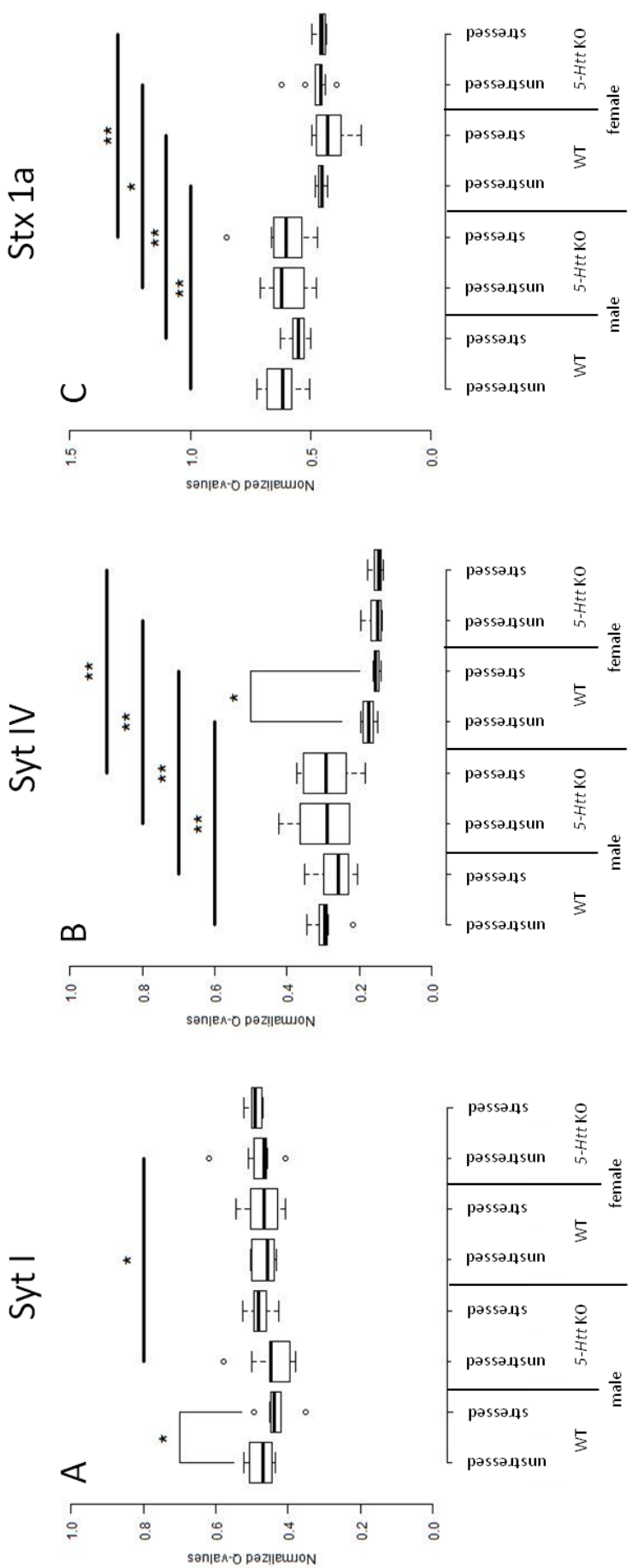


Figure 3.2.7: Expression of Syt I (A), Syt IV (B) and Stx 1a (C) in cortex of male and female stressed and unstressed WT and 5-*Htt* KO mice. Strong gender differences could be found concerning the expression of Syt IV (B) and Stx 1a (C). In both cases the expression was stronger in male mice than in female mice. These effects could not be seen concerning the expression of Syt I (A). Stress effects on Syt I could be found in male WT mice (A) and on female WT mice (B).

Kruskal-Wallis test Syt I: p = 0,06384, Kruskal-Wallis test Syt IV: p = 0,00000006, Kruskal-Wallis test Stx 1a: p = 0,0000006;
Wilcoxon Rank sum test: **: p < 0,004 (significant with Bonferroni correction), *: 0,05 > p > 0,004 (tendency). Thin lines: stress effects and genotype effects; thick lines: gender effects.

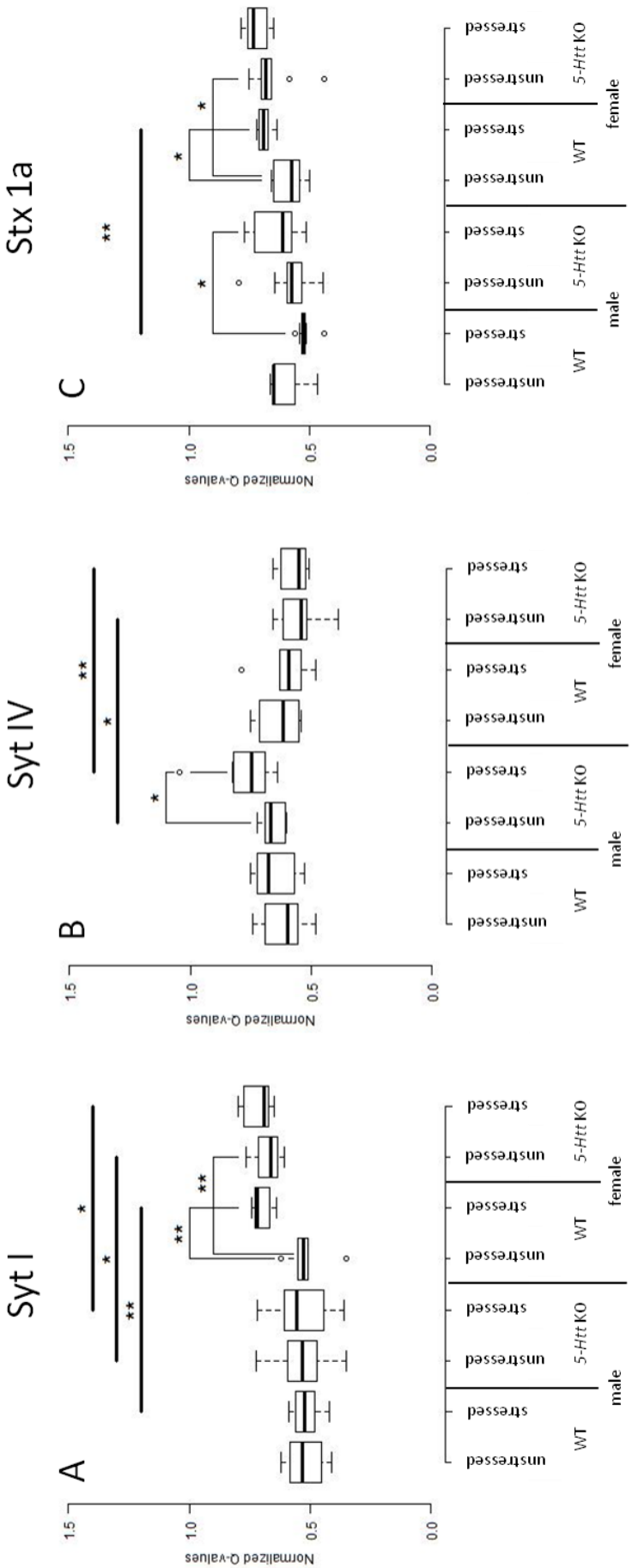


Figure 3.2.8: Expression of Syt I (A), Syt IV (B) and Stx 1a (C) in the hippocampus of male and female stressed and unstressed WT and *5-Htt* KO mice. Gender effects could be demonstrated concerning the expression of Syt I (A). In *5-Htt* KO mice, the expression of Syt IV was stronger in males than in females (B). Stress influenced the expression of Syt I in female WT mice (A), the expression of Syt IV in male *5-Htt* KO mice (B) as well as the expression of Stx 1a in female WT mice (C). Also gender effects could be shown in the expression of Syt I (A) and Stx 1a (C).

Kruskal-Wallis test Syt I: $p = 0{,}00003$, Kruskal-Wallis test Syt IV: $p = 0{,}0016$, Kruskal-Wallis test Stx 1a: $p = 0{,}00014$; Wilcoxon Rank sum test: **: $p < 0{,}004$ (significant with Bonferroni correction), *: $0{,}05 > p > 0{,}004$ (tendency). Thin lines: stress effects and genotype effects; thick lines: gender effects.

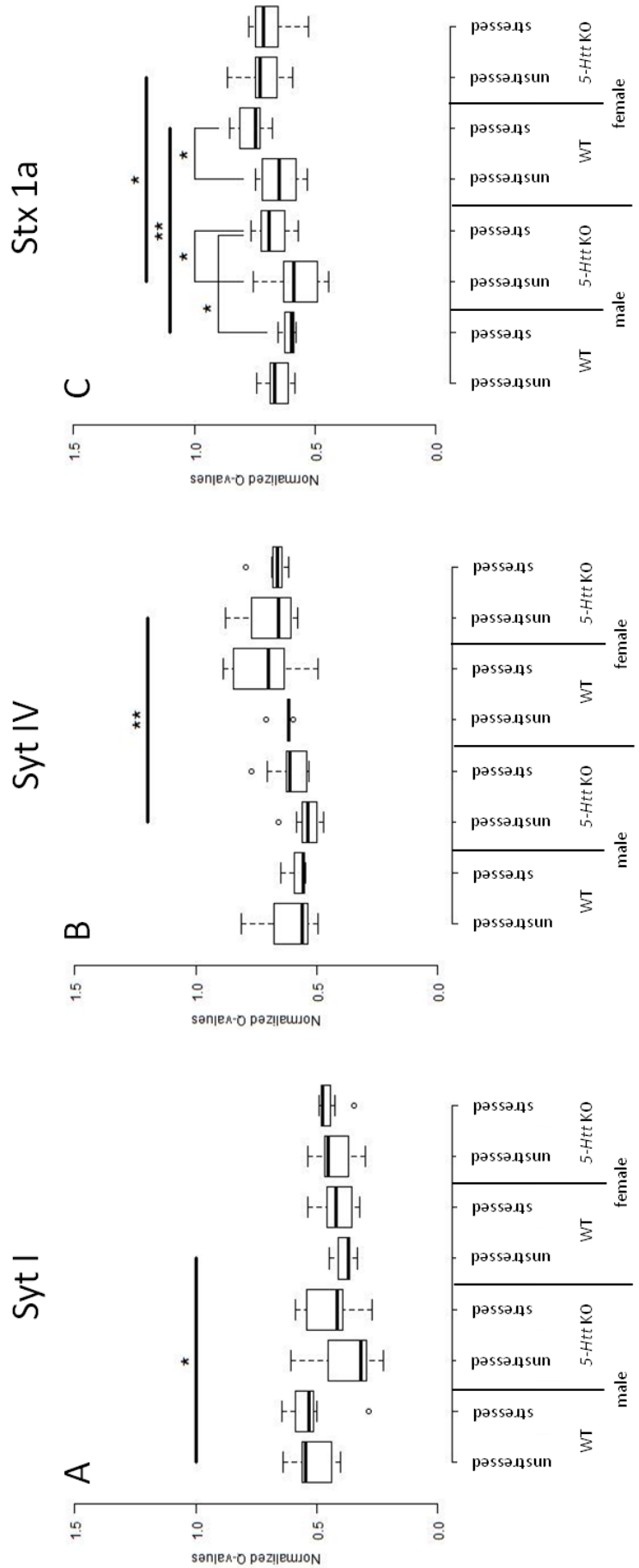


Figure 3.2.9: Expression of Syt I (A), Syt IV (B) and Stx 1a (C) in the hypothalamus of male and female stressed and unstressed WT and *5-Htt* KO mice. Gender effects could be demonstrated concerning the expression of Syt I (A), Syt IV (B) and Stx 1a (C). Stress influenced the expression of Stx 1a in hypothalamus of male *5-Htt* KO mice as well as its expression in female WT mice (C). Also an influence of genotype on the expression of Stx 1a could be shown in stressed male mice (C).

Kruskal-Wallis test Syt I: p = 0,0456, Kruskal-Wallis test Syt IV: p = 0,0024, Kruskal-Wallis test Stx 1a: p = 0,0009; Wilcoxon Rank sum test: **: p < 0,004 (significant with Bonferroni correction), *: 0,05 > p > 0,004 (tendency). Thin lines: stress effects and genotype effects; thick lines: gender effects.

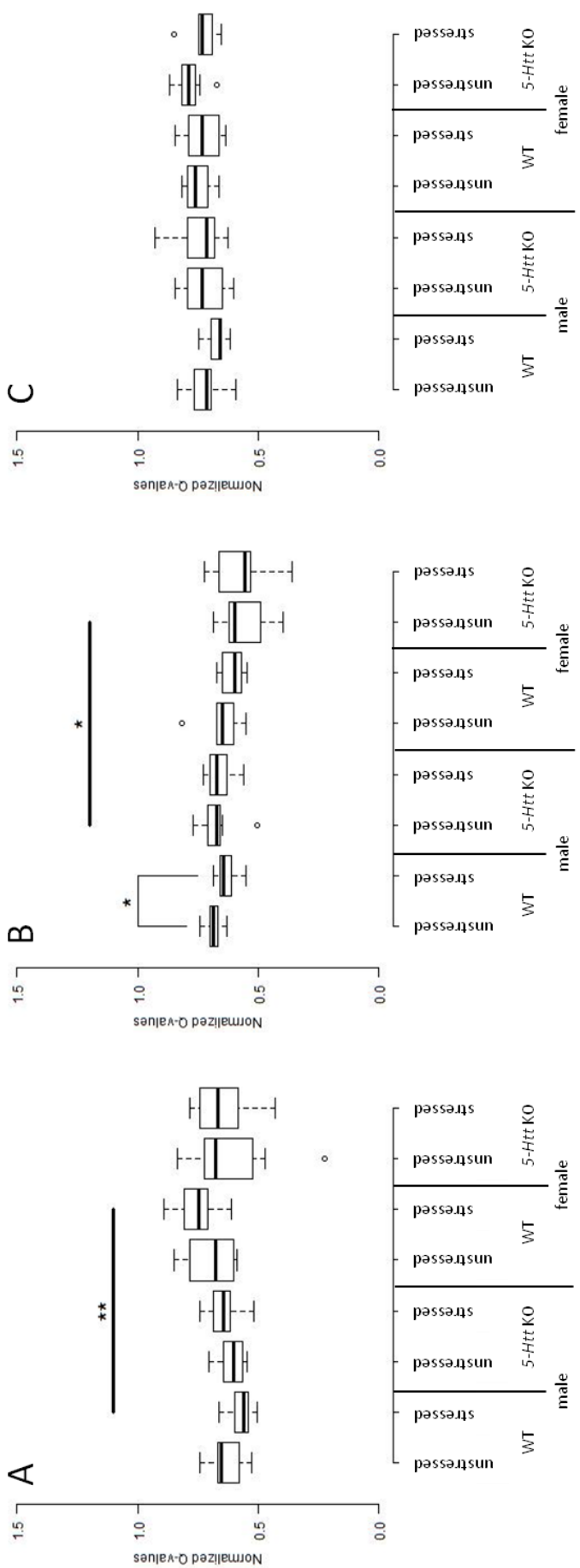


Figure 3.2.10: Expression of Syt I (A), Syt IV (B) and Stx 1a (C) in the amygdala of male and female stressed and unstressed WT and *5-Htt* KO mice. Single gender effects could be demonstrated concerning the expression of Syt I (A) and Syt IV (B). Stress influenced the expression of Syt IV in the amygdala of male WT KO mice (B).

Kruskal-Wallis test Syt I: p = 0,0182, Kruskal-Wallis test Syt IV: p = 0,0109, Kruskal-Wallis test Stx 1a: p = 0,1979; Wilcoxon Rank sum test: **: p < 0,004 (significant with Bonferroni correction), *: 0,05 > p > 0,004 (tendency). Thin lines: stress effects and genotype effects; thick lines: gender effects.

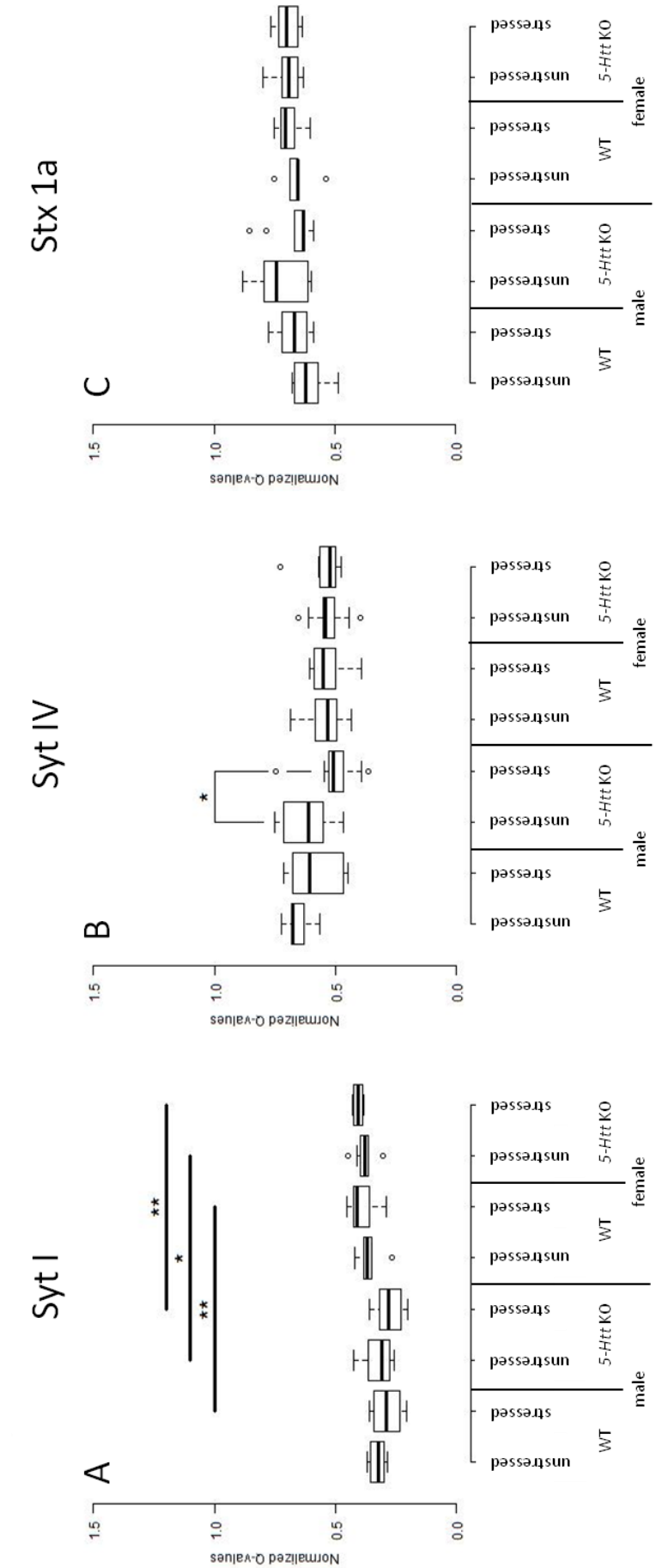


Figure 3.2.11: Expression of Syt I (A), Syt IV (B) and Stx 1a (C) in raphe of male and female stressed and unstressed WT and *5-Htt* KO mice. Gender effects could be demonstrated concerning the expression of Syt I (A). Stress influenced the expression of Syt IV in raphe of male *5-Htt* KO mice (B).

Kruskal-Wallis test Syt I: p = 0,00002, Kruskal-Wallis test Syt IV: p = 0,0366, Kruskal-Wallis test Stx 1a: p = 0,21; Wilcoxon Rank sum test: **: p < 0,004 (significant with Bonferroni correction), *: 0,05 > p > 0,004 (tendency). Thin lines: stress effects and genotype effects; thick lines: gender effects.

Normalized Q-values of Syt I in cortex:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,4354	0,4451	**0,4699**	0,4759	0,5072	0,5214
Male WT stressed	7	0,3520	0,4211	**0,4374**	0,4316	0,4477	0,4944
Male KO unstressed	10	0,3799	0,3993	**0,4471**	0,4454	0,4505	0,5799
Male KO stressed	7	0,4274	0,4598	**0,4831**	0,4775	0,4939	0,5250
Female WT unstressed	6	0,4324	0,4428	**0,4566**	0,4650	0,4911	0,5036
Female WT stressed	8	0,4091	0,4287	**0,4656**	0,4682	0,5028	0,5435
Female KO unstressed	9	0,4072	0,4602	**0,4677**	0,4835	0,4933	0,6186
Female KO stressed	8	0,4688	0,4732	**0,4904**	0,4896	0,5005	0,5214

Normalized Q-values of SytIV in cortex:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,2192	0,2883	**0,2960**	0,2943	0,3122	0,3440
Male WT stressed	7	0,2069	0,2304	**0,2595**	0,2685	0,3002	0,3520
Male KO unstressed	10	0,2259	0,2331	**0,2900**	0,3031	0,3618	0,4235
Male KO stressed	7	0,1843	0,2374	**0,2933**	0,2904	0,3537	0,3730
Female WT unstressed	6	0,1487	0,1647	**0,1760**	0,1750	0,1890	0,1953
Female WT stressed	8	0,1417	0,1493	**0,1568**	0,1539	0,1594	0,1627
Female KO unstressed	8	0,1371	0,1400	**0,1510**	0,1564	0,1641	0,1958
Female KO stressed	8	0,1331	0,1423	**0,1472**	0,1512	0,1586	0,1789

Normalized Q-values of Stx 1A in cortex:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,5036	0,5793	**0,6158**	0,6228	0,6796	0,7222
Male WT stressed	7	0,4990	0,5275	**0,5499**	0,5540	0,5742	0,6258
Male KO unstressed	10	0,4787	0,5362	**0,6208**	0,6017	0,6502	0,7073
Male KO stressed	7	0,4699	0,5374	**0,6017**	0,6144	0,6525	0,8490
Female WT unstressed	6	0,4294	0,4479	**0,4518**	0,4549	0,4629	0,4831
Female WT stressed	8	0,2893	0,3848	**0,4285**	0,4180	0,4677	0,4967
Female KO unstressed	9	0,3906	0,4550	**0,4581**	0,4772	0,4798	0,6186
Female KO stressed	8	0,4324	0,4413	**0,4544**	0,4545	0,4592	0,4944

Normalized Q-values of Syt I in the hippocampus:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,4123	0,4549	**0,5316**	0,5202	0,5838	0,6205
Male WT stressed	8	0,4219	0,4838	**0,5236**	0,5167	0,5523	0,5871
Male KO unstressed	9	0,3515	0,4736	**0,5303**	0,5330	0,5925	0,7211
Male KO stressed	9	0,3606	0,4439	**0,5567**	0,5422	0,6092	0,7194
Female WT unstressed	6	0,3499	0,5096	**0,5294**	0,5149	0,5493	0,6220
Female WT stressed	7	0,6380	0,6663	**0,7194**	0,6984	0,7278	0,7431
Female KO unstressed	8	0,6064	0,6434	**0,6605**	0,6738	0,7084	0,7675
Female KO stressed	8	0,6469	0,6724	**0,6909**	0,7142	0,7676	0,7983

Normalized Q-values of Syt IV in the hippocampus:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,4824	0,5556	**0,5953**	0,6156	0,6886	0,7431
Male WT stressed	8	0,5291	0,5849	**0,6776**	0,6532	0,7207	0,7535
Male KO unstressed	9	0,6036	0,6092	**0,6682**	0,6582	0,6885	0,7211
Male KO stressed	9	0,6409	0,6885	**0,7448**	0,7638	0,8207	1,0460
Female WT unstressed	6	0,5415	0,5644	**0,6170**	0,6315	0,6917	0,7500
Female WT stressed	8	0,4824	0,5573	**0,5919**	0,5994	0,6307	0,7891
Female KO unstressed	9	0,3882	0,5170	**0,5415**	0,5480	0,6148	0,6590
Female KO stressed	8	0,5111	0,5255	**0,5492**	0,5697	0,6086	0,6574

Normalized Q-values of Stx 1A in the hippocampus:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,4692	0,5605	**0,6469**	0,5994	0,6476	0,6636
Male WT stressed	8	0,4398	0,5194	**0,5218**	0,5179	0,5282	0,5593
Male KO unstressed	9	0,4449	0,5316	**0,5723**	0,5802	0,5912	0,7946
Male KO stressed	9	0,5134	0,5737	**0,6106**	0,6336	0,7295	0,7711
Female WT unstressed	6	0,4983	0,5475	**0,5751**	0,5829	0,6334	0,6574
Female WT stressed	7	0,6351	0,6713	**0,6901**	0,6862	0,7080	0,7194
Female KO unstressed	9	0,4398	0,6590	**0,6822**	0,6554	0,6998	0,7500
Female KO stressed	8	0,6469	0,6827	**0,7338**	0,7198	0,7505	0,7818

Normalized Q-values of Syt I in the hypothalamus:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,4002	0,4383	**0,5442**	0,5109	0,5584	0,6388
Male WT stressed	7	0,2847	0,5120	**0,5342**	0,5232	0,5872	0,6449
Male KO unstressed	9	0,2258	0,2943	**0,3156**	0,3794	0,4526	0,6063
Male KO stressed	9	0,2734	0,3902	**0,4142**	0,4539	0,5417	0,5897
Female WT unstressed	6	0,3313	0,3628	**0,3712**	0,3821	0,4015	0,4473
Female WT stressed	8	0,3207	0,3566	**0,4201**	0,4154	0,4487	0,5389
Female KO unstressed	9	0,2973	0,3705	**0,4551**	0,4262	0,4650	0,5365
Female KO stressed	7	0,3452	0,4434	**0,4746**	0,4517	0,4819	0,4914

Normalized Q-values of Syt IV in the hypothalamus:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,4952	0,5375	**0,5597**	0,6135	0,6755	0,8138
Male WT stressed	8	0,5482	0,5526	**0,5571**	0,5755	0,5936	0,6489
Male KO unstressed	10	0,4739	0,5039	**0,5381**	0,5394	0,5565	0,6579
Male KO stressed	9	0,5319	0,5394	**0,6096**	0,6118	0,6268	0,7681
Female WT unstressed	6	0,5971	0,6125	**0,6174**	0,6290	0,6192	0,7117
Female WT stressed	7	0,4963	0,6344	**0,7022**	0,7104	0,8230	0,8864
Female KO unstressed	8	0,5767	0,6144	**0,6566**	0,6905	0,7526	0,8782
Female KO stressed	7	0,6181	0,6445	**0,6640**	0,6753	0,6804	0,7952

Normalized Q-values of Stx 1A in the hypothalamus:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,5834	0,6107	**0,6687**	0,6558	0,6867	0,7436
Male WT stressed	8	0,5808	0,5909	**0,5999**	0,6089	0,6243	0,6549
Male KO unstressed	10	0,4432	0,4938	**0,5896**	0,5744	0,6297	0,7540
Male KO stressed	9	0,5688	0,6253	**0,6922**	0,6742	0,7233	0,7663
Female WT unstressed	6	0,5319	0,5967	**0,6481**	0,6455	0,7005	0,7471
Female WT stressed	8	0,6780	0,7279	**0,7472**	0,7633	0,8055	0,8542
Female KO unstressed	9	0,5943	0,6595	**0,7283**	0,7111	0,7454	0,8621
Female KO stressed	8	0,5283	0,6792	**0,7158**	0,6915	0,7432	0,7770

Normalized Q-values of Syt I in the amygdala:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,5296	0,5770	**0,6521**	0,6306	0,6682	0,7421
Male WT stressed	7	0,5034	0,5401	**0,5598**	0,5710	0,5955	0,6627
Male KO unstressed	10	0,5458	0,5657	**0,6037**	0,6061	0,6394	0,7053
Male KO stressed	9	0,5175	0,6155	**0,6446**	0,6378	0,6876	0,7404
Female WT unstressed	6	0,5890	0,6110	**0,6788**	0,6974	0,7702	0,8486
Female WT stressed	8	0,6126	0,7119	**0,7464**	0,7532	0,7951	0,8907
Female KO unstressed	9	0,2242	0,5236	**0,6766**	0,6207	0,7218	0,8369
Female KO stressed	8	0,4282	0,5891	**0,6661**	0,6494	0,7375	0,7844

Normalized Q-values of Syt IV in the amygdala:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,6298	0,6652	**0,6876**	0,6849	0,7013	0,7438
Male WT stressed	7	0,5496	0,6123	**0,6446**	0,6323	0,6605	0,6861
Male KO unstressed	10	0,5046	0,6596	**0,6743**	0,6751	0,7070	0,7701
Male KO stressed	8	0,5624	0,6451	**0,6735**	0,6627	0,7005	0,7269
Female WT unstressed	6	0,5509	0,6078	**0,6478**	0,6554	0,6688	0,8159
Female WT stressed	8	0,5483	0,5733	**0,5989**	0,6067	0,6449	0,6719
Female KO unstressed	8	0,3977	0,5233	**0,5996**	0,5632	0,6190	0,6876
Female KO stressed	8	0,3601	0,5330	**0,5534**	0,5730	0,6478	0,7235

Normalized Q-values of Stx 1A in the amygdala:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,5931	0,6950	**0,7135**	0,7226	0,7631	0,8349
Male WT stressed	7	0,6140	0,6513	**0,6581**	0,6732	0,6961	0,7456
Male KO unstressed	10	0,6000	0,6525	**0,7311**	0,7203	0,7873	0,8427
Male KO stressed	9	0,6241	0,6829	**0,7152**	0,7424	0,7954	0,9307
Female WT unstressed	6	0,6642	0,7182	**0,7588**	0,7499	0,7877	0,8159
Female WT stressed	8	0,6372	0,6733	**0,7325**	0,7307	0,7757	0,8427
Female KO unstressed	9	0,6704	0,7612	**0,7899**	0,7812	0,8178	0,8664
Female KO stressed	8	0,6551	0,6904	**0,7345**	0,7309	0,7473	0,8486

Normalized Q-values of Syt I in raphe:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,2869	0,3001	**0,3221**	0,3271	0,3549	0,3708
Male WT stressed	8	0,2053	0,2371	**0,2901**	0,2868	0,3370	0,3615
Male KO unstressed	10	0,2586	0,2786	**0,3104**	0,3229	0,3615	0,4230
Male KO stressed	7	0,2020	0,2287	**0,2784**	0,2757	0,3157	0,3607
Female WT unstressed	6	0,2671	0,3519	**0,3701**	0,3600	0,3810	0,4221
Female WT stressed	8	0,2883	0,3613	**0,4096**	0,3914	0,4223	0,4523
Female KO unstressed	9	0,3040	0,3649	**0,3786**	0,3803	0,3984	0,4492
Female KO stressed	8	0,3848	0,3918	**0,4060**	0,4069	0,4235	0,4289

Normalized Q-values of Syt IV in raphe:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,5634	0,6297	**0,6762**	0,6551	0,6817	0,7230
Male WT stressed	8	0,4471	0,4680	**0,6074**	0,5830	0,6620	0,7131
Male KO unstressed	10	0,4661	0,5531	**0,6115**	0,6125	0,6966	0,7520
Male KO stressed	9	0,3666	0,4683	**0,5089**	0,5043	0,5269	0,7468
Female WT unstressed	6	0,4339	0,5038	**0,5324**	0,5440	0,5719	0,6856
Female WT stressed	8	0,3938	0,5103	**0,5493**	0,5338	0,5810	0,6066
Female KO unstressed	9	0,3984	0,5031	**0,5404**	0,5314	0,5505	0,6532
Female KO stressed	8	0,4759	0,5019	**0,5209**	0,5465	0,5627	0,7281

Normalized Q-values of Stx 1A in raphe:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,4859	0,5686	**0,6222**	0,6077	0,6661	0,6762
Male WT stressed	8	0,5873	0,6213	**0,6652**	0,6695	0,7119	0,7749
Male KO unstressed	10	0,5969	0,6126	**0,7400**	0,7200	0,7949	0,8840
Male KO stressed	9	0,5859	0,6251	**0,6309**	0,6731	0,6669	0,8559
Female WT unstressed	6	0,5379	0,6532	**0,6547**	0,6563	0,6795	0,7503
Female WT stressed	8	0,6010	0,6680	**0,7025**	0,6925	0,7153	0,7503
Female KO unstressed	8	0,6280	0,6601	**0,6921**	0,6943	0,7128	0,7967
Female KO stressed	8	0,6338	0,6577	**0,6984**	0,6959	0,7200	0,7660

3.2.3. Gene expression of CRH, CRHR1 and CRHR2 in different brain regions – strong gender effects in the hypothalamus

Although neither stress nor genotype had clear and consistent effects on the expression of CRH and its two receptors in the here investigated brain regions, it was surprising that gender seems to have a strong influence on the expression of CRH as well as CRHR1 and also CRHR2, not only in the hypothalamus, where gender effects were strongest, but also in the cortex, the hippocampus and the amygdala where the expression of CRH as well as the expression of CRHR1 were influenced clearly by gender. Results of qRT-PCR were described in detail in the following chapters:

3.2.3.1. Gene expression of CRH, CRHR1 and CRHR2 in cortex

In cortex, the expression of CRH (Figure 3.2.12 A) was tendentially increased after stress in male *5-Htt* WT mice (p = **0.0376**) but in contrast to this tendentially decreased after stress in female *5-Htt* KO mice (p = **0.0193**) whereas no significant stress effects could be found in female *5-Htt* WT mice and in male *5-Htt* KO mice. Only a possible decrease of CRH expression could be demonstrated in female *5-Htt* WT mice (p = 0.0875). The expression of CRHR1 in cortex (Figure 3.2.12 B) wasn't changed at all in consequence of stress of genotype. Cortical CRHR2 expression (Figure 3.2.12 C) was not changed in consequence of stress, genotype or gender.

There were effects of the *5-Htt* genotype on the expression levels of CRH in male *5-Htt* KO mice (p = 0.0549) and in female *5-Htt* KO mice (p = 0.0599) but these effects were not significant and not even tendential. Cortical CRH was expressed more strongly in male stressed *5-Htt* WT mice than in female stressed *5-Htt* WT mice (p = **0.003**). The similar effect did exist in stressed *5-Htt* KO mice (p = 0.0784) but this difference in gene expression was neither statistically significant nor statistically tendential.

A strong influence of gender on the expression of CRHR1 in stressed *5-Htt* WT mice (p = **0.0233**) and in unstressed *5-Htt* KO mice (p = **0.0015**) could be detected. In both cases, the expression was stronger in male mice than in female mice. The same effect could be shown in unstressed *5-Htt* WT mice (p = 0.0528) but this difference was not statistically clear.

3.2.3.2. Gene expression of CRH, CRHR1 and CRHR2 in the hippocampus

In the hippocampus, the expression of CRH wasn't changed in consequence of stress (Figure 3.2.13 A) whereas the expression of CRHR1 in hippocampus (Figure 3.2.13 B) was increased by stress in female *5-Htt* WT mice (p = **0.0449**) and to a minor degree in male *5-Htt* WT mice (p = 0.0839). The hippocampal expression of CRHR2 (Figure 3.2.13 C) was not changed by stress, genotype or gender.

In contrast to stress, the *5-Htt* genotype changed the expression of CRH in unstressed female mice (p = **0.0449**) with increased CRH mRNA levels in *5-Htt* KO mice compared to *5-Htt* WT mice.

An influence of gender on the expression of CRH was found in *5-Htt* WT mice (unstressed: p = **0.0127**, stressed: p = **0.0252**) with higher expression levels in male mice compared to female mice. More

gender effects could be demonstrated regarding the expression of CRHR1, e.g. in stressed *5-Htt* WT mice (p = **0.0048**) which showed higher expression levels of CRHR1 mRNA in male mice compared to female mice.

3.2.3.3. Gene expression of CRH, CRHR1 and CRHR2 in the hypothalamus

In the hypothalamus, the expression of CRH (Figure 3.2.14 A) was changed by stress only in male *5-Htt* WT mice (p = **0.039**) with increased expression after stress. The expression of CRHR1 in hypothalamus (Figure 3.2.14 B) was not influenced by stress but the expression of CRHR2 in hypothalamus (Figure 3.2.14 C) was increased by stress in female *5-Htt* WT mice (p = **0.0196**).

An effect of *5-Htt* genotype on the expression of CRHR1 could be demonstrated in stressed *5-Htt* KO mice (p = **0.0428**) with higher mRNA levels in *5-Htt* WT mice than in *5-Htt* KO mice.

Regarding the expression of CRH, expression levels were always higher in female mice compared to male mice (unstressed *5-Htt* WT: p = **0.003**, stressed *5-Htt* WT: p = **0.0009**, unstressed *5-Htt* KO: p = **0.0004**, stressed *5-Htt* KO: p = **0.0009**).

Regarding the expression of CRHR1 additional gender effects could be found in unstressed *5-Htt* WT mice (p = **0.0159**), stressed *5-Htt* WT mice (p = **0.0014**) and in stressed *5-Htt* KO mice (p = **0.0159**). In contrast to the expression of CRH, the expression of CRHR1 here was higher in male mice than in female mice. Concerning the expression of CRHR2 there was an effect of gender on the expression of CRHR2 mRNA in all groups, whereas mRNA levels were shown to be higher in female mice than in male mice (unstressed *5-Htt* WT: p = **0.0065**, stressed *5-Htt* WT: p = **0.0009**, unstressed *5-Htt* KO: p = **0.0002**, stressed *5-Htt* KO: p = **0.0006**) like it was in the case of CRH expression in the hypothalamus.

3.2.3.4. Gene expression of CRH, CRHR1 and CRHR2 in the amygdala

In the amygdala, the expression of CRH (Figure 3.2.15 A) was increased after stress in male *5-Htt* KO mice (p = **0.0451**) and it was decreased after stress in female *5-Htt* WT mice (p = **0.0444**). The expression of CRHR1 in the amygdala (Figure 3.2.15 B) was not influenced neither by stress nor by genotype in male animals, but in female mice stress decreased the expression of CRHR1 in *5-Htt* WT mice (p = **0.0055**) but not in *5-Htt* KO mice. By contrast to CRH and CRHR1 the expression of CRHR2 in the amygdala (Figure 3.2.15 C) was not changed by stress, genotype or gender.

The expression of CRH was also influenced by genotype in unstressed male mice (p = **0.0244**) and in stressed female mice (p = **0.0443**). In male unstressed mice, the expression of CRH was higher in *5-Htt* WT mice than in *5-Htt* KO mice. Contrary to this, the expression of CRH was higher in *5-Htt* KO mice than in *5-Htt* WT mice in female stressed animals. In female stressed mice an additional genotype effect could be shown as stressed female *5-Htt* KO mice expressed CRHR1 more strongly than stressed female *5-Htt* WT mice (p = **0.0115**).

There was a gender dependent difference in stressed *5-Htt* WT mice (p = **0.0234**) with higher expression of CRH in males than in females. Moreover, in *5-Htt* KO mice but not in *5-Htt* WT mice the expression of CRHR1 was influenced by stress: female *5-Htt* KO mice expressed CRHR1 in the amygdala more strongly than male mice both in unstressed (p = **0.0028**) and in stressed (p = **0.0074**) mice. The same effect existed in unstressed *5-Htt* WT mice (p = 0.0626) but this difference was neither significant nor tendential.

3.2.3.5. Gene expression of CRH, CRHR1 and CRHR2 in raphe

Raphe could be shown to be a region in which the expression of CRH, CRHR1 and CRHR2 didn't react to stress, genotype or gender as in raphe, the expression of CRH was not influenced by stress, genotype and gender (Figure 3.2.16 A) and also the expression of CRHR1 (Figure 3.2.16 B) was not significantly nor tendentially influenced by each factor. There was only a very slight influence of gender on the expression of CRHR1 in stressed *5-Htt* WT mice (p = 0.065), unstressed *5-Htt* KO mice (p = 0.0572) as well as in stressed *5-Htt* KO mice (p = 0.0504). In all cases, the expression of CRHR1 seemed to be higher in female mice than in male mice.

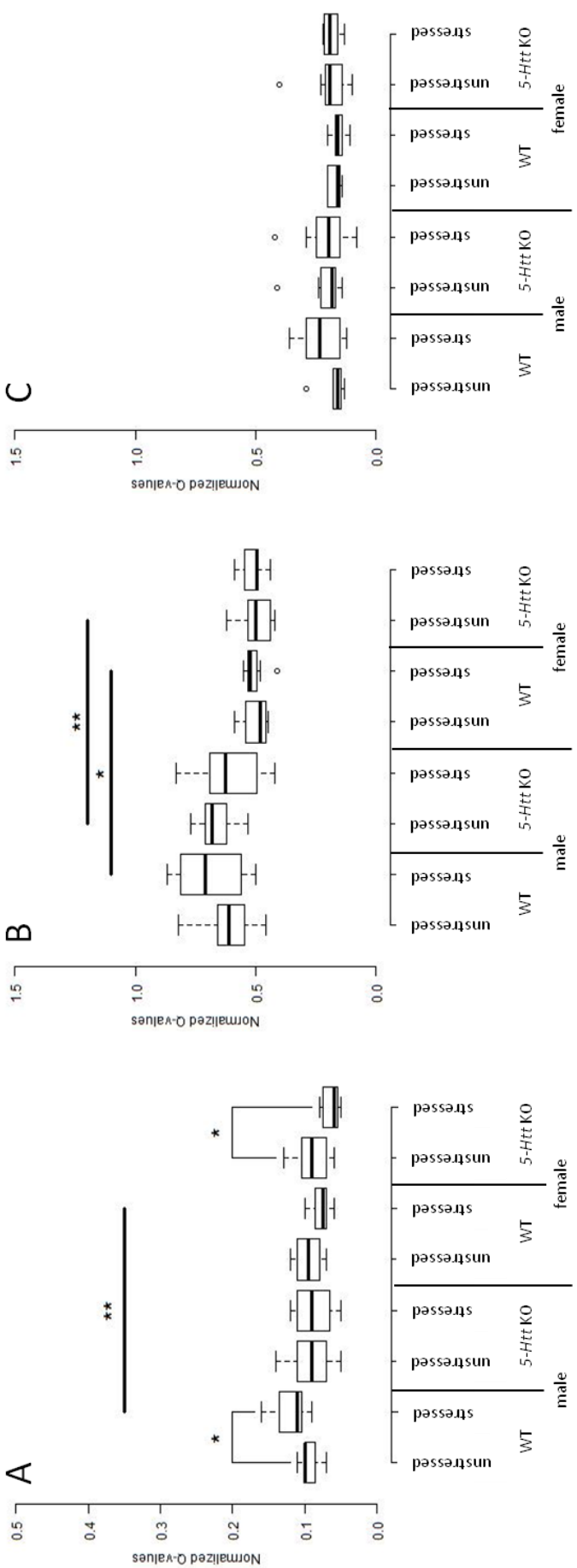


Figure 3.2.12: Expression of CRH (A), CRHR1 (B) and CRHR2 (C) in cortex of male and female stressed and unstressed WT and *5-Htt* KO mice. Gender effects could be demonstrated concerning the expression of CRH (A) and CRHR1 (B). Stress influenced the expression of CRH in cortex of male WT mice and female *5-Htt* KO mice (A).

Kruskal-Wallis test CRHSyt I: p = 0,005, Kruskal-Wallis test CRHR1: p = 0,0007, Kruskal-Wallis test CRHR2: p = 0,4408; Wilcoxon Rank sum test: **: p < 0,004 (significant with Bonferroni correction), *: 0,05 > p > 0,004 (tendency). Thin lines: stress effects and genotype effects; thick lines: gender effects.

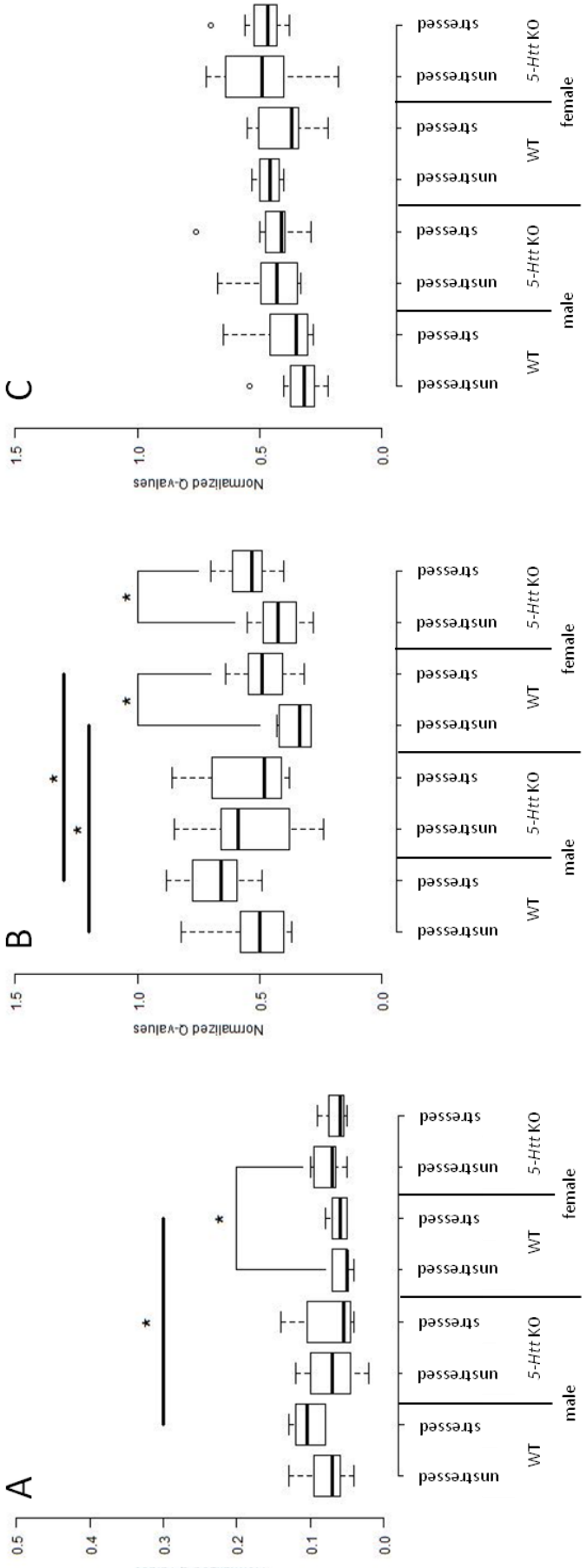


Figure 3.2.13: Expression of CRH (A), CRHR1 (B) and CRHR2 (C) in the hippocampus of male and female stressed and unstressed WT and 5-*Htt* KO mice. Gender effects could be demonstrated concerning the expression of CRH (A) and CRHR1 (B). Stress influenced the expression of CRHR1 in hippocampus of female WT mice and female 5-*Htt* KO mice (B). Genotype influenced the expression of CRH in hippocampus of female unstressed mice (A).

Kruskal-Wallis test CRH: p = 0,0768, Kruskal-Wallis test CRHR1: p = 0,0007, Kruskal-Wallis test CRHR2: p = 0,4408; Wilcoxon Rank sum test: **: p < 0,004 (significant with Bonferroni correction), *: 0,05 > p > 0,004 (tendency). Thin lines: stress effects and genotype effects; thick lines: gender effects.

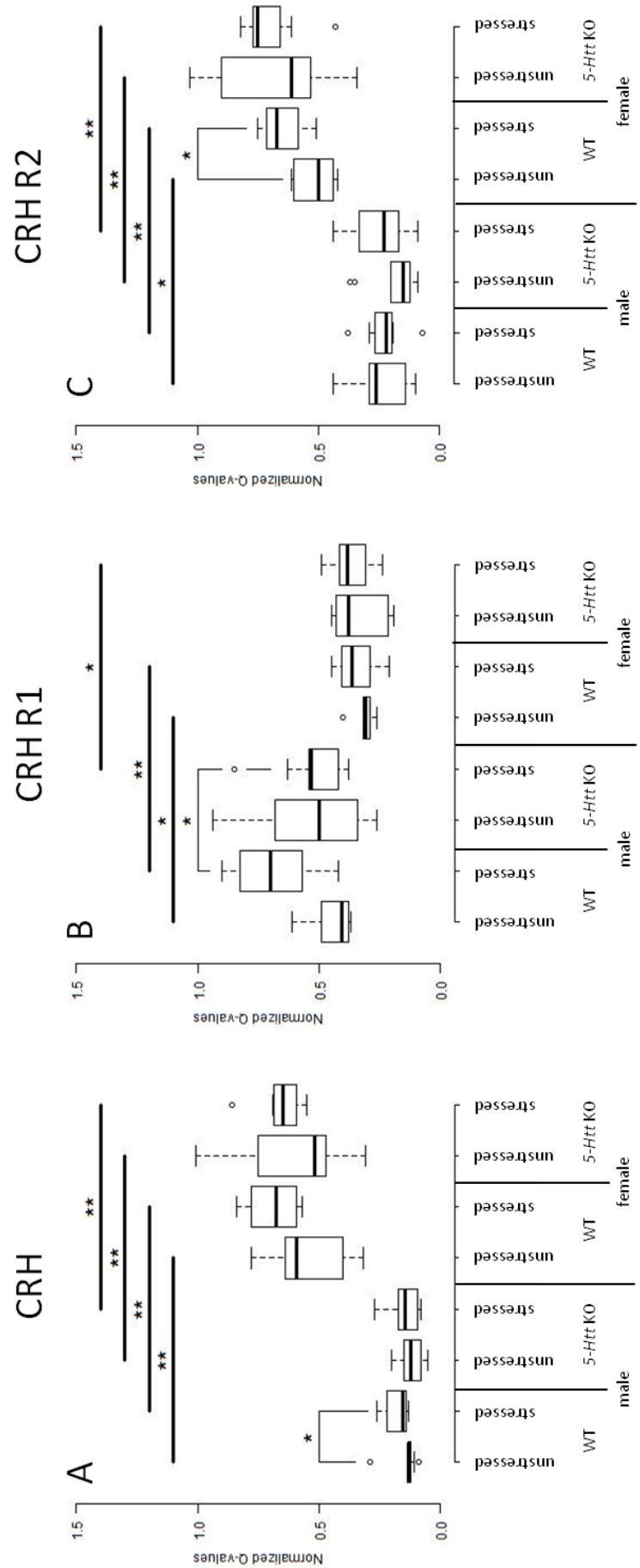


Figure 3.2.14: Expression of CRH (A), CRHR1 (B) and CRHR2 (C) in the hypothalamus of male and female stressed and unstressed WT and *5-Htt* KO mice. Gender effects could be demonstrated concerning the expression of CRH (A), CRHR1 (B) and CRHR2 (C). Stress influenced the expression of CRHR2 in hypothalamus of female WT mice (C). Genotype influenced the expression of CRHR1 in hypothalamus of male stressed mice (B).

Kruskal-Wallis test CRH: p = 0,00000003, Kruskal-Wallis test CRHR1: p = 0,0003, Kruskal-Wallis test CRHR2: p = 0,00000003; Wilcoxon Rank sum test: **: p < 0,004 (significant with Bonferroni correction), *: 0,05 > p > 0,004 (tendency). Thin lines: stress effects and genotype effects; thick lines: gender effects.

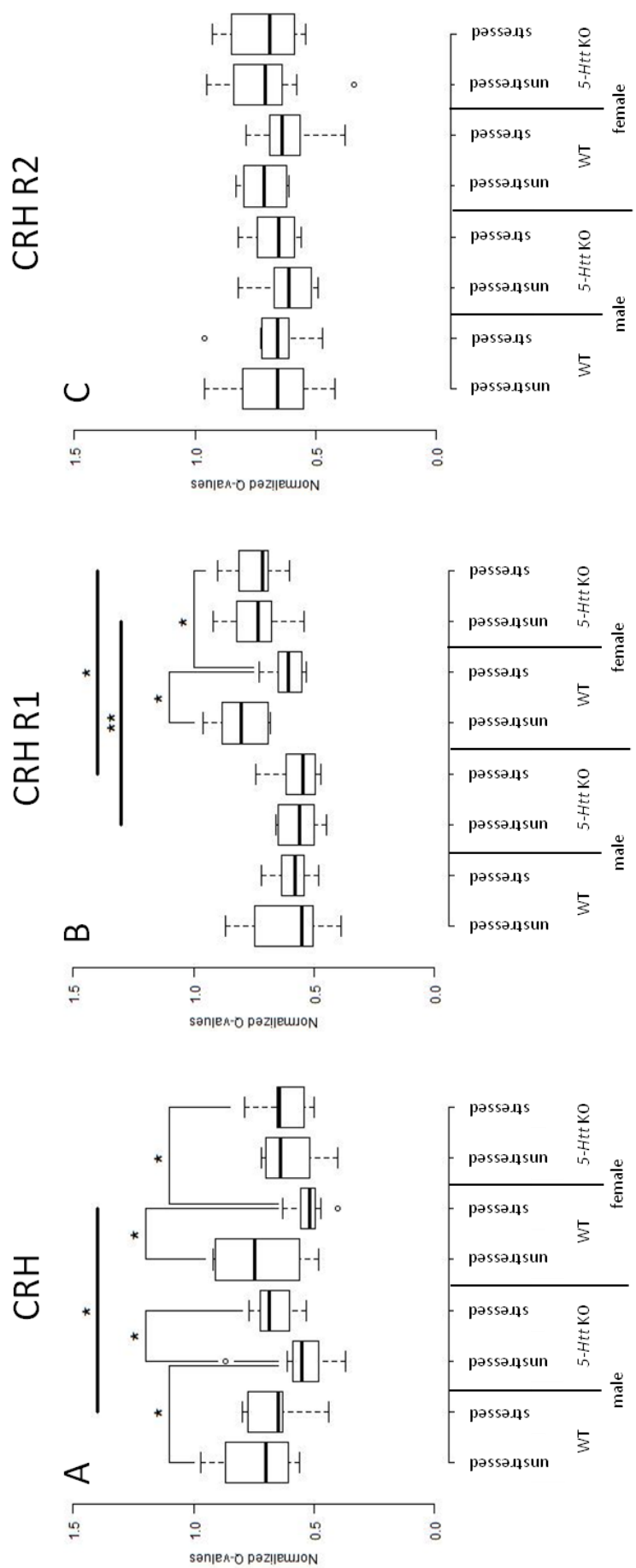


Figure 3.2.15: Expression of CRH (A), CRHR1 (B) and CRHR2 (C) in the amygdala of male and female stressed and unstressed WT and 5-*Htt* KO mice. Gender effects could be demonstrated concerning the expression of CRH (A) and CRHR1 (B). Stress influenced the expression of CRH in male 5-*Htt* KO mice, female WT mice and female 5-*Htt* KO mice (A). Also the expression of CRHR1 was influenced by stress in female WT mice (B). An effect of genotype on the expression of CRH could be shown in unstressed male mice and stressed female mice (A). The expression of CRHR1 was influenced by genotype in stressed female mice (B).

Kruskal-Wallis test CRH: p = 0,0158, Kruskal-Wallis test CRHR1: p = 0,0004, Kruskal-Wallis test CRHR2: p = 0,6798; Wilcoxon Rank sum test: **: p < 0,004 (significant with Bonferroni correction), *: 0,05 > p > 0,004 (tendency). Thin lines: stress effects and genotype effects; thick lines: gender effects.

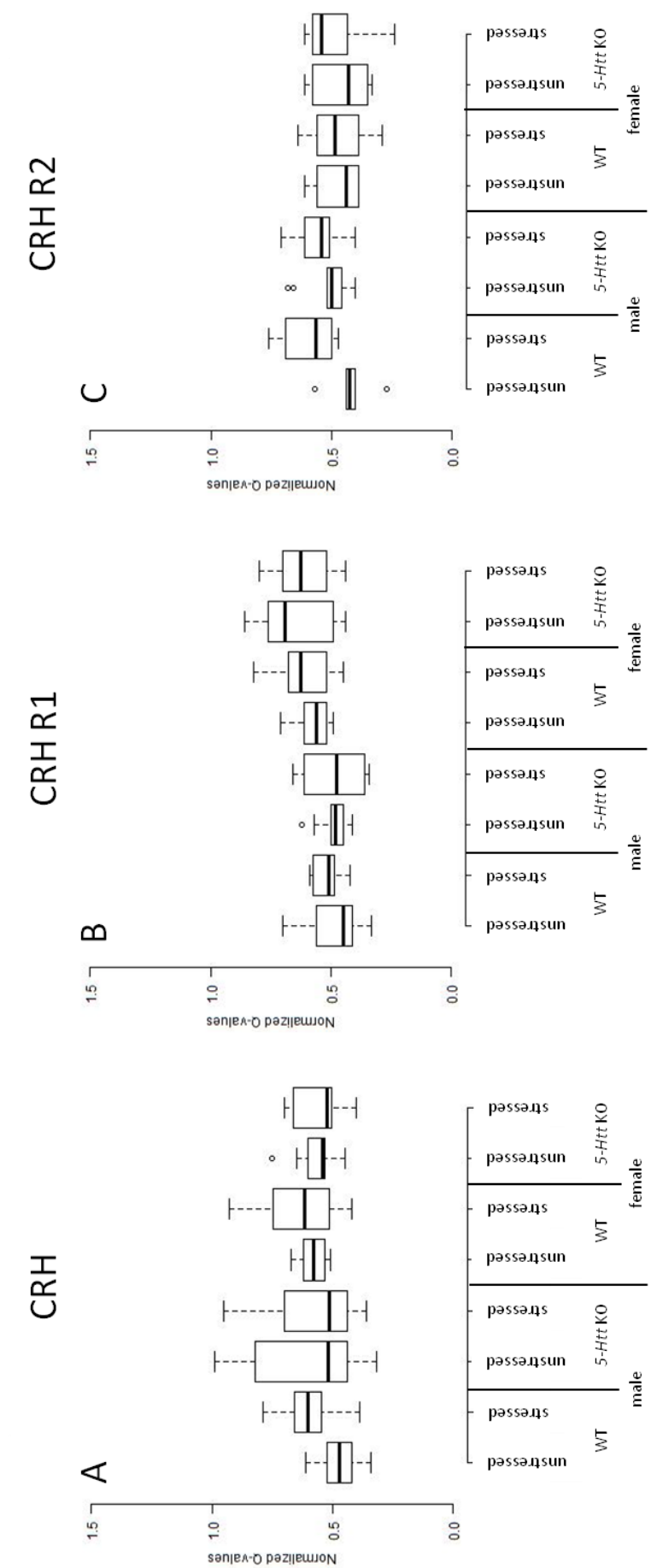


Figure 3.2.16: Expression of CRH (A), CRHR1 (B) and CRHR2 (C) in raphe of male and female stressed and unstressed WT and *5-Htt* KO mice. The expression of CRH and of both receptors was not significantly influenced by stress, genotype or gender.

Kruskal-Wallis test CRH: p = 0,5098, Kruskal-Wallis test CRHR1: p = 0,0366, Kruskal-Wallis test CRHR2: p = 0,1851; Wilcoxon Rank sum test: **: p < 0,004 (significant with Bonferroni correction), *: 0,05 > p > 0,004 (tendency). Thin lines: stress effects and genotype effects; thick lines: gender effects.

Normalized Q-values of CRH in cortex:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0.0700	0.0850	0.1000	0.0929	0.1000	0.1100
Male WT stressed	7	0.0900	0.1050	0.1100	0.1200	0.1350	0.1600
Male KO unstressed	10	0,0500	0,0700	**0,0900**	0,0920	0,1100	0,1400
Male KO stressed	8	0,0500	0,0675	**0,0900**	0,0875	0,1050	0,1200
Female WT unstressed	6	0,0700	0,0825	**0,0950**	0,0950	0,1075	0,1200
Female WT stressed	8	0,0600	0,0700	**0,0750**	0,0775	0,0825	0,1000
Female KO unstressed	8	0,0600	0,0700	**0,0900**	0,0900	0,1025	0,1300
Female KO stressed	8	0,0500	0,0575	**0,0600**	0,0638	0,0725	0,0800

Normalized Q-values of CRHR1 in cortex:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,4600	0,5450	**0,6100**	0,6143	0,6600	0,8200
Male WT stressed	6	0,5000	0,5950	**0,7100**	0,6933	0,7875	0,8700
Male KO unstressed	9	0,5300	0,6200	**0,6800**	0,6678	0,7100	0,7700
Male KO stressed	7	0,4200	0,5125	**0,6250**	0,6088	0,6800	0,8300
Female WT unstressed	6	0,4500	0,4650	**0,4800**	0,5000	0,5250	0,5900
Female WT stressed	8	0,4100	0,5025	**0,5250**	0,5075	0,5300	0,5500
Female KO unstressed	9	0,4200	0,4400	**0,5000**	0,5000	0,5300	0,6200
Female KO stressed	8	0,4400	0,4900	**0,4950**	0,5112	0,5425	0,5900

Normalized Q-values of CRHR2 in cortex:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,1300	0,1450	**0,1600**	0,1757	0,1800	0,2900
Male WT stressed	6	0,1200	0,1675	**0,2350**	0,2317	0,2800	0,3600
Male KO unstressed	8	0,1400	0,1700	**0,1850**	0,2150	0,2250	0,4100
Male KO stressed	7	0,0800	0,1650	**0,1950**	0,2112	0,2300	0,4200
Female WT unstressed	6	0,1400	0,1500	**0,1600**	0,1683	0,1925	0,2000
Female WT stressed	8	0,1100	0,1450	**0,1600**	0,1562	0,1700	0,2000
Female KO unstressed	9	0,1000	0,1400	**0,1900**	0,1967	0,2100	0,4000
Female KO stressed	8	0,1300	0,1600	**0,1900**	0,1850	0,2125	0,2200

Normalized Q-values of CRH in the hippocampus:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,0400	0,0600	**0,0700**	0,0786	0,0950	0,1300
Male WT stressed	6	0,0800	0,0850	**0,1050**	0,1033	0,1175	0,1300
Male KO unstressed	8	0,0200	0,0525	**0,0700**	0,0713	0,0950	0,1200
Male KO stressed	8	0,0400	0,0475	**0,0550**	0,0738	0,0975	0,1400
Female WT unstressed	6	0,0400	0,0500	**0,0500**	0,0550	0,0650	0,0700
Female WT stressed	7	0,0500	0,0500	**0,0600**	0,0614	0,0700	0,0800
Female KO unstressed	8	0,0500	0,0675	**0,0700**	0,0763	0,0925	0,1000
Female KO stressed	8	0,0500	0,0575	**0,0600**	0,0650	0,0725	0,0900

Normalized Q-values of CRHR1 in the hippocampus:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,3700	0,4000	**0,5000**	0,5214	0,5800	0,8200
Male WT stressed	7	0,4900	0,5950	**0,6600**	0,6814	0,7750	0,8800
Male KO unstressed	9	0,2400	0,3800	**0,5900**	0,5244	0,6600	0,8500
Male KO stressed	7	0,3800	0,4100	**0,4800**	0,5513	0,6575	0,8600
Female WT unstressed	6	0,2900	0,2950	**0,3350**	0,3500	0,4050	0,4300
Female WT stressed	7	0,3200	0,4050	**0,4900**	0,4786	0,5450	0,6400
Female KO unstressed	8	0,2800	0,3600	**0,4250**	0,4188	0,4775	0,5500
Female KO stressed	8	0,4000	0,4900	**0,5300**	0,5450	0,6050	0,7000

Normalized Q-values of CRHR2 in the hippocampus:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,2200	0,2750	**0,3200**	0,3400	0,3750	0,5400
Male WT stressed	7	0,2800	0,3050	**0,3500**	0,4014	0,4600	0,6500
Male KO unstressed	8	0,3300	0,3475	**0,4300**	0,4425	0,4775	0,6700
Male KO stressed	6	0,2900	0,3950	**0,4100**	0,4571	0,4750	0,7600
Female WT unstressed	6	0,4000	0,4200	**0,4600**	0,4617	0,5000	0,5300
Female WT stressed	7	0,2200	0,3400	**0,3700**	0,4043	0,5050	0,5500
Female KO unstressed	9	0,1800	0,4000	**0,4900**	0,5022	0,6400	0,7200
Female KO stressed	8	0,3800	0,4350	**0,4650**	0,4900	0,5075	0,7000

Normalized Q-values of CRH in the hypothalamus:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,0900	0,1200	**0,1300**	0,1443	0,1300	0,2900
Male WT stressed	8	0,1300	0,1400	**0,1550**	0,1775	0,2050	0,2600
Male KO unstressed	9	0,0500	0,0800	**0,1200**	0,1211	0,1500	0,2000
Male KO stressed	8	0,0800	0,0975	**0,1450**	0,1475	0,1625	0,2700
Female WT unstressed	6	0,3200	0,4400	**0,5950**	0,5550	0,6375	0,7800
Female WT stressed	8	0,5700	0,6025	**0,6750**	0,6887	0,7650	0,8400
Female KO unstressed	9	0,3100	0,4700	**0,5200**	0,5878	0,7500	1,0100
Female KO stressed	8	0,5500	0,5975	**0,6500**	0,6587	0,6825	0,8600

Normalized Q-values of CRHR1 in the hypothalamus:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	6	0,3700	0,3850	**0,4050**	0,4433	0,4700	0,6100
Male WT stressed	8	0,4200	0,5750	**0,7000**	0,6887	0,8075	0,9000
Male KO unstressed	9	0,2600	0,3400	**0,5000**	0,5144	0,6800	0,9400
Male KO stressed	8	0,3800	0,4200	**0,5300**	0,5233	0,5400	0,8500
Female WT unstressed	6	0,2600	0,2925	**0,3100**	0,3150	0,3200	0,4000
Female WT stressed	8	0,2100	0,3100	**0,3650**	0,3475	0,4025	0,4500
Female KO unstressed	8	0,1900	0,2175	**0,3800**	0,3362	0,4250	0,4500
Female KO stressed	8	0,2400	0,3300	**0,3850**	0,3687	0,4125	0,4900

Normalized Q-values of CRHR2 in the hypothalamus:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,1000	0,1400	**0,2600**	0,2371	0,2900	0,4400
Male WT stressed	8	0,0700	0,1975	**0,2200**	0,2263	0,2525	0,3800
Male KO unstressed	9	0,0900	0,1200	**0,1500**	0,1867	0,2000	0,3700
Male KO stressed	9	0,0900	0,1825	**0,2300**	0,2440	0,3075	0,4400
Female WT unstressed	6	0,4200	0,4550	**0,5000**	0,5117	0,5750	0,6100
Female WT stressed	8	0,5100	0,5925	**0,6700**	0,6500	0,6975	0,7500
Female KO unstressed	9	0,3400	0,5300	**0,6100**	0,6678	0,9000	1,0300
Female KO stressed	8	0,4300	0,6850	**0,7500**	0,7013	0,7700	0,8200

Normalized Q-values of CRH in the amygdala:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,5600	0,6050	**0,7000**	0,7400	0,8700	0,9700
Male WT stressed	7	0,4400	0,6300	**0,6500**	0,6714	0,7750	0,8000
Male KO unstressed	10	0,3700	0,4800	**0,5500**	0,5560	0,5875	0,8700
Male KO stressed	8	0,5300	0,6250	**0,6850**	0,6650	0,7225	0,7700
Female WT unstressed	6	0,4800	0,5900	**0,7450**	0,7267	0,8850	0,9200
Female WT stressed	8	0,4000	0,5075	**0,5200**	0,5212	0,5425	0,6300
Female KO unstressed	9	0,4000	0,5200	**0,6400**	0,6033	0,7000	0,7200
Female KO stressed	8	0,5000	0,5500	**0,6450**	0,6213	0,6525	0,7900

Normalized Q-values of CRHR1 in the amygdala:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,3900	0,5050	**0,5500**	0,6157	0,7450	0,8700
Male WT stressed	7	0,4800	0,5400	**0,5800**	0,5900	0,6350	0,7200
Male KO unstressed	10	0,4500	0,5025	**0,5600**	0,5620	0,6400	0,6600
Male KO stressed	7	0,4700	0,5025	**0,5450**	0,5650	0,5925	0,7400
Female WT unstressed	6	0,6800	0,7100	**0,8050**	0,8033	0,8700	0,9600
Female WT stressed	8	0,5300	0,5550	**0,6050**	0,6088	0,6500	0,7300
Female KO unstressed	8	0,5400	0,6875	**0,7350**	0,7400	0,7950	0,9200
Female KO stressed	8	0,6000	0,6900	**0,7150**	0,7412	0,7950	0,9000

Normalized Q-values of CRHR2 in the amygdala:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,4200	0,5500	**0,6600**	0,6786	0,8050	0,9600
Male WT stressed	7	0,4700	0,6100	**0,6600**	0,6800	0,7250	0,9600
Male KO unstressed	10	0,4900	0,5325	**0,6100**	0,6190	0,6650	0,8200
Male KO stressed	7	0,5600	0,5950	**0,6550**	0,6687	0,7150	0,8200
Female WT unstressed	6	0,6100	0,6325	**0,7150**	0,7150	0,7900	0,8300
Female WT stressed	8	0,3800	0,5775	**0,6400**	0,6200	0,6900	0,7900
Female KO unstressed	9	0,3400	0,6400	**0,7100**	0,7111	0,8400	0,9500
Female KO stressed	8	0,5400	0,5950	**0,6900**	0,7163	0,8200	0,9300

Normalized Q-values of CRH in raphe:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,3400	0,4200	**0,4700**	0,4729	0,5250	0,6100
Male WT stressed	8	0,3900	0,5525	**0,6000**	0,5988	0,6400	0,7900
Male KO unstressed	9	0,3200	0,4400	**0,5200**	0,6067	0,8200	0,9900
Male KO stressed	10	0,3600	0,4400	**0,5150**	0,5780	0,6575	0,9500
Female WT unstressed	6	0,5100	0,5375	**0,5800**	0,5817	0,6150	0,6700
Female WT stressed	8	0,4200	0,5175	**0,6150**	0,6375	0,7175	0,9300
Female KO unstressed	9	0,4500	0,5300	**0,5400**	0,5722	0,6000	0,7500
Female KO stressed	8	0,4000	0,5125	**0,5250**	0,5613	0,6625	0,7000

Normalized Q-values of CRHR1 in raphe:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	7	0,3300	0,4100	**0,4500**	0,4886	0,5600	0,7000
Male WT stressed	8	0,4200	0,4875	**0,5100**	0,5187	0,5725	0,5900
Male KO unstressed	9	0,4100	0,4500	**0,4800**	0,4922	0,5000	0,6200
Male KO stressed	9	0,3400	0,3700	**0,4750**	0,4840	0,5900	0,6600
Female WT unstressed	6	0,4900	0,5275	**0,5600**	0,5750	0,6000	0,7100
Female WT stressed	8	0,4500	0,5250	**0,6250**	0,6138	0,6625	0,8200
Female KO unstressed	9	0,4400	0,4900	**0,6900**	0,6433	0,7600	0,8600
Female KO stressed	8	0,4400	0,5450	**0,6250**	0,6162	0,6750	0,8000

Normalized Q-values of CRHR2 in raphe:

Treatment group	n_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Male WT unstressed	6	0,2700	0,4050	**0,4250**	0,4217	0,4375	0,5700
Male WT stressed	6	0,4700	0,5150	**0,5650**	0,5917	0,6600	0,7600
Male KO unstressed	9	0,4000	0,4600	**0,5000**	0,5144	0,5200	0,6800
Male KO stressed	8	0,4000	0,5100	**0,5400**	0,5667	0,6100	0,7100
Female WT unstressed	6	0,3900	0,3950	**0,4400**	0,4717	0,5375	0,6100
Female WT stressed	8	0,2900	0,4000	**0,4850**	0,4750	0,5550	0,6400
Female KO unstressed	9	0,3300	0,3500	**0,4300**	0,4633	0,5800	0,6100
Female KO stressed	8	0,2400	0,4475	**0,5400**	0,4950	0,5800	0,6100

3.2.4. Summary of the gene expression study

In the following tables give an overview on all results of the qRT-PCR study. Test results of post-hoc Wilcoxon rank rum tests are listed per investigated brain region:

Gene expression in cortex									
Group1	Group2	c-Fos	Fra-2	Syt I	Syt IV	Stx 1a	CRH	CRHR1	CRHR2
Male *5-Htt* WT unstressed	Male *5-Htt* WT stressed	Kruskal-Wallis Test: P > 0,05	↑ **	↓ (*)	ns	↓ (*)	↑ *	ns	Kruskal-Wallis Test: P > 0,05
Male *5-Htt* KO unstressed	Male *5-Htt* KO stressed		ns	↑ (*)	ns	ns	ns	ns	
Male *5-Htt* WT unstressed	Male *5-Htt* KO unstressed		ns	ns	ns	ns	ns	ns	
Male *5-Htt* WT stressed	Male *5-Htt* KO stressed		↓ **	↑ *	ns	ns	↓ (*)	ns	
Female *5-Htt* WT unstressed	Female *5-Htt* WT stressed		ns	ns	↓ *	ns	↓ (*)	ns	
Female *5-Htt* KO unstressed	Female *5-Htt* KO stressed		ns	ns	ns	ns	↓ *	ns	
Female *5-Htt* WT unstressed	Female *5-Htt* KO unstressed		ns	ns	ns	ns	ns	ns	
Female *5-Htt* WT stressed	Female *5-Htt* KO stressed		ns	ns	ns	ns	↓ (*)	ns	
Male *5-Htt* WT unstressed	Female *5-Htt* WT unstressed		ns	ns	↓**	↓ **	ns	↓ (*)	
Male *5-Htt* WT stressed	Female *5-Htt* WT stressed		ns	ns	↓ **	↓**	↓**	↓*	
Male *5-Htt* KO unstressed	Female *5-Htt* KO unstressed		↑ **	↑*	↓ **	↓**	ns	↓ **	
Male *5-Htt* KO stressed	Female *5-Htt* KO stressed		↑**	ns	↓ **	↓ **	↓ (*)	ns	

Table 3.2.1: Comparisons of gene expression in cortex. Changes of gene expression are coloured in grey. ns: not significant; ↓: gene expression is lower in Group2 than in Group1. ↑: gene expression is higher in Group2 than in Group1;
**: $p < 0.004$, * : $0.004 < p < 0.05$; (*): $0.05 < p < 0.1$.

Gene expression in hippocampus									
Group1	Group2	c-Fos	Fra-2	Syt I	Syt IV	Stx 1a	CRH	CRHR1	CRHR2
Male *5-Htt* WT unstressed	Male *5-Htt* WT stressed	↑ (*)	ns	ns	ns	ns	ns	↑ (*)	Kruskal-Wallis Test: P > 0,05
Male *5-Htt* KO unstressed	Male *5-Htt* KO stressed	↑ *	↑ **	ns	↑ *	ns	ns	ns	
Male *5-Htt* WT unstressed	Male *5-Htt* KO unstressed	ns	ns	ns	↑ (*)	ns	ns	ns	
Male *5-Htt* WT stressed	Male *5-Htt* KO stressed	↑ *	↑ *	ns	ns	↑ *	ns	ns	
Female *5-Htt* WT unstressed	Female *5-Htt* WT stressed	ns	↑ *	↑ **	ns	↑ *	ns	↑ *	
Female *5-Htt* KO unstressed	Female *5-Htt* KO stressed	ns	↑ **	ns	ns	ns	ns	↑ *	
Female *5-Htt* WT unstressed	Female *5-Htt* KO unstressed	ns	ns	↑ **	ns	↑ *	↑ *	ns	
Female *5-Htt* WT stressed	Female *5-Htt* KO stressed	↑ (*)	↑ (*)	ns	ns	ns	ns	ns	
Male *5-Htt* WT unstressed	Female *5-Htt* WT unstressed	↑ *	ns	ns	ns	ns	ns	↓ *	
Male *5-Htt* WT stressed	Female *5-Htt* WT stressed	ns	↑*	↑**	ns	↑ **	↑ **	↓ **	
Male *5-Htt* KO unstressed	Female *5-Htt* KO unstressed	ns	↑*	↑ *	↓ *	↑ (*)	ns	ns	
Male *5-Htt* KO stressed	Female *5-Htt* KO stressed	↑ *	ns	↑ *	↓ **	↑ (*)	↓ (*)	ns	

Table 3.2.2: Comparisons of gene expression in the hippocampus. Changes of gene expression are coloured in grey. ns: not significant; ↓: gene expression is lower in Group2 than in Group1. ↑: gene expression is higher in Group2 than in Group1; **: $p < 0.004$, * : $0.004 < p < 0.05$; (*): $0.05 < p < 0.1$.

Gene expression in hypothalamus									
Group1	Group2	c-Fos	Fra-2	Syt I	Syt IV	Stx 1a	CRH	CRHR1	CRHR2
Male *5-Htt* WT unstressed	Male *5-Htt* WT stressed	↑ **	↑ **	ns	ns	ns	↑ *	ns	ns
Male *5-Htt* KO unstressed	Male *5-Htt* KO stressed	↑ *	↑ **	ns	↑ (*)	↑ *	ns	ns	ns
Male *5-Htt* WT unstressed	Male *5-Htt* KO unstressed	ns	↓ (*)	↓ (*)	ns	↓ (*)	ns	ns	ns
Male *5-Htt* WT stressed	Male *5-Htt* KO stressed	ns	ns	ns	ns	↑ *	ns	↓ *	ns
Female *5-Htt* WT unstressed	Female *5-Htt* WT stressed	↑ **	↑ **	ns	ns	↑ *	ns	ns	↑ *
Female *5-Htt* KO unstressed	Female *5-Htt* KO stressed	↑ *	↑ *	ns	ns	ns	ns	ns	ns
Female *5-Htt* WT unstressed	Female *5-Htt* KO unstressed	ns	↑ *	ns	ns	ns	ns	ns	ns
Female *5-Htt* WT stressed	Female *5-Htt* KO stressed	ns		ns	ns	ns	ns	ns	ns
Male *5-Htt* WT unstressed	Female *5-Htt* WT unstressed	↑ *	ns	↓ *	ns	ns	↑ **	↓ *	↑ *
Male *5-Htt* WT stressed	Female *5-Htt* WT stressed	↑**	↑ **	↓ (*)	↑(*)	↑ **	↑**	↓ **	↑**
Male *5-Htt* KO unstressed	Female *5-Htt* KO unstressed	↑ **	↑ **	ns	↑ **	↑ *	↑ **	ns	↑ **
Male *5-Htt* KO stressed	Female *5-Htt* KO stressed	↑ **	↑ **	ns	↑(*)	ns	↑ **	↓ *	↑ **

Table 3.2.3: Comparisons of gene expression in the hypothalamus. Changes of gene expression are coloured in grey. ns: not significant; ↓: gene expression is lower in Group2 than in Group1. ↑: gene expression is higher in Group2 than in Group1; **: $p < 0.004$, * : $0.004 < p < 0.05$; (*): $0.05 < p < 0.1$.

Gene expression in the amygdala									
Group1	Group2	c-Fos	Fra-2	Syt I	Syt IV	Stx 1a	CRH	CRHR1	CRHR2
Male *5-Htt* WT unstressed	Male *5-Htt* WT stressed	↑ *	↑ *	ns	↓ *	Kruskal-Wallis Test: P > 0,05	ns	ns	Kruskal-Wallis Test: P > 0,05
Male *5-Htt* KO unstressed	Male *5-Htt* KO stressed	↑ **	↑ **	ns	ns		↑ *	ns	
Male *5-Htt* WT unstressed	Male *5-Htt* KO unstressed	ns	ns	ns	ns		↓ *	ns	
Male *5-Htt* WT stressed	Male *5-Htt* KO stressed	ns	ns	↑ (*)	ns		ns	ns	
Female *5-Htt* WT unstressed	Female *5-Htt* WT stressed	ns	↑ (*)	ns	ns		↓ *	↓ *	
Female *5-Htt* KO unstressed	Female *5-Htt* KO stressed	ns	ns	ns	ns		ns	ns	
Female *5-Htt* WT unstressed	Female *5-Htt* KO unstressed	ns	ns	ns	ns		ns	ns	
Female *5-Htt* WT stressed	Female *5-Htt* KO stressed	ns	ns	ns	ns		↑ *	↑ *	
Male *5-Htt* WT unstressed	Female *5-Htt* WT unstressed	↑ *	ns	ns	ns		ns	↑ (*)	
Male *5-Htt* WT stressed	Female *5-Htt* WT stressed	↑*	ns	↑ **	ns		↓ *	ns	
Male *5-Htt* KO unstressed	Female *5-Htt* KO unstressed	↑ **	↑ **	ns	↓*		ns	↑**	
Male *5-Htt* KO stressed	Female *5-Htt* KO stressed	↑ (*)	ns	ns	↓ (*)		ns	↑*	

Table 3.2.4: Comparisons of gene expression in the amygdala. Changes of gene expression are coloured in grey. ns: not significant; ↓: gene expression is lower in Group2 than in Group1. ↑: gene expression is higher in Group2 than in Group1; **: $p < 0.004$, * : $0.004 < p < 0.05$; (*): $0.05 < p < 0.1$.

Gene expression in raphe									
Group1	Group2	c-Fos	Fra-2	Syt I	Syt IV	Stx 1a	CRH	CRHR1	CRHR2
Male *5-Htt* WT unstressed	Male *5-Htt* WT stressed	↑ **	↑ **	ns	ns	Kruskal-Wallis Test: P > 0,05	Kruskal-Wallis Test: P > 0,05	ns	Kruskal-Wallis Test: P > 0,05
Male *5-Htt* KO unstressed	Male *5-Htt* KO stressed	↑ **	↑ **	ns	↓ *			ns	
Male *5-Htt* WT unstressed	Male *5-Htt* KO unstressed	ns	ns	ns	ns			ns	
Male *5-Htt* WT stressed	Male *5-Htt* KO stressed	↓ *	↓ **	ns	ns			ns	
Female *5-Htt* WT unstressed	Female *5-Htt* WT stressed	↑ *	↑ **	ns	ns			ns	
Female *5-Htt* KO unstressed	Female *5-Htt* KO stressed	↑ (*)	ns	↑ (*)	ns			ns	
Female *5-Htt* WT unstressed	Female *5-Htt* KO unstressed	ns	↑(*)	ns	ns			ns	
Female *5-Htt* WT stressed	Female *5-Htt* KO stressed	ns	ns	ns	ns			ns	
Male *5-Htt* WT unstressed	Female *5-Htt* WT unstressed	ns	ns	ns	↓ (*)			ns	
Male *5-Htt* WT stressed	Female *5-Htt* WT stressed	ns	↑ *	↑ **	ns			↑ (*)	
Male *5-Htt* KO unstressed	Female *5-Htt* KO unstressed	↑ *	↑ **	↑ *	↓ (*)			↑ (*)	
Male *5-Htt* KO stressed	Female *5-Htt* KO stressed	ns	↑ *	↑ **	ns			↑ (*)	

Table 3.2.5: Comparisons of gene expression in raphe. Changes of gene expression are coloured in grey. ns: not significant; ↓: gene expression is lower in Group2 than in Group1. ↑: gene expression is higher in Group2 than in Group1;
**: $p < 0.004$, * : $0.004 < p < 0.05$; (*): $0.05 < p < 0.1$.

3.3. The influence of stress and the 5-Htt on neuronal morphology – a morphological study

The influence of social stress on the neuronal morphology of pyramidal neurons in prefrontal cortical regions (IL and CG) as well as its influence on the hippocampus was investigated in this study.

Our hypothesis was, that stress and/or the *5-Htt* genotype have an influence on neuronal morphology of pyramidal neurons in prefrontal cortical regions as well as on CA1 pyramidal neurons in the hippocampus. We postulated that the morphology of these neurons would be changed in consequence of stress and/or the loss of the 5-Htt.

Brains were stained using Golgi-Cox impregnation and afterwards pyramidal neurons were identified by an experimenter blind to the stress condition and the genotype of the animals. The neurons were reconstructed with the computer-based microscopy system Neurolucida (Microbrightfield Inc.). As shown in Figure 3.3.1, the typical pyramidal neurons which were analyzed within these three areas have some characteristic features. IL pyramidal neurons (Figure 3.3.1 A) had somata which were in a distance of about 400 µm from the surface of the IL measured along the apical dendrites which are set at right angles to the brain's surface. CG pyramidal neurons (Figure 3.3.1 B) were chosen in the same way as the IL neurons with somata placed about 400 µm away from the surface of the brain. Both kinds of pyramidal neurons have one apical dendrite with one main branch and numerous side branches emanating from the main branch and they end up in a brush-like manner which is called the apical tuft. The hippocampal (Figure 3.3.1 C) pyramidal neurons of the CA1 region have many side branches sprouting from the apical dendrite and they look more complex than the pyramidal neurons in the prefrontal cortex (Figure 3.3.1 D). Additionally, spines were counted on apical dendrites of IL pyramidal neurons as well as on single apical dendritic segments of hippocampal pyramidal neurons. For each neuron, the total dendritic length of the apical dendrite as well as the number of branch points (= nodes) and the number of nodes per dendritic length in µm (= branch packing) of the whole apical dendrite were analyzed. For IL apical dendrites also the total spine number as well as the total spine density were analyzed. Basal dendrites were evaluated by looking at the mean length of all basal dendritic trees, as each cell possesses 4 to 10 basal dendritic trees, but as not all dendritic trees were undamaged, some of the basal dendritic trees could not be well reconstructed. All apical dendrites were analyzed using Sholl analysis. Different parameters were evaluated: the number of crossing points (= intersections) of dendrites with spheres of different radii, the amount of dendritic material between neighbouring spheres and the number of nodes between neighbouring spheres. For IL also the number of spines between neighbouring spheres as well as the amount of spines per dendritic length of dendrites in the space between neighbouring spheres were investigated. Additionally, dendrograms of CG pyramidal neurons were evaluated as well as the mean spine density of segments on hippocampal apical dendrites and the density of spines in relation to the branch order of apical dendrites in IL.

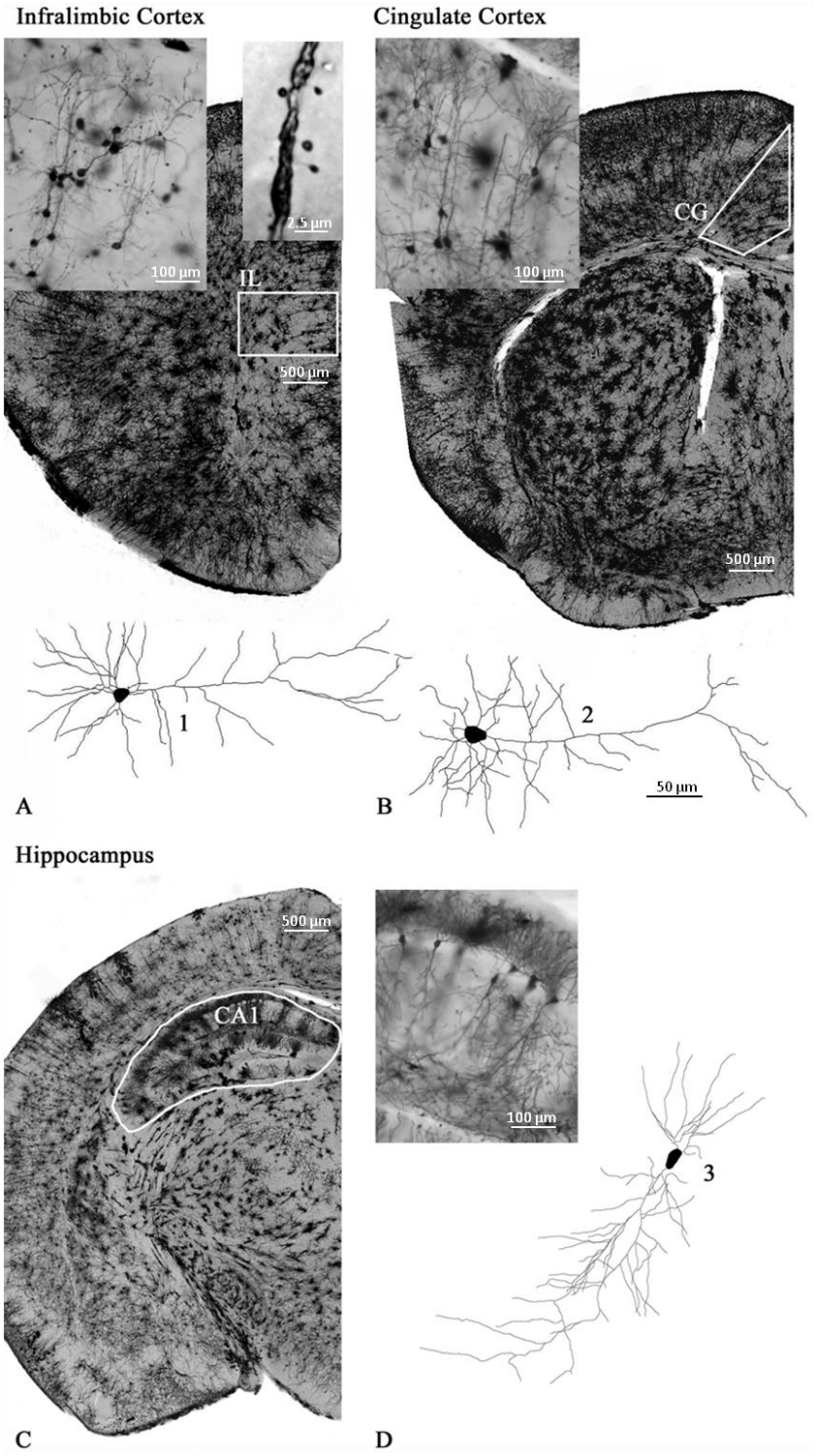


Figure 3.3.1: Mouse brains were stained with Golgi-Cox mercury impregnation as shown in the overview screens and in the details. In the so stained coronal sections, pyramidal neurons were reconstructed and morphologically analyzed in the IL (A, 1) [Bregma 2.1 to 1.3] as well as in the CG (B, 2) [Bregma 1.3 to -0.82] and in the CA1 region on the hippocampus (C, 3) [Bregma -1.7 to -2.8]. The neurons were reconstructed with the computer-based microscopy system Neurolucida (Microbrightfield, Inc.). Afterwards, morphological features of the single neurons were analyzed with the Neurolucida Explorer (Microbrightfield, Inc.).
IL: infralimbic cortex; CG: cingulate cortex; CA1: cornu ammonis region 1; Bregma was specified according to Paxinos and Watson (1997).

3.3.1. Morphological analyses of pyramidal neurons in CG

In the CG no differences between neurons of mice with different genotype and/or stress experience were found concerning total length of apical dendrite (Figure 3.3.2 A), the total amount of nodes in apical dendrites (Figure 3.3.2 B), the amount of nodes per total length of apical dendrites (Figure 3.3.2 C) and the total length of basal dendrites (Figure 3.3.2 D). But it seems that basal dendrites shrink after stress although these differences are not significant at all (Figure 3.3.2 D).

As the amount of reconstructed basal dendrites per neuron was different for each cell (Figure 3.3.3 A), the differences in the total length of basal dendrites (Figure 3.3.3 B) are presumably a consequence of this unequal amount of basal dendritic trees. Therefore the mean length of basal dendrites is more trustworthy than the total length of the dendritic material of basal dendritic trees.

No differences of groups were found using the Sholl analysis of the intersections of apical dendrites with the spheres around the soma (Figure 3.3.4 A), the length of apical dendritic material between neighboring spheres (Figure 3.3.4 B) and the amount of nodes within sectors between neighbouring spheres (Figure 3.3.4 C).

Parameter	Treatment group	N_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Total length in µm (AD)	WT control	6	1889	1902	**1995**	2037	2178	2236
"	WT loser	6	1832	1991	**2131**	2157	2253	2609
"	KO control	6	1630	1787	**2148**	2018	2223	2268
"	KO loser	6	1852	2006	**2198**	2144	2315	2318
Nodes (AD)	WT control	6	17.70	18.25	**19.56**	19.67	21.20	21.62
"	WT loser	6	17.56	18.23	**19.74**	19.91	20.78	23.56
"	KO control	6	14.75	17.20	**20.01**	19.26	21.08	23.10
"	KO loser	6	18.00	19.48	**20.59**	20.57	22.02	22.62
Branch packing (AD)	WT control	6	4.04	4.28	**4.36**	4.49	4.60	5.24
"	WT loser	6	4.01	4.29	**4.55**	4.60	4.69	5.55
"	KO control	6	3.53	4.11	**4.79**	4.63	4.89	5.85
"	KO loser	6	4.07	4.56	**4.80**	4.77	5.07	5.34
Mean length in µm (BD)	WT control	6	44.80	53.55	**65.80**	63.54	69.66	84.45
"	WT loser	6	31.57	41.71	**46.79**	44.83	50.63	51.87
"	KO control	5	35.04	47.26	**64.51**	58.63	70.32	76.00
"	KO loser	6	37.22	42.10	**48.65**	47.82	53.21	57.79

Table 3.3.1: Analysis of total apical and basal dendrites in CG. Rounded values were used for boxplots in Figure 3.3.2; Min.: minimum; 1st Q.: first quartile; Mean: arithmetic mean; 3rd Q.: third quartile; Max.: maximum; AD: apical dendrites; BD: basal dendrites.

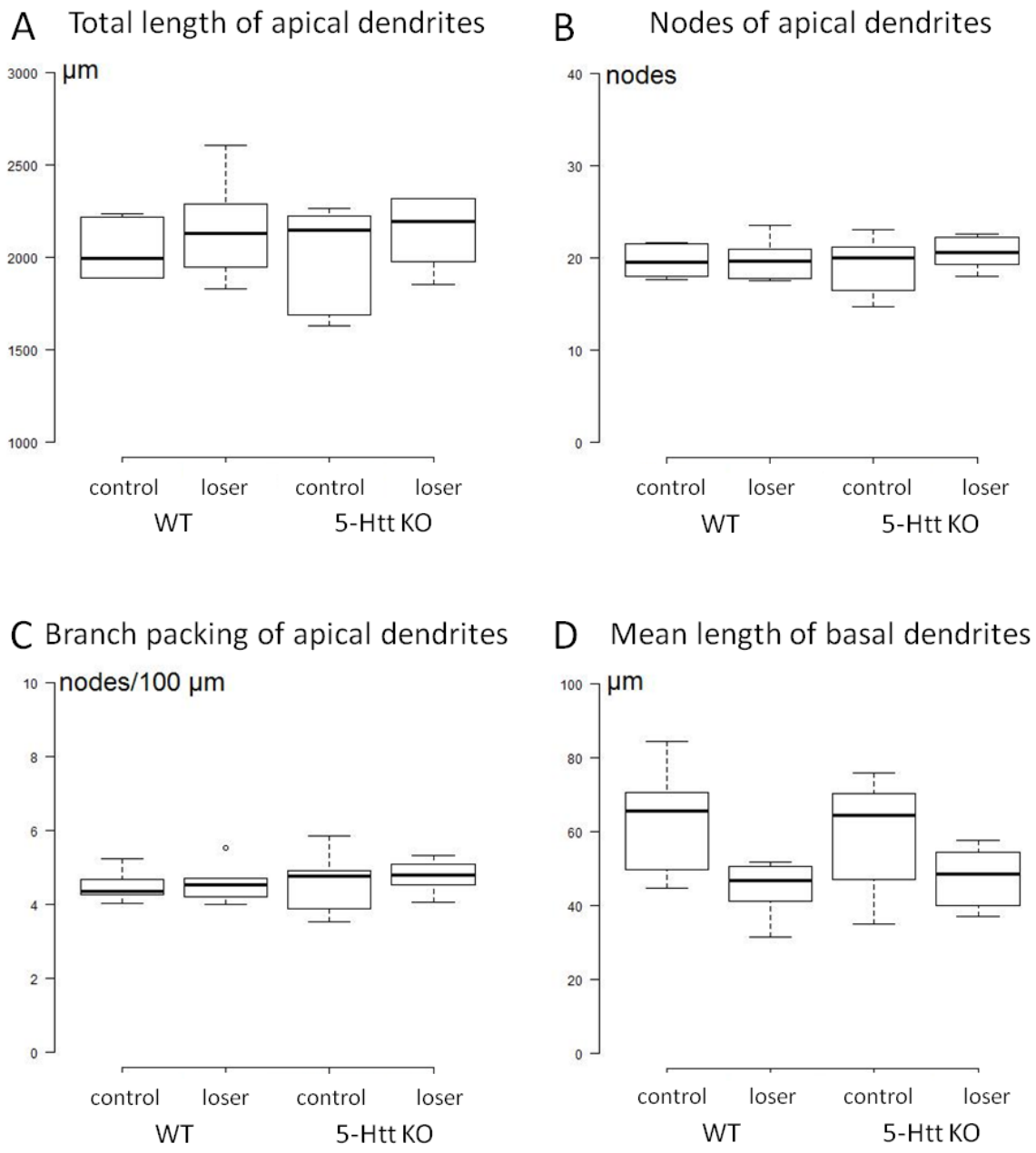


Figure 3.3.2: Analysis of total apical and basal dendrites in CG. In CG no differences were detected concerning the total length of apical dendrites (A), the mean amount of nodes of total apical dendrites (B), the branch packing of total apical dendrites (C) and the mean total length of basal dendrites (D). $n_{WT\_control} = 6$, $n_{WT\_loser} = 6$, $n_{5\text{-Htt KO}\_control} = 6$, $n_{5\text{-Htt KO}\_loser} = 6$; values: Table 3.3.1.

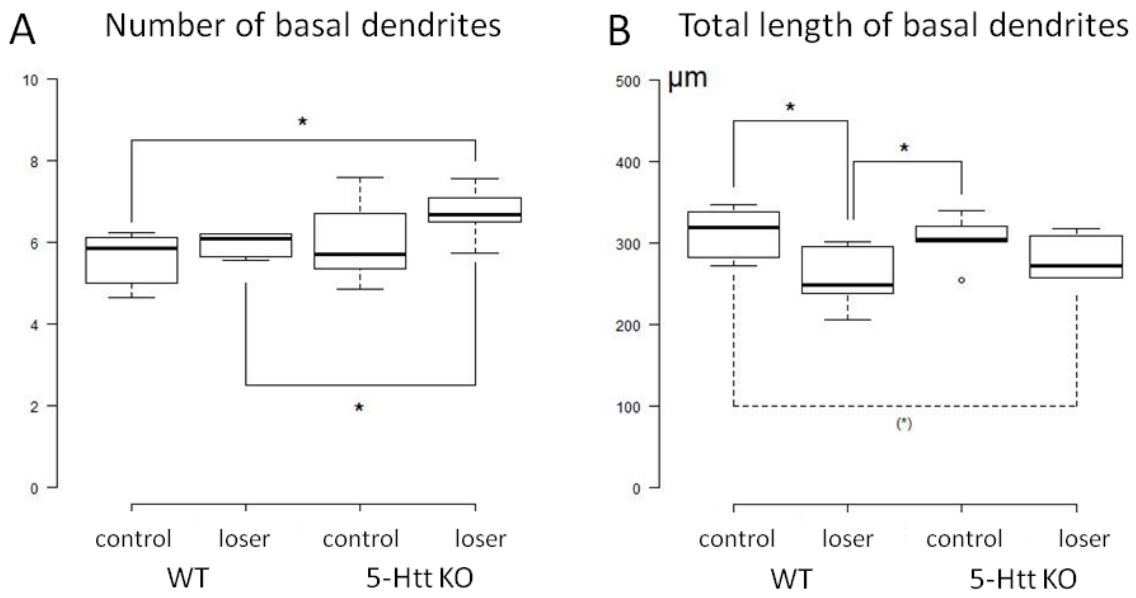


Figure 3.3.3: Analysis of number and total length of basal dendrites in CG. As the number of basal dendritic trees varied from group to group, the total length of basal dendritic trees could not be evaluated in CG as the differences here found are a consequence of the unequal number of basal dendrites. $n_{WT\_control} = 6$, $n_{WT\_loser} = 6$, $n_{5\text{-Htt KO}\_control} = 6$, $n_{5\text{-Htt KO}\_loser} = 6$; values: Table 3.3.2.

Parameter	Treatment group	N_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Dendrites (BD)	WT control	6	4.67	5.18	**5.85**	5.62	6.08	6.25
"	WT loser	6	5.57	5.77	**6.10**	5.98	6.19	6.22
"	KO control	6	4.86	5.38	**5.70**	5.99	6.53	7.60
"	KO loser	6	5.75	6.51	**6.67**	6.71	7.03	7.56
Total length in µm (BD)	WT control	6	272	289	**320**	314	338	347
"	WT loser	6	207	240	**249**	257	285	302
"	KO control	5	255	303	**305**	305	321	341
"	KO loser	6	257	260	**273**	281	301	318

Table 3.3.2: Analysis of basal dendrites in CG. Rounded values of number of basal dendrites and of total dendritic length (in µm) were used for boxplots in Figure 3.3.3; Min.: minimum; 1st Q.: first quartile; Mean: arithmetic mean; 3rd Q.: third quartile; Max.: maximum; BD: basal dendrites.

Moreover, no differences could be found concerning details in dendrogram structure of apical dendritic trees (Figure 3.3.5) as there were no differences concerning the number of side branches emanating from the main apical dendrite in this region.

No differences could be shown concerning the length of the terminal branches of apical dendrites (data not shown).

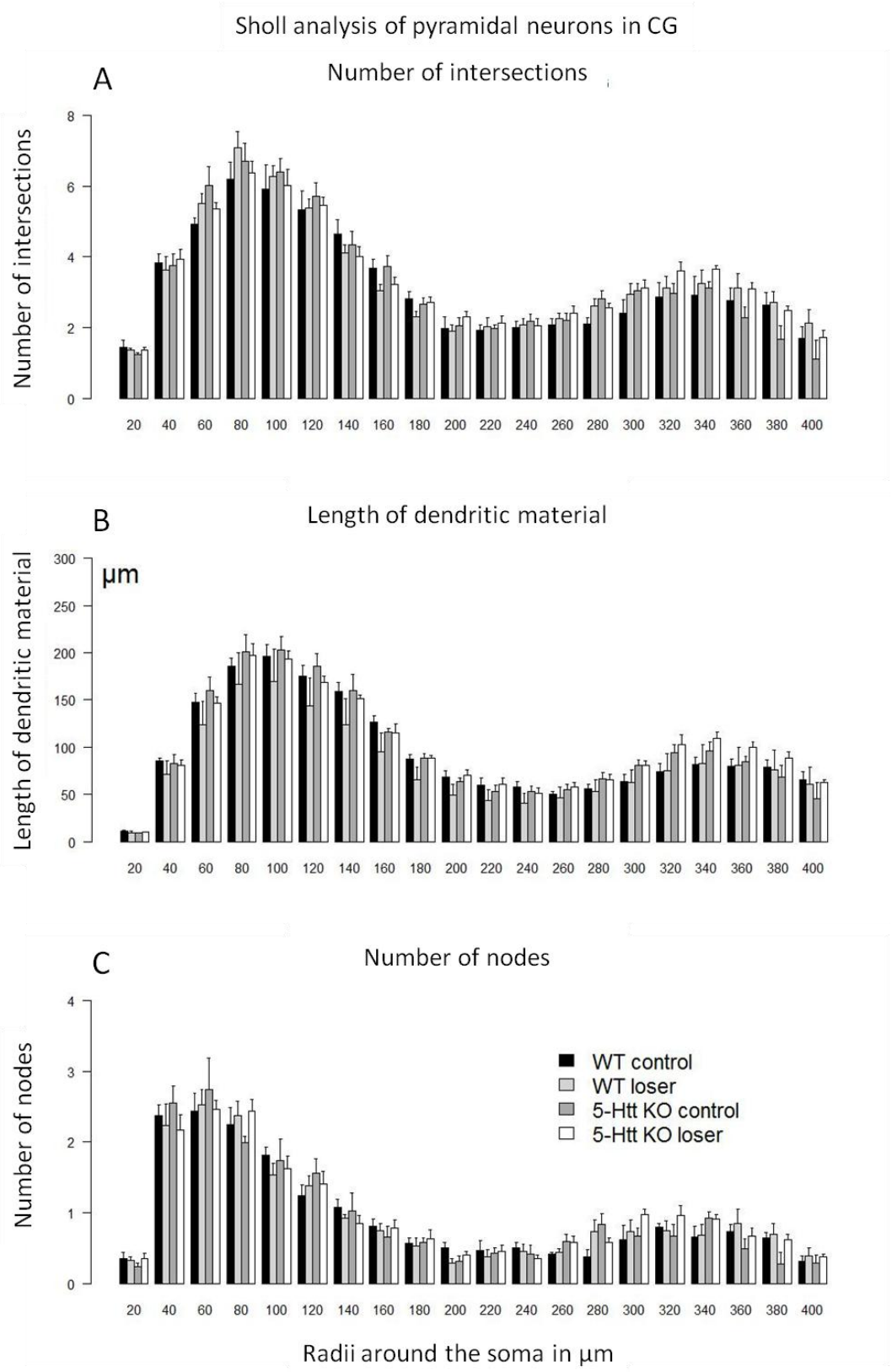


Figure 3.3.4: Sholl analysis of apical dendrites in CG. Sholl analysis of (A) the amount of intersections with apical dendrites and concentric spheres of different radii around the soma, (B) length of dendritic material between neighbouring spheres and (C) number of nodes of apical dendrites. Bars: Arithmetic means of all animals per group; error bars: SEM. $n_{WT\_control} = 6$, $n_{WT\_loser} = 6$, $n_{5\text{-}Htt\ KO\_control} = 6$, $n_{5\text{-}Htt\ KO\_loser} = 6$.

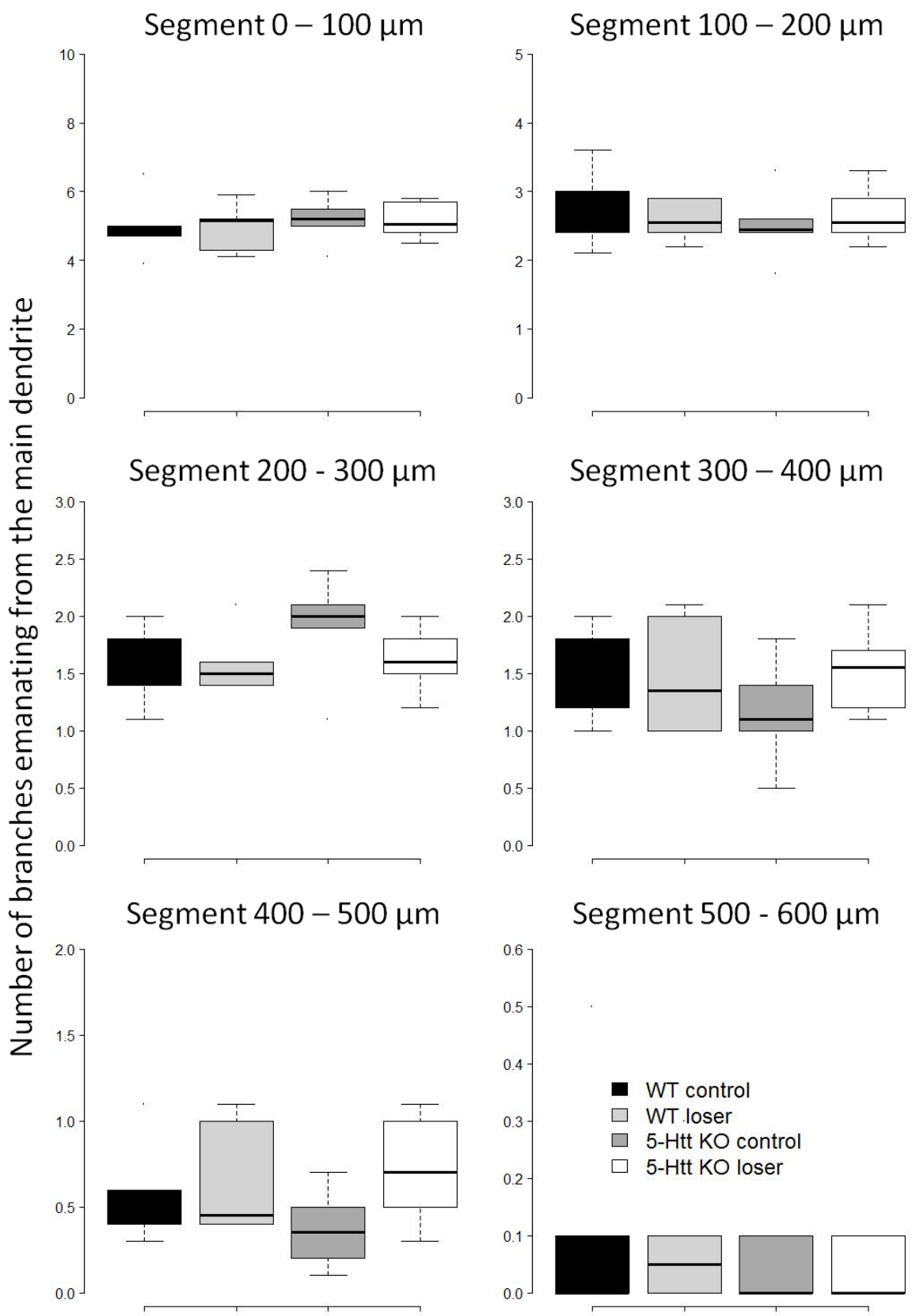


Figure 3.3.5: Dendrogram analysis of apical dendrites in CG. No differences could be detected in the number of side branches emanating directly from the main apical dendrites at any distance from the soma. $n_{WT\_control} = 6$, $n_{WT\_loser} = 6$, $n_{5\text{-Htt KO}\_control} = 6$, $n_{5\text{-Htt KO}\_loser} = 6$; values: Table 3.3.3.

Segment	Treatment group	N_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
0-100 µm	WT control	6	3.90	4.75	**4.95**	5.00	5.00	6.50
"	WT loser	6	4.10	4.50	**5.15**	4.97	5.20	5.90
"	KO control	6	4.10	5.03	**5.20**	5.17	5.45	6.00
"	KO loser	6	4.50	4.85	**5.05**	5.15	5.55	5.80
100-200 µm	WT control	6	2.10	2.45	**2.60**	2.72	2.90	3.60
"	WT loser	6	2.20	2.40	**2.55**	2.58	2.85	2.90
"	KO control	6	1.80	2.40	**2.45**	2.50	2.58	3.30
"	KO loser	6	2.20	2.40	**2.55**	2.65	2.85	3.30
200-300 µm	WT control	6	1.10	1.40	**1.45**	1.53	1.73	2.00
"	WT loser	6	1.40	1.43	**1.50**	1.58	1.58	2.10
"	KO control	6	1.10	1.93	**2.00**	1.92	2.08	2.40
"	KO loser	6	1.20	1.53	**1.60**	1.62	1.75	2.00
300-400 µm	WT control	6	1.00	1.25	**1.60**	1.53	1.80	2.00
"	WT loser	6	1.00	1.08	**1.35**	1.47	1.85	2.10
"	KO control	6	0.50	1.00	**1.10**	1.15	1.35	1.80
"	KO loser	6	1.10	1.28	**1.55**	1.53	1.68	2.10
400-500 µm	WT control	6	0.30	0.43	**0.55**	0.58	0.60	1.10
"	WT loser	6	0.40	0.40	**0.45**	0.63	0.88	1.10
"	KO control	6	0.10	0.20	**0.35**	0.37	0.50	0.70
"	KO loser	6	0.30	0.50	**0.70**	0.72	0.98	1.10
500-600 µm	WT control	6	0.00	0.00	**0.00**	0.10	0.08	0.50
"	WT loser	6	0.00	0.00	**0.05**	0.08	0.10	0.30
"	KO control	6	0.00	0.00	**0.00**	0.03	0.08	0.10
"	KO loser	6	0.00	0.00	**0.00**	0.03	0.08	0.10

Table 3.3.3: Analysis of dendrogram structure of apical dendrites in CG. Rounded values of number of side branches emanating from the main apical dendrites were used for boxplots in Figure 3.3.5; Min.: minimum; 1st Q.: first quartile; Mean: arithmetic mean; 3rd Q.: third quartile; Max.: maximum.

Summing it all up it can be said that the hypothesis could not be corroborated for pyramidal neurons in CG as neither stress nor genotype had an influence on the investigated morphological parameters.

3.3.2. Morphological analyses of pyramidal neurons in IL

In IL, no differences between groups could be detected concerning the total length of apical dendrite (Figure 3.3.6 A), the total number of nodes of apical dendrites (Figure 3.3.6 B), the branch packing of apical dendrites (Figure 3.3.6 C) and the mean length of basal dendrites (Figure 3.3.6 D). Regarding the total number of spines on apical dendrites (Figure 3.3.7 A) the p-value of the Kruskal-Wallis test performed over all four groups was > 0.1, but the p-value of Wilcoxon rank sum test here was < 0.05 comparing WT control mice und *5-Htt* KO control mice. Spine density on apical dendrites was not different between groups (Figure 3.3.7 B).

Additionally, spine density was analyzed in relation to branch order (Figure 3.3.8 and Figure 3.3.9). But no differences could be found with this kind of analysis. Spine density increased with branch order as shown in Figure 3.3.8, Figure 3.3.9 and in Table 3.3.9.

Sholl analysis of pyramidal apical dendrites in IL revealed no differences concerning the number of crossings (= intersections) of apical dendrites with the spheres around the soma (data not shown) and the number of nodes between the spheres of neighboring radii (data not shown). Differences could be found between unstressed and stressed WT mice in the length of dendritic material (Figure 3.3.10 A). Within the sectors between spheres of 80µm and 100 µm ($p = 0.0173$) radii around the middle of the soma and between spheres of 140 µm and 160 µm ($p = 0.0303$) around the soma, stress tended to increase the length of dendritic material, as with the Bonferroni correction the level of significance had to be adjusted to $p = 0.0083$ and therefore differences could not be termed significant. The same effect could be seen in all sectors between 60 and 200 µm around the soma, although differences were not significant in the other sectors. For better understanding, the analyzed sectors between neighbouring spheres are named after the radius of the bigger sphere, e.g. sector 100 µm means the sector between the spheres of 80 µm radius and 100 µm radius. Differences between groups are summarized in Table 3.3.7.

Concerning the amount of spines, no differences were found as the p-values of the Kruskal-Wallis test were > 0.1 (Figure 3.3.10). Regarding spine density (amount of spines/µm of dendritic material), possible differences could be detected in the sectors at a distance between 280 µm and 300 µm from the soma (Figure 3.3.10 C). In this region apical dendrites of pyramidal neurons of unstressed WT mice always had more spines per µm of dendritic material than the apical dendrites of pyramidal neurons of unstressed *5-Htt* KO mice but this effect was neither significant not tendential.

In summary we can say that in IL we can corroborate our hypothesis as there are some changes in the morphology of neurons after stress and between genotypes as shown by the Sholl analysis (Figure 3.3.10).

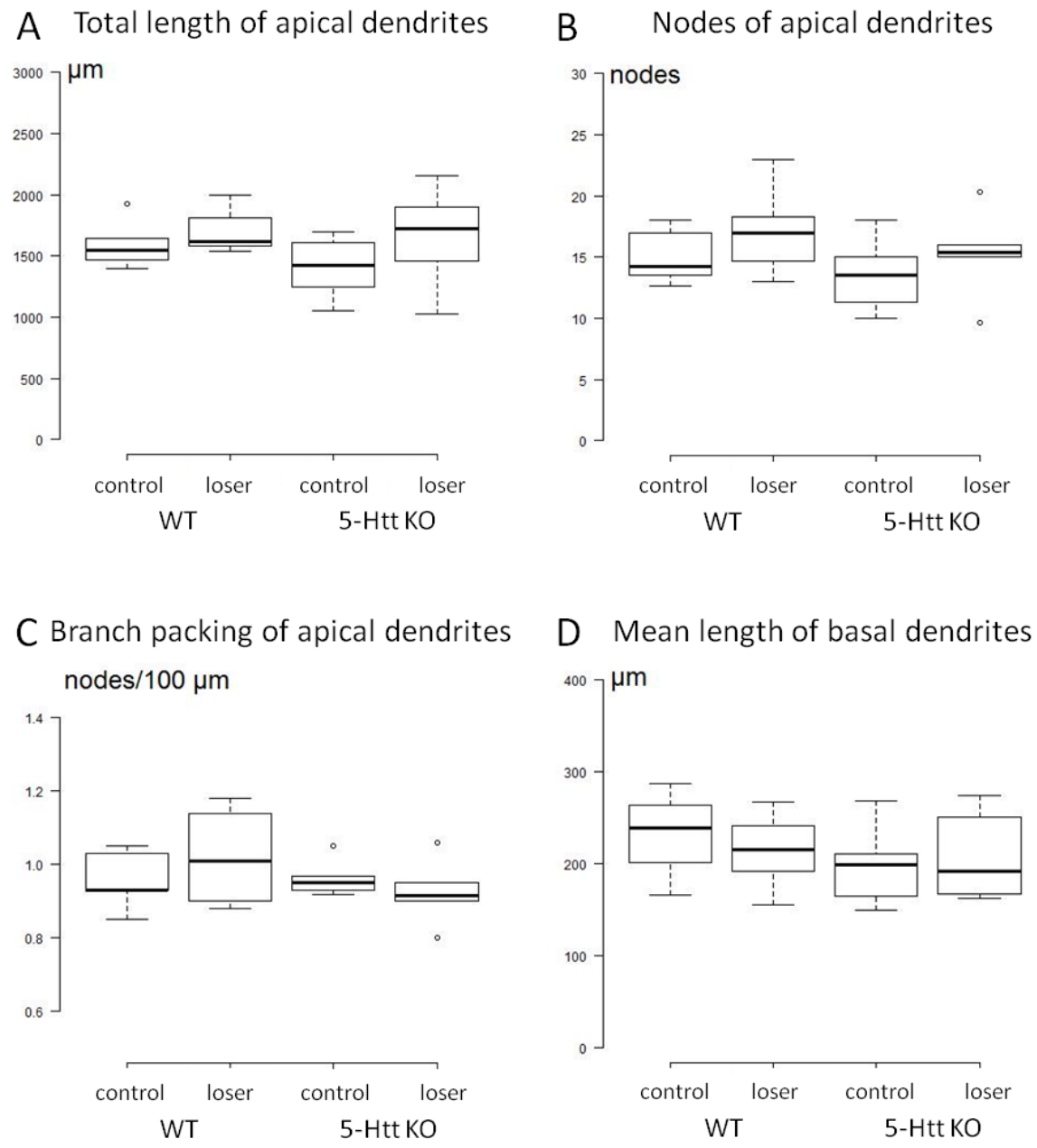


Figure 3.3.6: Analysis of total apical and basal dendrites in IL. In IL no differences were detected concerning the total length of apical dendrites (A), mean number of nodes of apical dendrites (B), branch packing of apical dendrites (C) and mean total length of basal dendrites (D). $n_{WT\_control}$ = 5, n_{WT_loser} = 6, $n_{5\text{-Htt KO\_control}}$ = 6, $n_{5\text{-Htt KO\_loser}}$ = 6; values: Table 3.3.4.

Parameter	Treatment group	N_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Total length in µm (AD)	WT control	5	1399	1466	**1543**	1597	1648	1929
"	WT loser	6	1539	1584	**1613**	1692	1769	1993
"	KO control	6	1055	1264	**1425**	1409	1585	1700
"	KO loser	6	1027	1519	**1720**	1664	1859	2159
Nodes (AD)	WT control	5	12.67	13.50	**14.25**	15.08	17.00	18.00
"	WT loser	6	13.00	15.25	**17.00**	17.17	18.00	23.00
"	KO control	6	10.00	11.62	**13.55**	13.57	14.90	18.00
"	KO loser	6	9.67	15.06	**15.38**	15.29	15.88	20.33
Branch packing (AD)	WT control	5	0.85	0.93	**0.93**	0.96	1.03	1.05
"	WT loser	6	0.88	0.92	**1.01**	1.02	1.12	1.18
"	KO control	6	0.92	0.93	**0.95**	0.96	0.97	1.05
"	KO loser	6	0.80	0.90	**0.92**	0.92	0.94	1.06
Mean length in µm (BD)	WT control	5	167	227	**237**	232	241	288
"	WT loser	6	156	195	**207**	212	234	268
"	KO control	6	150	171	**190**	196	206	269
"	KO loser	5	163	167	**191**	209	251	274

Table 3.3.4: Analysis of total apical and basal dendrites in IL. Rounded values were used for boxplots in Figure 3.3.6; Min.: minimum; 1st Q.: first quartile; Mean: arithmetic mean; 3rd Q.: third quartile; Max.: maximum; AD: apical dendrites; BD: basal dendrites.

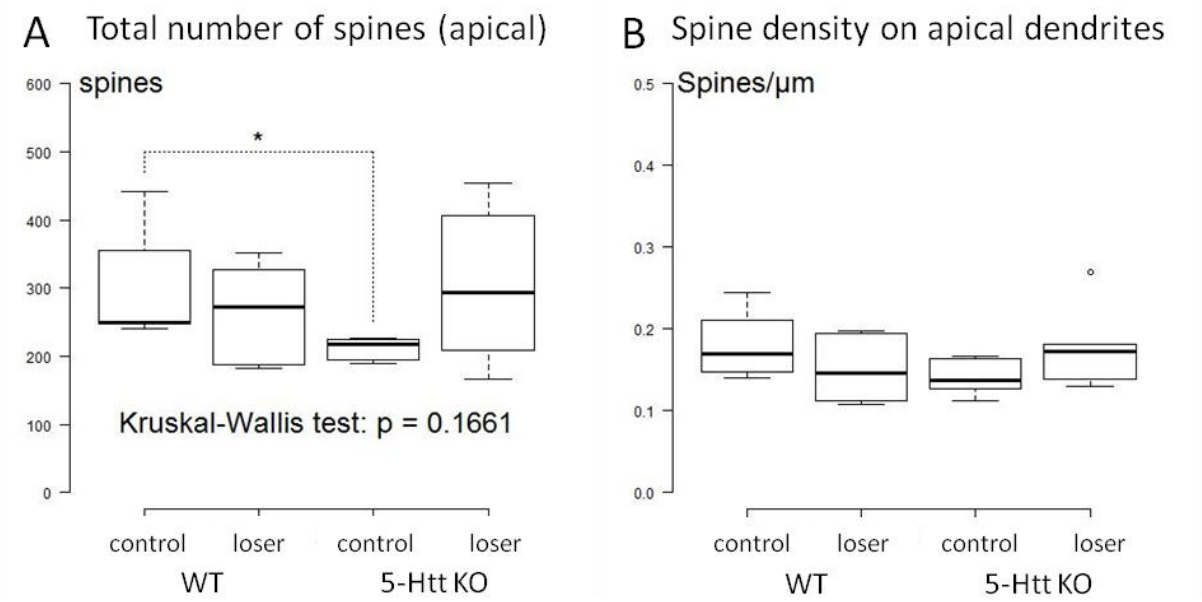


Figure 3.3.7: Analysis of spine number and spine density of apical dendrites in IL. No differences could be found in total spine number (A) and spine density (B). But possibly there was some difference between unstressed WT mice and unstressed *5-Htt* KO mice regarding total number of spines on apical dendrites in IL (A) although the p-value of Kruskal-Wallis test didn't allow any tests as it was > 0.1. No differences could be detected concerning the spine density of total apical dendrites (B). $n_{WT\_control}$ = 5, n_{WT_loser} = 6, $n_{5\text{-}Htt\ KO\_control}$ = 6, $n_{5\text{-}Htt\ KO\_loser}$ = 6; values: Table 3.3.5; *: p-value of Kruskal-Wallis test < 0.1 and p-value of Wilcoxon rank sum test < 0.05.

Parameter	Treatment group	N_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Spines (AD)	WT control	5	241	247	**250**	307	356	442
"	WT loser	6	182	205	**272**	265	318	352
"	KO control	6	189	200	**218**	212	224	227
"	KO loser	6	167.0	218	**290**	304	391	454
Spine density (AD)	WT control	5	0.14	0.15	**0.17**	0.18	0.21	0.24
"	WT loser	6	0.11	0.12	**0.15**	0.15	0.19	0.20
"	KO control	6	0.11	0.13	**0.14**	0.14	0.16	0.17
"	KO loser	6	0.13	0.15	**0.17**	0.18	0.18	0.27

Table 3.3.5: Analysis of total spine number and spine density in IL. Rounded values of spine number and of spine density were used for boxplots in Figure 3.3.7; Min.: minimum; 1st Q.: first quartile; Mean: arithmetic mean; 3rd Q.: third quartile; Max.: maximum; AD: apical dendrites.

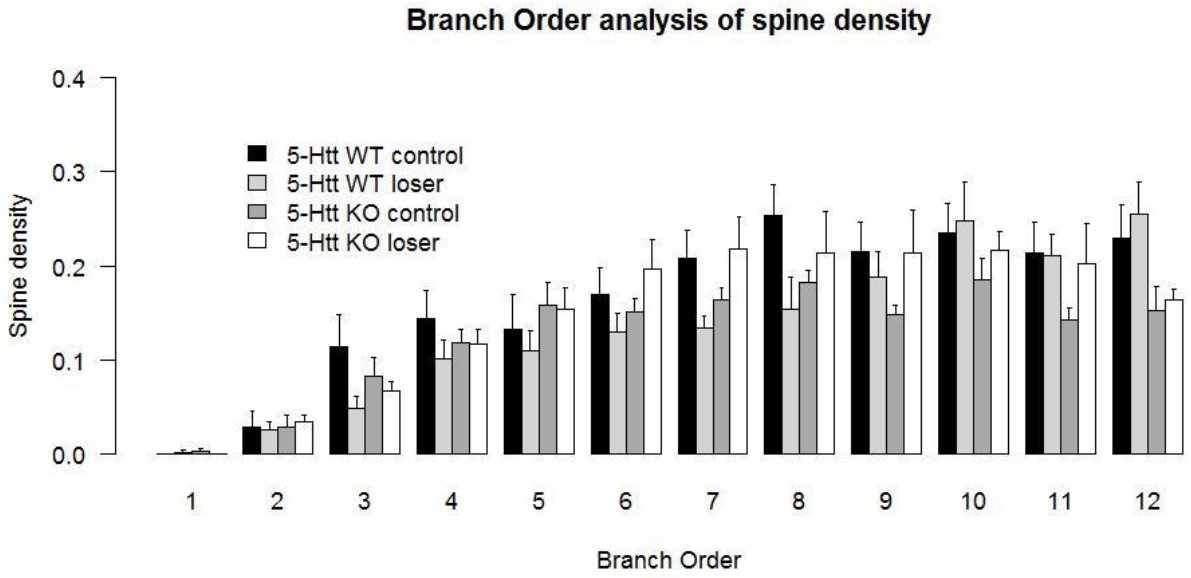


Figure 3.3.8: Mean spine density of apical dendrites depending on branch order in IL. Spine density was analyzed per branch order of the apical dendritic tree. No differences between groups could be found using this kind of analysis. But spine density increases with branching factor. Data are presented as arithmetic mean + SEM.

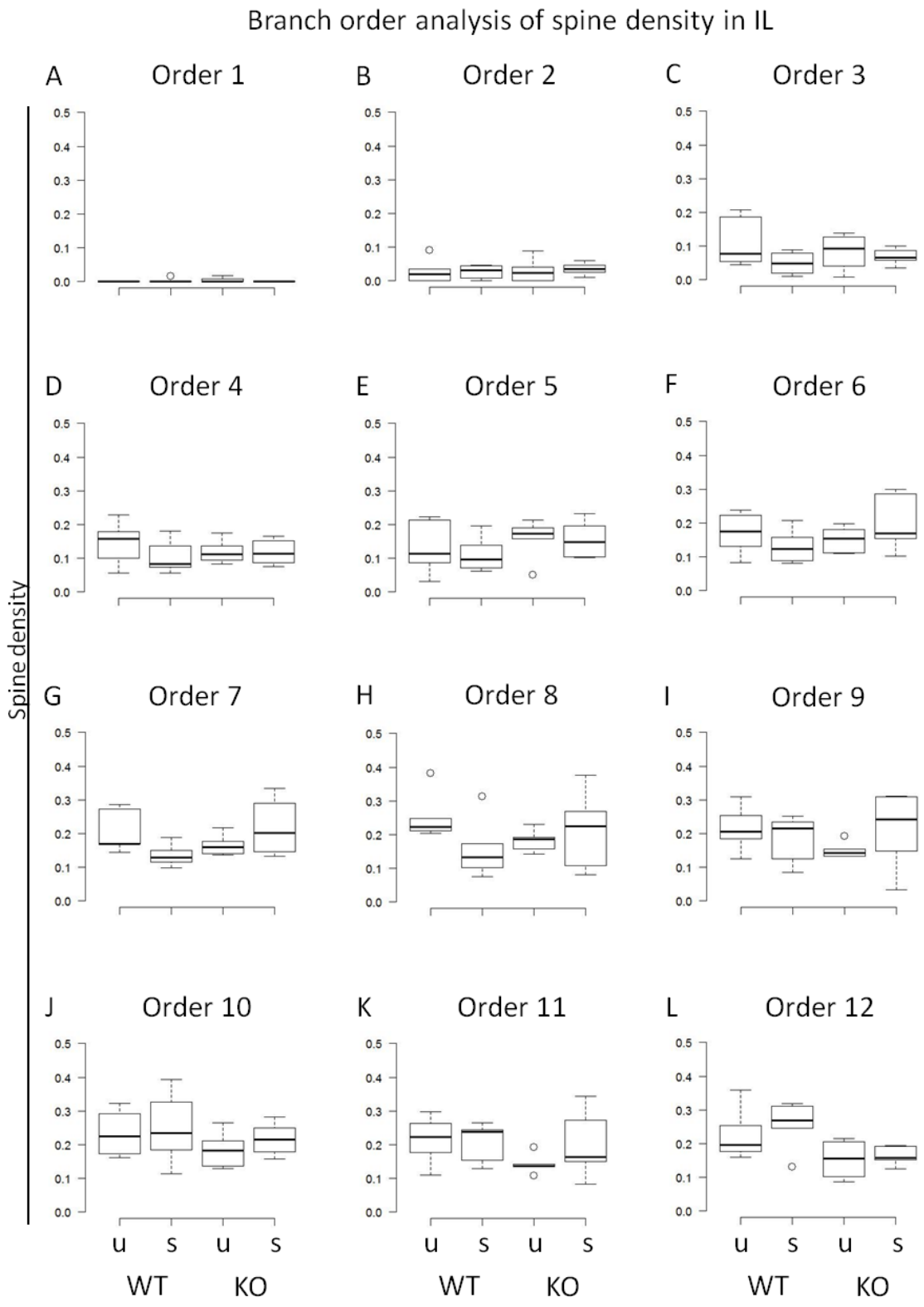


Figure 3.3.9: Spine density of apical dendrites depending on branch order in IL. No differences between groups can be seen concerning the spine density depending on branch order. Data are presented as boxplots for each group and branch order. u: unstressed control mice; s: stressed loser mice; WT: wild-type mice; KO: *5-Htt* knockout mice. Rounded values are shown in the Table 3.3.6 on the next page.

Branch Order	Treatment group	N_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Order 1	WT control	5	0.00	0.00	**0.00**	0.00	0.00	0.00
"	WT loser	6	0.00	0.00	**0.00**	0.00	0.00	0.00
"	KO control	6	0.00	0.00	**0.00**	0.00	0.00	0.00
"	KO loser	6	0.00	0.00	**0.00**	0.00	0.00	0.00
Order 2	WT control	5	0.00	0.00	**0.02**	0.03	0.03	0.09
"	WT loser	6	0.00	0.01	**0.03**	0.03	0.04	0.05
"	KO control	6	0.00	0.00	**0.02**	0.03	0.04	0.09
"	KO loser	6	0.01	0.02	**0.03**	0.04	0.05	0.06
Order 3	WT control	5	0.05	0.05	**0.06**	0.11	0.19	0.21
"	WT loser	6	0.01	0.02	**0.05**	0.05	0.07	0.09
"	KO control	6	0.01	0.05	**0.09**	0.08	0.12	0.14
"	KO loser	6	0.04	0.06	**0.07**	0.07	0.08	0.10
Order 4	WT control	5	0.06	0.1	**0.16**	0.14	0.18	0.23
"	WT loser	6	0.06	0.07	**0.08**	0.10	0.12	0.18
"	KO control	6	0.08	0.09	**0.11**	0.12	0.14	0.17
"	KO loser	6	0.07	0.09	**0.11**	0.12	0.15	0.17
Order 5	WT control	5	0.03	0.09	**0.11**	0.13	0.21	0.22
"	WT loser	6	0.07	0.07	**0.10**	0.11	0.13	0.20
"	KO control	6	0.05	0.16	**0.17**	0.16	0.19	0.21
"	KO loser	6	0.10	0.11	**0.15**	0.16	0.19	0.23
Order 6	WT control	5	0.08	0.13	**0.18**	0.17	0.22	0.24
"	WT loser	6	0.08	0.09	**0.12**	0.13	0.15	0.21
"	KO control	6	0.11	0.19	**0.15**	0.15	0.18	0.20
"	KO loser	6	0.10	0.16	**0.17**	0.20	0.26	0.30
Order 7	WT control	5	0.14	0.17	**0.17**	0.21	0.27	0.29
"	WT loser	6	0.10	0.12	**0.13**	0.13	0.14	0.19
"	KO control	6	0.14	0.14	**0.16**	0.17	0.17	0.22
"	KO loser	6	0.13	0.15	**0.20**	0.22	0.28	0.33
Order 8	WT control	5	0.20	0.21	**0.22**	0.25	0.25	0.38
"	WT loser	6	0.08	0.11	**0.13**	0.15	0.17	0.31
"	KO control	6	0.14	0.16	**0.19**	0.18	0.19	0.23
"	KO loser	6	0.08	0.13	**0.22**	0.21	0.27	0.38
Order 9	WT control	5	0.13	0.18	**0.21**	0.22	0.25	0.31
"	WT loser	6	0.09	0.15	**0.22**	0.19	0.23	0.25
"	KO control	6	0.13	0.14	**0.14**	0.15	0.15	0.19
"	KO loser	6	0.03	0.16	**0.24**	0.21	0.30	0.31
Order 10	WT control	5	0.16	0.17	**0.23**	0.24	0.29	0.32
"	WT loser	6	0.11	0.19	**0.24**	0.25	0.31	0.39
"	KO control	5	0.13	0.14	**0.18**	0.19	0.21	0.27
"	KO loser	5	0.16	0.18	**0.22**	0.22	0.25	0.28
Order 11	WT control	5	0.11	0.18	**0.22**	0.21	0.26	0.30
"	WT loser	6	0.13	0.18	**0.24**	0.21	0.24	0.27
"	KO control	5	0.11	0.13	**0.14**	0.14	0.14	0.19
"	KO loser	5	0.08	0.15	**0.16**	0.20	0.27	0.34
Order 12	WT control	5	0.16	0.18	**0.20**	0.23	0.25	0.36
"	WT loser	5	0.13	0.25	**0.27**	0.26	0.31	0.32
"	KO control	4	0.09	0.11	**0.16**	0.15	0.21	0.20
"	KO loser	5	0.13	0.15	**0.16**	0.16	0.19	0.19

Table 3.3.9: Analysis of spine density depending on branch order in IL. Rounded values of spine density were used for boxplots in Figure 3.3.9; Min.: minimum; 1st Q.: first quartile; Mean: arithmetic mean; 3rd Q.: third quartile; Max.: maximum; AD: apical dendrites.

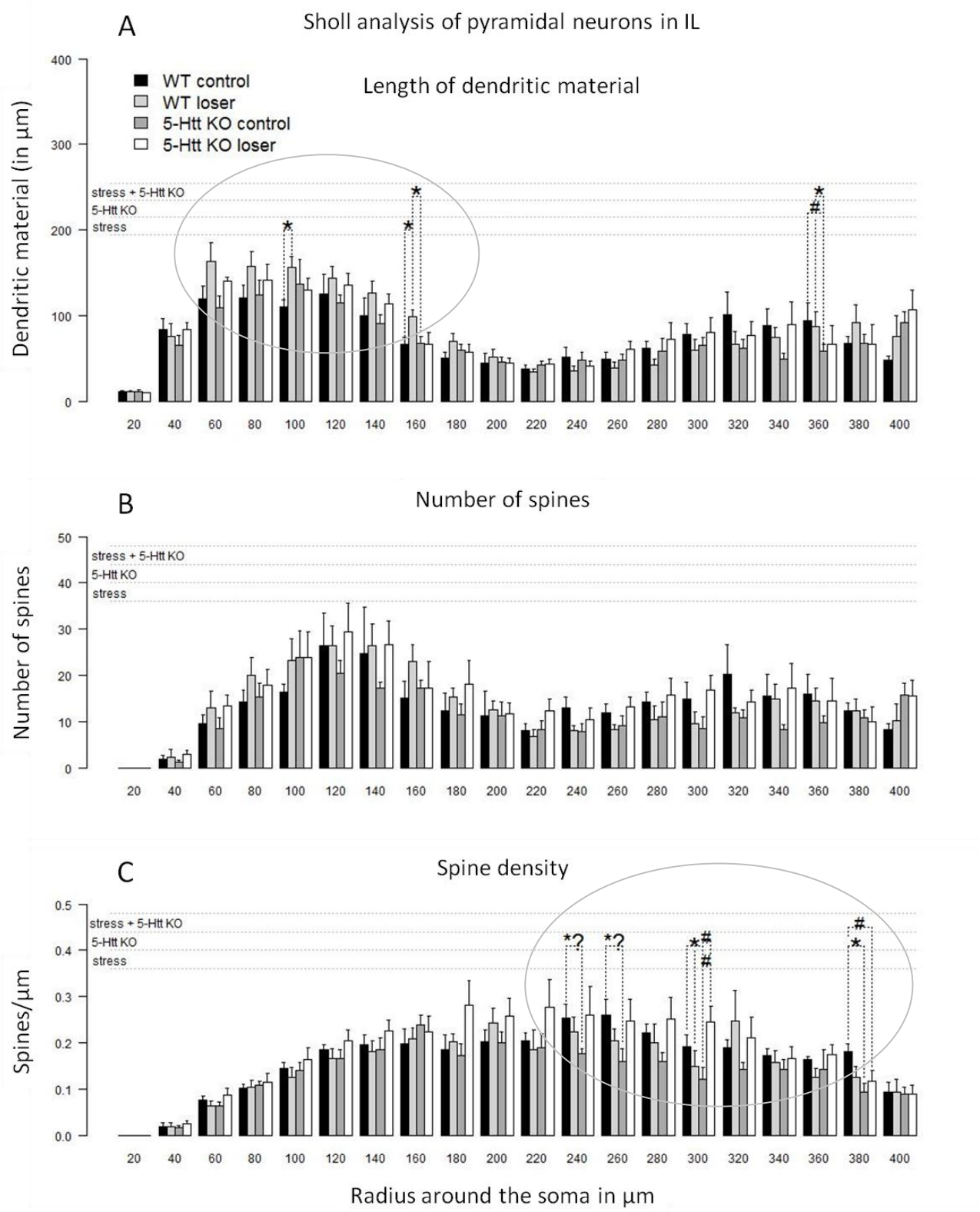


Figure 3.3.10: Sholl analysis of pyramidal neurons in IL. In IL, Sholl analysis of length of dendritic material (A) revealed stress effects in WT mice, as well as genotype effects. The number of spines within spheres was not influenced by stress or genotype (B) but the spine density was lower in unstressed *5-Htt* KO mice than in unstressed WT mice (C). Data was presented as arithmetic means (bars) and SEM (error bars). *: p-value of Kruskal-Wallis test < 0.1 and p-value of Wilcoxon test < 0.05 and > 0.0083; #: p-value of Kruskal-Wallis test < 0.1 and p-value of Wilcoxon test < 0.1 and > 0.05; *?: p-value of Kruskal-Wallis test > 0.1 but p-value of Wilcoxon test < 0.05; IL: infralimbic cortex.

Kruskal-Wallis test: p < 0.1	different Stress condition		different *5-Htt* Genotype		different Stress condition + different *5-Htt* Genotype	
Group 1	WT control	*5-Htt* KO control	WT control	WT loser	WT control	WT loser
Group 2	WT loser	*5-Htt* KO loser	*5-Htt* KO control	*5-Htt* KO loser	*5-Htt* KO loser	*5-Htt* KO control
p-values of Wilcoxon rank sum test **(length of dendritic material within sectors)**	100 µm: **0.01732** 160 µm: **0.0303**		360 µm: 0.052			160 µm: **0.026** 360 µm: **0.026**
p-values of Wilcoxon rank sum test **(spine density within sectors)**		300 µm: 0.0649	300 µm: **0.0303** 380 µm: **0.0087**	300 µm: 0.0931	380 µm: 0.0823	

Table 3.3.7: p-values of Sholl analysis in IL. Comparisons between 2 groups were carried out using the Wilcoxon rank sum test if the p-value of the Kruskal-Wallis test was < 0.1. Because 5 tests were performed on these data, the level of significance had to be corrected using the Bonferroni correction. But as none of the p-values was < 0.0083, also tendencies (0.05 > p > 0.0083) or possible tendencies (0.1 > p > 0.05) were taken into account.

3.3.3. Morphological analyses of pyramidal neurons in the hippocampus

In pyramidal neurons of the CA1 region of the hippocampus no differences between groups could be detected concerning the total length of apical dendrite (Figure 3.3.11 A), the total amount of nodes in apical dendrites (Figure 3.3.11 B), the total amount of nodes per total length of apical dendrites (Figure 3.3.11 C) and the total length of basal dendrites (Figure 3.3.11 D).

Additionally, no differences between groups could be detected concerning the spine density of segments of the apical dendrites in hippocampal pyramidal neurons (Figure 3.3.12 A). Also the average size of the somata was the same in each group (Figure 3.3.12 B).

No differences of groups were found using the Sholl analysis of the intersections of apical dendrites (Figure 3.3.13 A), the length of apical dendritic material between neighboring spheres (Figure 3.3.13 B) as well as the amount of nodes in the sectors between neighbouring spheres (Figure 3.3.13 C).

In the hippocampus, our hypothesis could not be corroborated as no morphological changes after stress and the loss of the *5-Htt* genotype could be demonstrated in our study.

Parameter	Treatment group	N_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Total length in µm (AD)	WT control	5	2628	2786	**2816**	2913	3099	3236
"	WT loser	6	2425	2500	**2666**	2663	2778	2961
"	KO control	6	2062	2503	**2683**	2756	2790	3842
"	KO loser	6	2132	2500	**2713**	2687	2878	3200
Nodes (AD)	WT control	5	27.50	29.50	**33.00**	32.20	34.75	36.25
"	WT loser	6	25.25	26.44	**30.25**	29.96	32.94	35.00
"	KO control	6	24.00	30.75	**31.38**	32.57	34.94	42.00
"	KO loser	6	25.67	28.67	**29.08**	30.04	32.50	34.25
Branch packing (AD)	WT control	5	0.98	1.12	**1.12**	1.10	1.13	1.16
"	WT loser	6	1.05	1.07	**1.12**	1.13	1.16	1.28
"	KO control	6	1.07	1.10	**1.13**	1.18	1.16	1.46
"	KO loser	6	1.04	1.06	**1.14**	1.13	1.18	1.22
Mean length in µm (BD)	WT control	5	427	438	**495**	514	531	679
"	WT loser	6	479	495	**536**	575	627	760
"	KO control	6	242	410	**456**	456	536	626
"	KO loser	6	363	502	**556**	547	627	670

Table 3.3.8: Analysis of total apical and basal dendrites in the hippocampus. Rounded values were used for boxplots in Figure 3.3.11; Min.: minimum; 1st Q.: first quartile; Mean: arithmetic mean; 3rd Q.: third quartile; Max.: maximum; AD: apical dendrites; BD: basal dendrites.

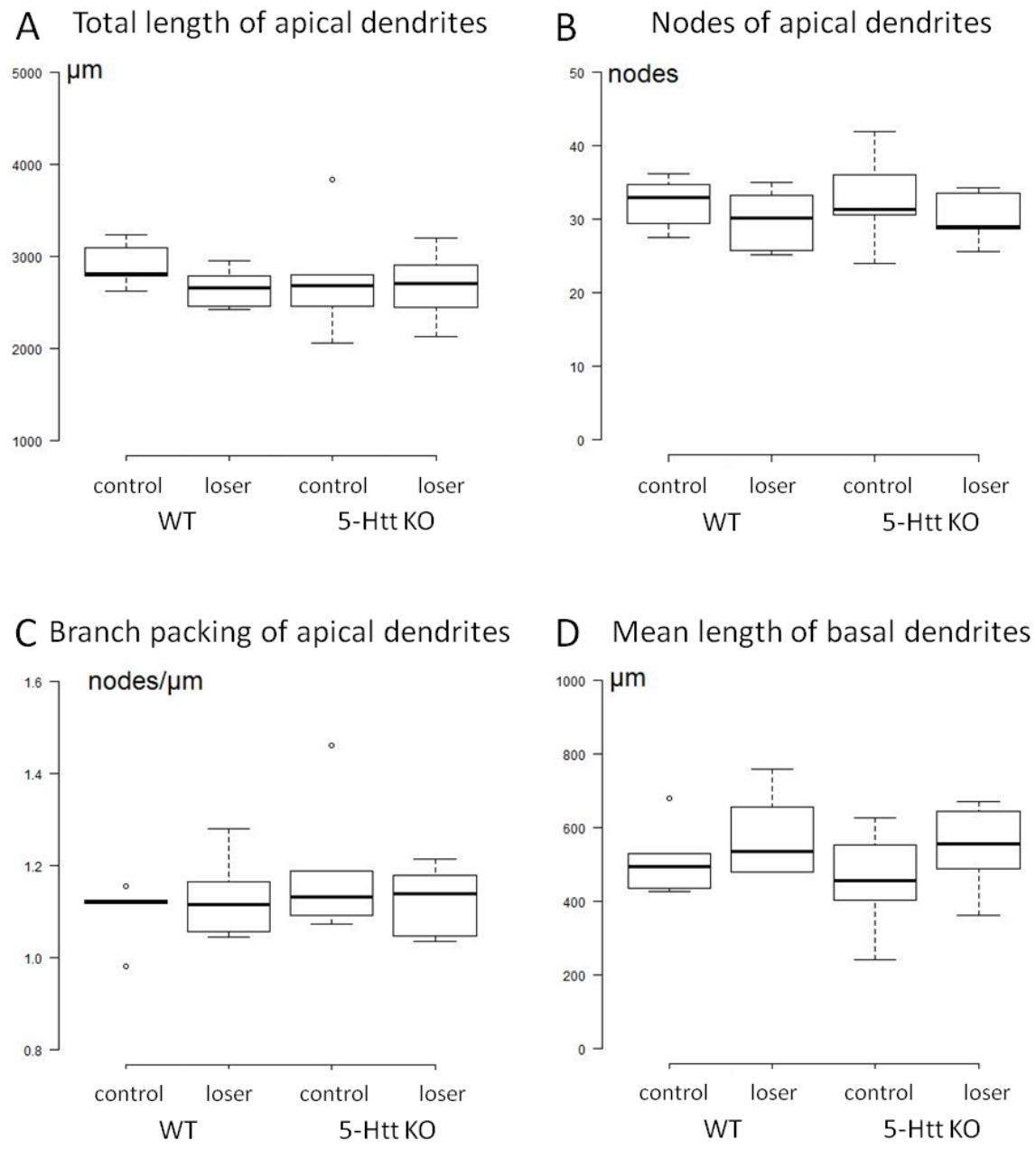


Figure 3.3.11: Analysis of total apical and basal dendrites in the hippocampus. In the hippocampus no differences were detected concerning the total length of apical dendrites (A), the mean amount of nodes of apical dendrites (B), the branch packing of apical dendrites (C) and the mean total length of basal dendrites (D). $n_{WT\_control} = 5$, $n_{WT\_loser} = 6$, $n_{5\text{-Htt KO}\_control} = 6$, $n_{5\text{-Htt KO}\_loser} = 6$; values: Table 3.3.8.

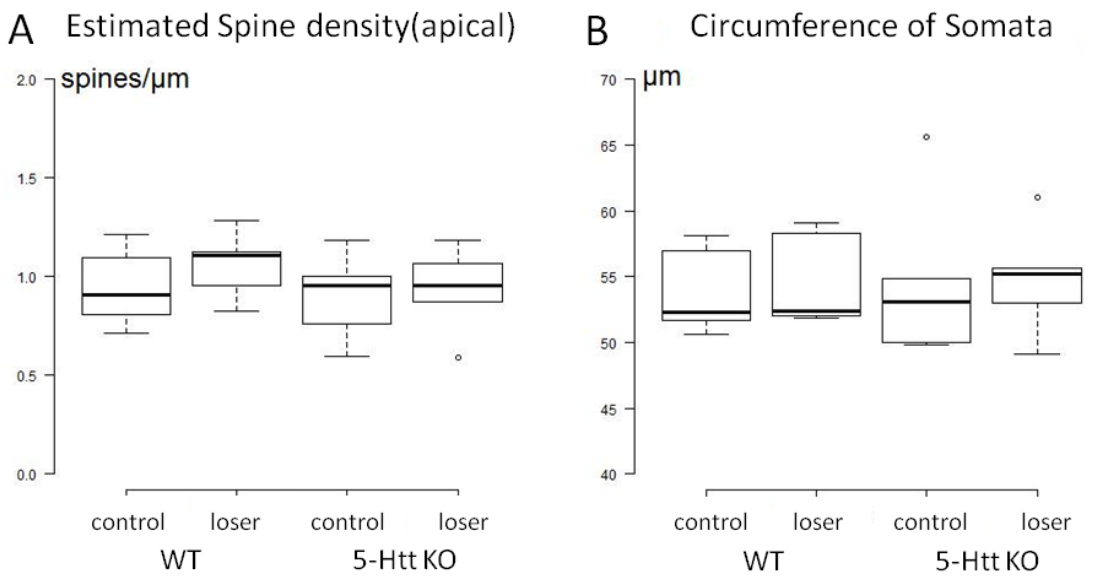


Figure 3.3.12: Analysis of spine density of apical dendrites and soma size in the hippocampus. No differences between groups could be detected concerning the spine density of segments of apical dendrites in the hippocampal pyramidal neurons (A). Also the average circumference of the somata (measured at (0|0|0), the centre of each soma) was the same in each group (B). $n_{WT\_control}$ = 5, n_{WT_loser} = 6, $n_{5\text{-Htt KO}\_control}$ = 6, $n_{5\text{-Htt KO}\_loser}$ = 6; values: Table 3.3.9.

Parameter	Treatment group	N_{values}	Min.	1st Q.	Median	Mean	3rd Q.	Max.
Spine density	WT control	5	0.71	0.81	**0.91**	0.95	1.10	1.21
"	WT loser	6	0.83	0.99	**1.11**	1.07	1.12	1.29
"	KO control	6	0.60	0.81	**0.96**	0.91	0.99	1.18
"	KO loser	6	0.59	0.89	**0.96**	0.94	1.04	1.19
Soma size	WT control	5	50.60	51.70	**52.25**	53.91	56.93	58.08
"	WT loser	6	51.88	52.08	**52.36**	54.34	56.84	59.12
"	KO control	6	49.83	50.42	**53.05**	54.41	54.78	65.60
"	KO loser	6	49.10	53.43	**55.20**	54.85	55.63	61.00

Table 3.3.9: Analysis of spine density and soma size in the hippocampus. Rounded values of mean spine density (of dendritic segments) and of the area of the soma (in µm) at the reference point (0|0|0) were used for boxplots in Figure 3.3.12; Min.: minimum; 1st Q.: first quartile; Mean: arithmetic mean; 3rd Q.: third quartile; Max.: maximum; AD: apical dendrites; BD: basal dendrites.

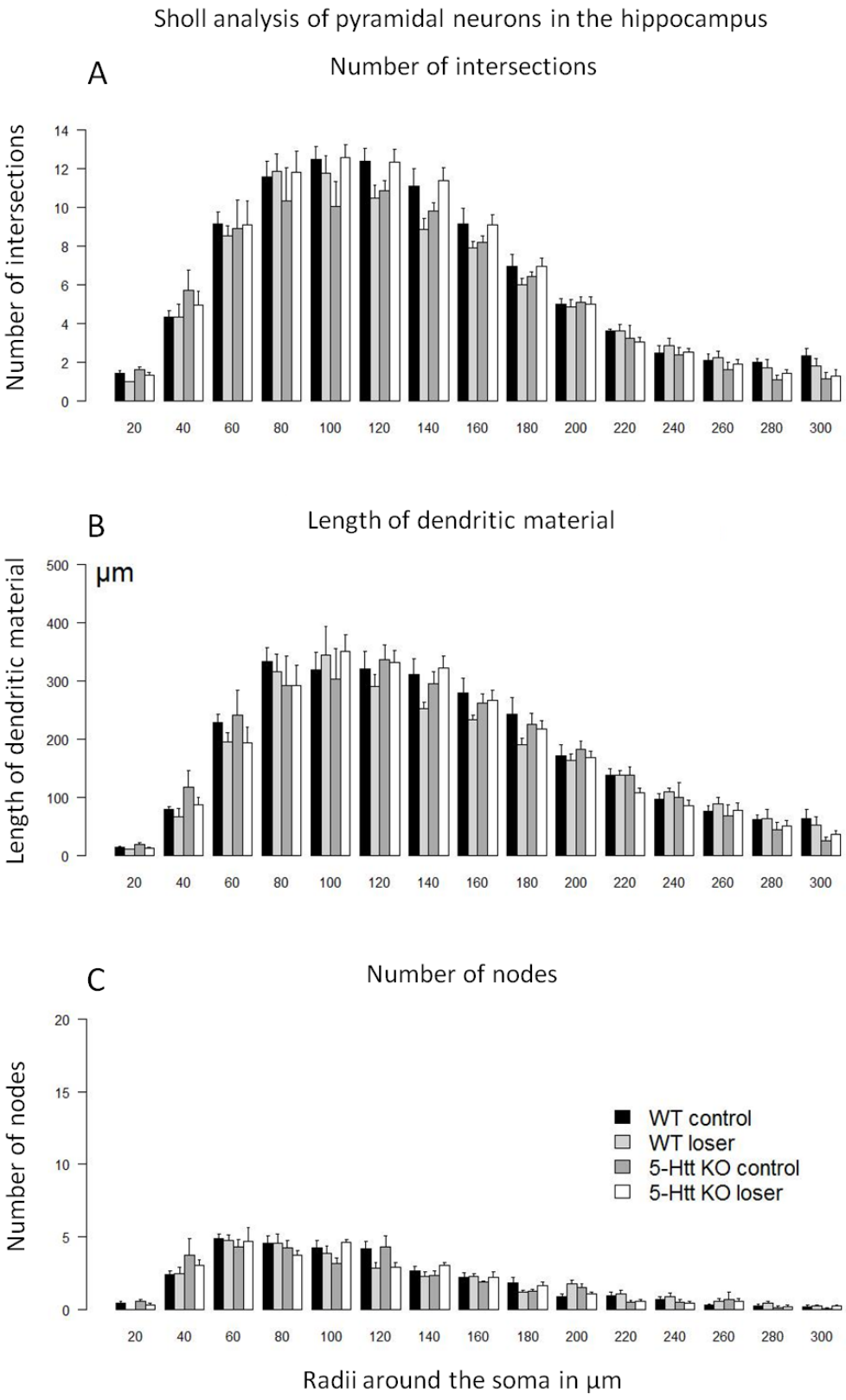


Figure 3.3.13: Sholl analysis of apical dendrites of CA1 pyramidal neurons. Sholl analysis of the amount of intersections (A) with apical dendrites and concentric spheres of different radii around the soma, (B) the length of dendritic material between neighbouring spheres and (C) number of nodes of apical dendrites (amount of nodes in sectors between adjacent spheres). Bars: Arithmetic mean of all animals per group; error bars: SEM. $n_{WT\_control} = 5$, $n_{WT\_loser} = 6$, $n_{5\text{-}Htt\ KO\_control} = 6$, $n_{5\text{-}Htt\ KO\_loser} = 6$

4. Discussion

4.1. Acute stress, *5-Htt* genotype and gender have an influence on stress reaction and gene expression in C57BL6/6J mice

In the here performed study, it could be shown that stress, *5-Htt* genotype and gender have an influence on the stress-dependent corticosterone release as well as on the expression of the two IEGs c-Fos and Fra-2 in different regions of the brain. It could be shown too, that these three factors (stress, *5-Htt* genotype and gender) have only little effect (in comparison to the other effects) on the expression of the three SPs Syt I, Syt IV and Stx 1A in these regions of the brain. Additionally, the assumption that Syt I and Syt IV have basically different functions in transmitter release and synaptic plasticity could be substantiated as well, as in the present study very different expression patterns of these both SPs could be found. The gene expression patterns which were found here are comparable to the gene expression patterns which Mittelsteadt et al. showed in their study (Mittelsteadt, Seifert et al. 2009). Moreover, various effects of gender and acute stress on the gene expression of these SPs could be demonstrated in the present study and these effects varied strongly concerning the expression of Syt I and Syt IV as summarized in Table 4.1. Both genes seem to be induced in a different way and therefore play different roles in synaptic plasticity and stress-induced gene expression. The expression of CRH, CRHR1 and CRHR2 was mainly influenced by gender and to a lesser extent by stress and *5-Htt* genotype.

4.1.1. Methodical considerations of the acute stress study

The effect of various external stimuli (such as acute or chronic stress) on the expression of different SPs is a wide field of interest which is the topic of many studies (Thompson 1996; Babity, Armstrong et al. 1997; Thome, Pesold et al. 2001; Gao, Bezchlibnyk et al. 2006; Fei, Guo et al. 2007) but is still not fully illuminated. Thome et al. investigated the influence of acute immobilization stress on the expression of synaptophysin and Syt III in rat hippocampus (Thome, Pesold et al. 2001). After 1 h of immobilization stress, they found an increased expression of Syt III. In contrast to this the expression of synaptophysin was decreased after acute stress. Same as in Thome's study, an immobilization stress of 1 hour was used in the study presented here. The mice were immediately sacrificed as it could be shown in a pretest that stress-induced changes in the expression of SP mRNA can be detected best directly after stress (Nietzer 2006, diploma thesis). As corticosterone is produced in a circadian rhythm over the day with a peak in the late afternoon and quite low basal corticosterone levels in the morning (Dixit and Buckley 1967; Zimmermann and Critchlow 1967), the experiments were performed between 9:00 and 12:00 a.m. and the treatment groups were broken up according to gender. As female mice tend to synchronize their female cycle when they are housed in female groups together with male mice in the same room (Whitten 1956) as our mice were, the texture of

the cervical mucus was not controlled to define the oestrus state because this procedure would have biased the scientific results of this study because the procedure of taking a smear is quite stressful for the mouse and would have changed the basal corticosterone levels of the female control animals.

4.1.2. *5-Htt* KO mice displayed a different stress reactivity compared to *5-Htt* WT mice

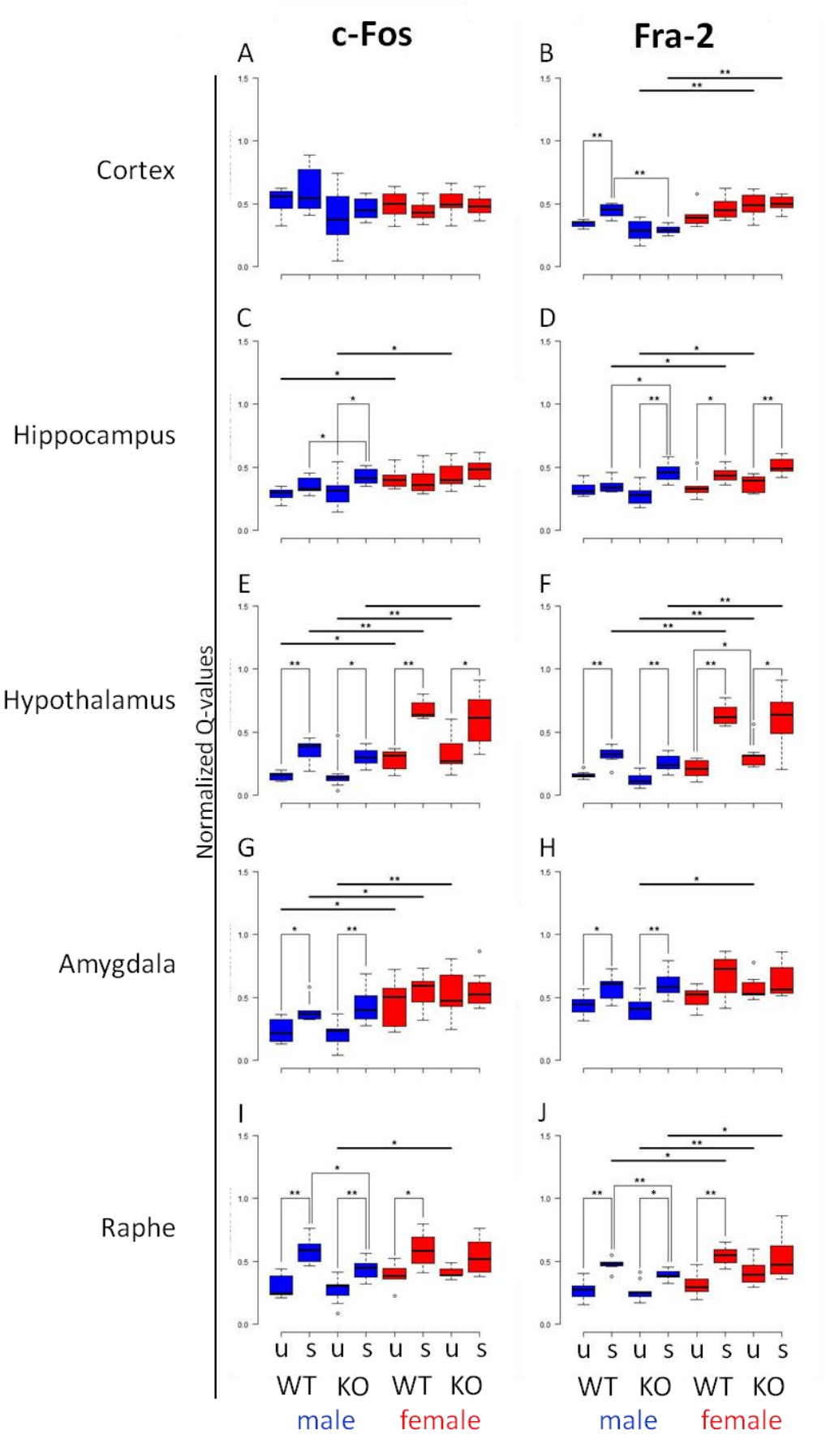
In all *5-Htt* genotypes (*5-Htt* WT, *5-Htt* HET and *5-Htt* KO), acute immobilization stress resulted in strongly increased (about 4-6 fold) plasma corticosterone levels. In stressed male animals tendentially lower genotype-dependent corticosterone levels were detected in the *5-Htt* KO mice compared to the *5-Htt* HET and the *5-Htt* WT mice, whereas genotype-dependent differences in stressed females couldn`t be found. These results are in accordance with the results shown in the study of Tjurmina et al. (Tjurmina, Armando et al. 2002), who investigated female mice only and couldn`t find a genotype effect on the corticosterone levels – with and without stress. It was reported that the basal plasma corticosterone levels in unstressed *5-Htt* KO mice were lower compared to the basal plasma corticosterone levels in *5-Htt* WT mice (Li, Wichems et al. 1999; Lanfumey, Mannoury La Cour et al. 2000). In accordance with Tjurmina and coworkers (Tjurmina, Armando et al. 2002) these results couldn't be confirmed in the here presented study as no differences could be found in the corticosterone levels between the unstressed control groups. In contrast to Li et al. an increased corticosterone level could be found after acute stress in *5-Htt* KO mice of both genders, whereas in the study of Li et al. 8-OH-DPAT increased corticosterone levels in *5-Htt* WT and *5-Htt* HET mice but not in *5-Htt* KO mice. The discrepancy between the current data and those published previously may not only be due to different activators of corticosterone release (acute immobilization stress versus 8-OH-DPAT injection) but also due to different genetic backgrounds, as the here used mice had a C57/Bl6 background, which is an inbred strain, while in contrast to this, Li and associates used CD1 mice, an outbred strain (Li, Wichems et al. 1999). It is well known that these differences in the genetic background produce different sensitivities to stress (Shanks, Griffiths et al. 1994; Flint and Tinkle 2001). The results of corticosterone measurements in the present study suggest an altered feedback inhibition in *5-Htt* KO mice as their amount of released corticosterone after stress was lower than the corticosterone levels of *5-Htt* WT and *5-Htt* HET mice after stress. In their study Li et al. discuss that the sensitivity of the adrenal gland to ACTH is altered by a deficiency in or lack of the 5-Htt function (Li, Wichems et al. 1999), as 5-Htt binding sites are known to be present in the adrenal gland (Blakely, Berson et al. 1991; Lefebvre, Contesse et al. 1998). In addition to the exaggerated adrenomedullary responses in mice with a targeted disruption of the *5-Htt* gene (Tjurmina, Armando et al. 2002), the reduced corticosterone response to immobilization can also be discussed as being intrinsically connected with the anxiety-like behaviours in these mice or more exactly, the less aggressive phenotype of these mice. One reason for the strong gender differences in the corticosterone release may be the influence of sexual hormones on

the HPA axis and other hormonal systems, especially in females. It seems to be that estrogens enhance the activity of the HPA axis (Burgess and Handa 1992; Handa, Burgess et al. 1994; Weiser and Handa 2009) and androgens inhibit its activity (Gaskin and Kitay 1970; Handa, Burgess et al. 1994; Weiser and Handa 2009). Further studies have to be carried out to elucidate the relation of sexual hormones and the HPA axis as well as the relation of sexual hormones, corticosterone and the expression of IEGs and SPs.

4.1.3. The expression of the IEGs c-Fos and Fra-2 is different in male and female mice

An interesting result of the present study was that in the hypothalamus the expression of both IEGs c-Fos and Fra-2 was nearly twice as high in female mice compared to male mice. This result was true in unstressed mice as well as in stressed mice of both *5-Htt* genotypes (*5-Htt* WT and *5-Htt* KO). As the same effect could be shown in the plasma corticosterone levels of male and female mice, it seems that there is a similar regulation mechanism both in the HPA axis and in the process of stress-induced IEG expression. It is well known that there is an interaction between the HPA axis and the HPG axis as e.g. the stress-induced IEG activation in the CNS is mostly suppressed on proestrus (Figueiredo, Dolgas et al. 2002). In rats, a unilateral injection of formalin induces bilateral c-Fos expression in the hippocampus of males and females, but the number of labeled neurons is twice as high in females compared to males. In the septum, an injection of formalin as well as restraint stress increases c-Fos expression, but this increase tends to be greater in males than in females (Aloisi, Zimmermann et al. 1997). In rats too, it could be shown that, induced by restraint stress, the c-Fos expression becomes increased by female hormones (estradiol benzoate) in the paraventricular nucleus of the hypothalamus and it becomes decreased by male hormones (dihydrotestosterone propionate) in the same region (Lund, Munson et al. 2004). How female hormones of the HPG axis increase the expression of IEGs in general has yet to be investigated.

Figure 4.1: Summary of the expression of c-Fos and Fra-2 in cortex (A, B), hippocampus (C, D), hypothalamus (E, F), amygdala (G, H) and raphe (I, J) of male and female *5-Htt* WT and *5-Htt* KO mice – with and without stress. Stress increased the expression of c-Fos and Fra-2 mainly in hypothalamus in both genders and in each genotype (E, F). Fra-2 was increased by stress also in hippocampus (D) and in the amygdala of male mice (H). Gender differences in the expression of c-Fos could be detected mainly in the hypothalamus (E) and the amygdala (G). Gender influenced the expression of Fra-2 mainly in the hypothalamus (F) and in the raphe (J) but also in the cortex (B) and in the hippocampus (D). In the raphe, significant stress effects on the expression of Fra-2 could be demonstrated only in *5-Htt* WT mice (J) while in the hippocampus, significantly increased Fra-2 expression after stress could be demonstrated in *5-Htt* KO mice (D). The data were presented as box-and-whisker diagrams and were analyzed using the open source program R (version 2.10.0). Kruskal-Wallis test cFos: $p = 0.0000000009$, Kruskal-Wallis test Fra-2: $p = 0.00000002$; **: $p < 0.004$ (significant with Bonferroni correction), *: $0.05 > p > 0.004$ (tendency). Thin lines: stress effects and genotype effects; thick lines: gender effects.


c-Fos
Fra-2
A
B
C
D
E
F
G
H
I
J
Cortex
Hippocampus
Hypothalamus
Amygdala
Raphe
Normalized Q-values
u s u s u s u s
WT KO WT KO
male female
u s u s u s u s
WT KO WT KO
male female

Moreover, in the present study we could demonstrate that the expression of c-Fos was increased after stress in the raphe of males and females (of both genotypes) but that this increase was significant only in males but not in females. Additionally, in the amygdala we could show nearly the same phenomenon. This could be a hint of the action of gonadal hormones on serotonergic circuits in the brain. Concerning the expression of Fra-2, similar results were obtained as males showed an increased expression of Fra-2 after stress in the amygdala while the expression of Fra-2 was not even tendential in female mice.

The amygdala plays a role in the processing of various external stimuli e.g. stressful stimuli and it induces the consequent vegetative reactions. The present data infer that male mice respond more acutely to stress than female mice which seem to be better protected from stress-dependent anxiety. But this phenomenon could be a consequence of the female cycle phase which was not diagnosed in this study. It could also be that during another phase of the female cycle this effect turns out differently or vanishes altogether. Larkin and associates could show that c-Fos expression was lower in female rats in consequence of ethanol-induced stress during metoestrus compared to the c-Fos expression during pro-oestrus (Larkin, Binks et al.).

Moreover, in the raphe of *5-Htt* WT mice, the expression of Fra-2 was increased significantly while in the hippocampus no effects or only tendential effects of stress on the expression of Fra-2 could be detected. This could point to a specific circuit between the raphe and the hippocampus in *5-Htt* WT mice. The median raphe nucleus and the dorsal hippocampus are involved in the expression of contextual conditioned fear (Andrade and Graeff 2001). It seems that in *5-Htt* KO mice the stress-induced increase in the expression of Fra-2 in the raphe independent of the expression of Fra-2 in their hippocampus. Hence this circuit could be impaired in the *5-Htt* KO mice, resulting in an increased hippocampal activity independent of the raphe activity. In contrast to this an increased activity in the raphe of *5-Htt* WT mice has an influence on the activity in the hippocampus. In *5-Htt* KO mice this is not so.

4.1.4. Acute stress affects the expression of SPs primarily in the hippocampus

But not only the expression of the two IEGs c-Fos and Fra-2 was changed after stress. The expression of the investigated SPs was also influenced, although to a lesser degree. In the hippocampus of female *5-Htt* WT mice it could be shown that the acute stress used here significantly increased the expression of Syt I whereas the expression of Stx 1A was only tendentially increased. Stress tendentially increased the expression of Syt IV in the hippocampus of male *5-Htt* knockout mice too. Stress-induced increases of Stx 1A could also be demonstrated in the hypothalamus of male *5-Htt* KO mice as well as of female *5-Htt* WT mice, although the differences between the genders here were not significant but tendential.

The hippocampus, together with the cingulate cortex, the hypothalamus, the thalamus and the amygdala is a part of the limbic system which is involved in stress-coping reactions. Acute immobilization leads to typical behavioural changes, such as increased forepaw treading, hind limb abduction, tremor and Straub tail scores (Katz 1979), as well as reduced rearing and significantly reduced locomotor activity. Thome and associates showed that these behavioural alterations tend to normalize after repeated chronic stress exposure (Thome, Pesold et al. 2001). As stress is a non-specific reaction to various stress factors which unbalance the organism, an acute stress reaction is essential to redress balance. Dysregulation of the HPA axis and an enhanced CRH activity also play a key role in these stress-related disorders. Stress stimulates the HPA axis and leads to an enhanced cortisol (in mice: corticosterone) level and it also leads to damage of neurons in the hippocampus, as Margarinos and coworkers could show in their study (Magarinos and McEwen 1995). Our findings support the notion that the hippocampus' synaptic plasticity is influenced by stress as this changes the expression of SPs somehow. Hence this region plays a role in stress coping mechanisms as it has been published previously (McEwen, Weiss et al. 1968). Moreover it could be shown that there is an influence of gender and genotype on the expression of the here analyzed SPs. The fact that in mice of different gender and *5-Htt* genotype different SPs are upregulated after stress (Syt I and Syt IV in female mice and Stx 1A in male mice) can be seen as an indicator that the here investigated proteins have very different functions. It is also well known that the hippocampus is involved in learning and memory and that acute stress at first facilitates learning, as illustrated by the Yerkes-Dodson law (Broadhurst 1957). In contrast to this there is a continuous impairment of learning if stress becomes chronic. This can be seen as an off-peak curve of an inversed U-shaped curve. An increased expression of SPs involved in signal transduction induced by unique stress exposure could be part of the mechanism causing the initial simplification of learning after acute stress. Concerning Syt I, a tendentially increased mRNA expression could be found in 5-Htt deficient female mice compared to female *5-Htt* WT animals. Additionally, an increased mRNA expression of Stx 1A could be demonstrated after stress in the hypothalamus of female *5-Htt* WT mice and male 5-Htt deficient mice. Stx 1A is a part of the SNARE-complex and it is shown to interact with some neurotransmitter transporters (5-HTT, DAT, GAT in a PKC-dependent manner; Glycine transporter) (Deken, Beckman et al. 2000; Geerlings, Lopez-Corcuera et al. 2000; Haase, Killian et al. 2001). Haase and coworkers provided evidence that Stx 1A interacts with 5-HTT and modulates the cell-surface expression which finally results in a reduction of 5-HT transport (Haase, Killian et al. 2001). Activation of protein kinase C (PKC) leads to an acute down-regulation of 5-HTT-mediated 5-HT uptake (Qian, Galli et al. 1997). Our data do not disagree with these findings, although we didn't find any genotype-dependent significant changes in the mRNA expression of Stx 1A. Compared to *5-Htt* WT mice of the same stress condition, only tendential differences between genotypes could be found in the hippocampus of

male stressed mice and in the hippocampus of female unstressed mice as well as in the hypothalamus of male stressed mice. In all cases, the expression of Stx 1A seemed to be increased in *5-Htt* KO mice compared to *5-Htt* WT mice. This could be part of a gender-dependent compensation mechanism in *5-Htt* KO mice, as the changes in expression were different in males and females.

4.1.5. Strong gender differences in the expression of SPs in cortex and raphe

Independent of stress, strong gender effects on the mRNA expression of Syt IV and Stx 1A could be found in the cortical regions. There was an increased gene expression in male mice compared to female mice. In contrast to these findings, there was an effect on the expression of Syt I in the raphe regions of female mice, where an increased expression of Syt I mRNA could be shown in female mice compared to male mice, and there were significant differences in *5-Htt* KO mice. There are connections of the raphe and the cortex (Celada, Puig et al. 2001) as there are mPFC neurons which project monosynaptically to 5-HT neurons (Hajos, Richards et al. 1998) and which possess postsynaptic 5-HT 1a receptors. Hence there is a feedback control of serotonergic activity via this pathway consisting of connections between cortical regions and raphe nuclei. It seems that these pathways are influenced by the *5-Htt* KO as these gender differences could be seen especially in the 5-Htt deficient mice.

4.1.6. Syt I and Syt IV have different functions although they are co-localized in some regions of the brain

In accordance to Mittelsteadt et al. (Mittelsteadt, Seifert et al. 2009) it could be shown in the present study that Syt I as well as Syt IV mRNA both are universally expressed in most brain areas whereas the expression intensity varies across the different brain regions.

Syt I acts as a Ca^{2+} sensor in fast synaptic vesicle exocytosis (Geppert, Goda et al. 1994) and triggers a fast release. The exact function of Syt IV is yet unknown, but it was once postulated to be an IEG (Vician, Lim et al. 1995). The present results argue against this assumption of Syt IV as an IEG, as the mRNA expression of Syt IV is not in the same way increased after stress as is the case concerning the expression of the IEGs c-Fos and Fra-2. The fact that one of the biggest differences concerning the expression pattern of Syt I and Syt IV was found in the cerebellum suggests that the function of Syt IV is different from the function of Syt I. In the cerebellum, Syt I is expressed in single granule cells but not in Purkinje cells whereas Syt IV is mostly expressed in Purkinje cells and to a lesser extent in granule cells (Nietzer 2006). This result is in accordance with the results Mittelsteadt et al. showed in their study (Mittelsteadt, Seifert et al. 2009). Additionally there are co-localisations of Syt I and Syt IV mRNA in the same cells in other brain regions e.g. in the hippocampus (Nietzer et al. unpublished) where Syt I and Syt IV are co-expressed in granule cells as well as in single cells of the hilus (polymorphic layer). Additionally, the qRT-PCR mRNA expression data showed that changes in the

mRNA expression of Syt I were not directly coupled with a change in the mRNA expression of Syt IV. This result strengthens the hypothesis that these SPs are probably expressed independently of each other's expression, although they are in some cells co-expressed. The gender-based differences concerning the gene expression of both SPs after stress is another sign of independent functions of these SPs. But the function of Syt I and Syt IV and their role in both genders have to be analyzed yet.

4.1.7. Syt IV as a compensatory IEG?

Syt IV is postulated to be an inhibitor of transmitter release as the overexpression of Syt IV leads to less transmitter release (Littleton, Serano et al. 1999; Wang, Grishanin et al. 2001). Thus it could be that stress increases transmitter release activity in the raphe and that in consequence of these changes the transmitter release in the hippocampus is decreased. This could be a compensation mechanism in *5-Htt* KO mice, as this circuit between their raphe and their hippocampus seems to be reinforced by stress. For this reason it could be suggested that Syt IV is not simply a normal IEG but that in some situations it could be mobilized into action as a compensatory IEG.

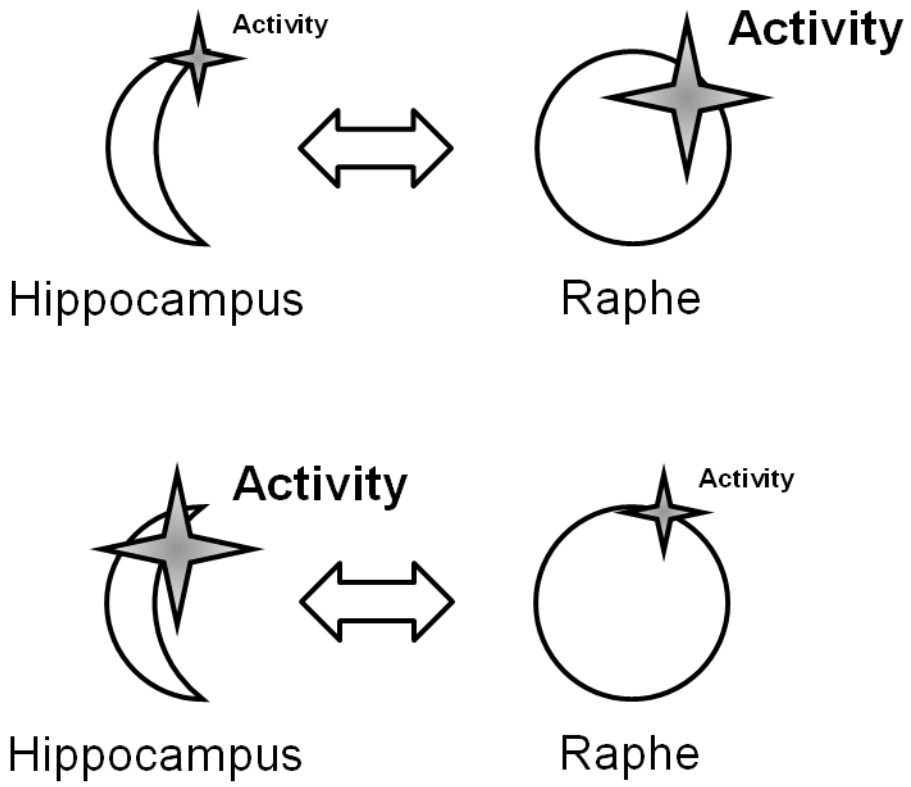


Figure 4.2: Possible activity circuit between the hippocampus and the raphe in male mice. The stronger the expression of SytIV (and hence the impairment of neuronal transmission) in the raphe the lesser the expression in the hippocampus. In the *5-Htt* KO mice, this circuit seems to be strengthened.

A

Syt I	male				female			
	WT		*5-Htt* KO		WT		*5-Htt* KO	
	unstressed	stressed	unstressed	stressed	unstressed	stressed	unstressed	stressed
Cortex	0,45	0,44	0,45	0,48 #	0,46	0,47	0,47 $	0,49
Hippocampus	0,53	0,52	0,53	0,56	0,53	0,72 ** $$	0,66 # $	0,69 $
Hypothalamus	0,54 $	0,53	0,32	0,41	0,37	0,42	0,46	0,48
Amygdala	0,65	0,56	0,60	0,65	0,68	0,75 $$	0,68	0,67
Raphe	0,32	0,29	0,31	0,28	0,37	0,41 $$	0,38 $	0,41 $

B

Syt IV	male				Female			
	WT		*5-Htt* KO		WT		*5-Htt* KO	
	unstressed	stressed	unstressed	stressed	unstressed	Stressed	unstressed	stressed
Cortex	0,30 $$	0,26 $$	0,29 $$	0,29 $$	0,18 *	0,16	0,15	0,15
Hippocampus	0,60	0,68	0,67 $	0,75 * $$	0,62	0,59	0,54	0,55
Hypothalamus	0,56	0,56	0,54	0,61	0,62	0,70	0,66 $$	0,66
Amygdala	0,69 *	0,65	0,67 $	0,67	0,65	0,60	0,60	0,55
Raphe	0,68	0,61	0,61 *	0,51	0,53	0,55	0,54	0,52

C

Stx 1A	male				female			
	WT		*5-Htt* KO		WT		*5-Htt* KO	
	unstresse d	stressed	unstressed	stressed	unstressed	stressed	unstressed	stressed
Cortex	0,62 $$	0,55 $$	0,62 $$	0,60 $$	0,45	0,43	0,46	0,45
Hippocampus	0,65	0,52	0,57	0,61 #	0,58	0,69 * $	0,68 #	0,73
Hypothalamus	0,67	0,60	0,60	0,69 * #	0,65	0,75 * $$	0,73 $	0,72
Amygdala	0,71	0,66	0,73	0,72	0,76	0,73	0,79	0,74
Raphe	0,62	0,67	0,74	0,63	0,66	0,70	0,69	0,70

Table 4.1: Summary of the expression changes of Syt I, Syt IV and Stx 1a in different brain regions of male and female *5-Htt* WT and *5-Htt* KO mice - with and without acute immobilization stress.

*, **: gene expression is increased by stress (same genotype and gender); #, ##: the expression is increased by genotype (same stress condition and gender); $, $$: the expression is increased by gender (same genotype and stress condition)

**, ##, $$: $p < 0.004$ (significant with Bonferroni correction), *, #, $: $0.05 > p > 0.004$ (tendency). Thin lines: stress effects and genotype effects; thick lines: gender effects.

4.1.8. The expression of CRH, CRHR1 and CRHR2 in the hypothalamus is influenced mostly by gender and only to a feeble extent by stress

As the hypothalamus is the first region of the brain where CRH is released after stress, it's a very important part of the HPA axis. So it is assumed that the CRH expression would increase rapidly in this region after stress. But in the present study, the expression of CRH was increased after stress only tendentially in male *5-Htt* WT mice and not in all other groups. Also Greetfeld and collegues could show a rapid stress-induced increase in CRH mRNA expression in the mouse paraventricular nucleus (PVN) of the hypothalamus (Greetfeld, Schmidt et al. 2009). As they used only male C57BL/6J WT mice in their study, we could confirm these results, as in the total hypothalamus of our mice at least a tendential increase of CRH mRNA after stress could be found in male *5-Htt* WT mice. Since it was not possible to differentiate between different hypothalamic nuclei while dissecting the brain for the here used qRT-PCR, the mRNA level we measured in our study was the summary of all hypothalamic nuclei pooled together. This could be one reason why any increase in CRH expression was not significant but only tendential.

CRH is mainly expressed in the parvocellular neurons of the hypothalamus. Its basic expression in the hypothalamus is quite strong as shown by ISH in a previous study (Jakob 2008). In another study it could be shown that chronic stress increases the expression of CRH in the hypothalamus (Herman, Cullinan et al. 1992). Herman and associates could demonstrate that the maximum of the CRH expression occurs 2 h after onset of stress and decreases again (Herman, Cullinan et al. 1992). Its basal level is reached again 4 h after stress (Greetfeld, Schmidt et al. 2009). This could be a second reason why the increase of CRH mRNA was not clearly detected in the present study as the mice were already sacrificed 60 min after the onset of stress. Interestingly, a stress-induced increase of CRH expression in the hypothalamus of male *5-Htt* WT mice could be detected neither in female *5-Htt* WT mice nor in the *5-Htt* KO mice of both genders. This points to an interaction of 5-Htt and gender as discussed in Chapter 4.1.10. But strong gender effects concerning the expression of CRH, CRHR1 and CRHR2 could be found in the hypothalamus: CRH as well as CRHR2 were expressed more strongly in female mice than in male mice whereas CRHR1 was expressed more strongly in male mice. This is another hint to a gender-dependent regulation of the HPA axis mediated by steroid hormones. This has already been demonstrated in previous studies: In male rats it could be shown that gonadectomy increases hypothalamic CRH immunoreactivity (Bingaman, Van de Kar et al. 1995). Additionally, another study has shown some evidence of a direct regulation of human CRH gene expression by estrogen (Vamvakopoulos and Chrousos 1993). We can suggest from all these data that the interactions on the hypothalamic level may contribute to the sexual dimorphism of stress response (Gaillard and Spinedi 1998; Spinedi and Gaillard 1998).

CRHR1 is shown to be expressed rather feebly in the hypothalamus of mice (Van Pett, Viau et al. 2000). But other investigators have detected stronger expression levels of CRHR1 in the hypothalamus (Jakob 2008). CRHR2 was also shown to be expressed only feebly in the hypothalamus, but there are some single cells in the hypothalamus which express CRHR2 quite strongly at basal levels (Van Pett, Viau et al. 2000). The here presented data don't disagree with all these findings, as the Ct-values of the qRT-PCR measured here were quite high, meaning that the mRNA levels were quite low in the investigated tissue. The role of the CRHR1 in the PVN is not yet fully understood. There is a "ultra short positive feedback loop" proposed by Ono and associates (Ono, Bedran de Castro et al. 1985) which supposes that CRH stimulates its own expression via the CRHR1 in the PVN. But in our study we couldn't find any increased CRHR1 mRNA level in consequence of stress. Also other investigators couldn't find any stress-induced increase of CRHR1 expression in the hypothalamus (Imaki, Katsumata et al. 2003; Greetfeld, Schmidt et al. 2009) so these data have yet to be verified.

Greetfeld and associates could show that stress has antagonistic effects on the expression of CRHR1 and CRHR2 of male mice. This supports the hypothesis that the CRHR2-specific ligand urocortin and CRHR2 may represent an 'antiparallel' stress system to the CRH/CRHR1 system (Greetfeld 2005). They suppose that CRH appears to play a stimulatory role in stress responsivity through activation of CRHR1, whereas specific actions of the CRHR2-selective ligands urocortin II or urocortin III may be important for damping stress sensitivity (Skelton, Nemeroff et al. 2000). As in all these studies single nuclei of the hypothalamus were investigated separately, the results of our study are not really comparable to these previous studies: in our male *5-Htt* WT mice no increase/decrease in the expression of CRHR1 and CRHR2 could be found in the hypothalamus. But in contrast to them we investigated the total hypothalamus because it was not possible to dissect single nuclei from frozen tissue. For this reason small differences in single nuclei of the hypothalamus couldn't be detected but only changes in the summary of all hypothalamic nuclei.

As former studies only investigated male animals (Jakob 2008; Greetfeld, Schmidt et al. 2009) it is interesting to see that there are differences in stress-dependent gene expression between males and females: In our study, the expression of CRHR2 was tendentially increased by stress in the hypothalamus of female *5-Htt* WT mice but not in male mice. CRHR2-related action is known to be involved in stress-coping reactions (Bale, Contarino et al. 2000; Skelton, Nemeroff et al. 2000; Coste, Heard et al. 2006) as male - but not female - *CRHR2* KO mice exhibit enhanced anxious behaviour in several tests of anxiety (Kishimoto, Radulovic et al. 2000) in contrast to mice lacking the CRHR1 (Timpl, Spanagel et al. 1998). The here found increase of CRHR2 expression in females could be a compensation mechanism in female mice to reduce the stress reaction. Additionally, it is known that estradiol impairs the sensitivity of the HPA axis to glucocorticoid negative feedback (Weiser and

Handa 2009). Moreover, corticosterone inhibits CRH and AVP expression in PVN neurons (Kovacs and Mezey 1987; Swanson and Simmons 1989) and the corticosterone level is much higher in female mice than in male mice (this study). Therefore the higher CRH expression levels in female mice could be connected with their elevated corticosterone levels as more CRH has to be produced to counteract the effects of the corticosterone. The lower CRHR1 expression level in females compared to males can be explained in that way: CRHR1 is the main receptor of CRH and it's supposed to stimulate the release of CRH (Ono, Bedran de Castro et al. 1985) which is not required any more in the females. CRHR2 is supposed to be involved in the down-regulation of stress reaction (Skelton, Nemeroff et al. 2000) and for this reason may be expressed proportionally to the CRH expression, as shown in the present study, at least in the hypothalamus, the most important region of the brain involved in the action of the HPA axis.

4.1.9. CRHR1 – the main receptor of CRH is influenced by stress in the limbic system

Although in the cortex, no stress-dependent differences in the expression of CRHR1 could be found in the here performed study, the expression of CRHR1 could be shown to be increased tendentially by stress in the hippocampus of female *5-Htt* WT and *5-Htt* KO mice. The expression of CRHR1 here was tendentially lower in female *5-Htt* WT mice than in male *5-Htt* WT mice – with and without stress. A rapid stress-induced down-regulation of CRHR1 mRNA was detected previously in the hippocampal pyramidal cell layer (CA1) of male WT mice by Greetfeld and associates (Greetfeld, Schmidt et al. 2009). In this study, it could also be shown that CRHR1 mRNA levels in the hippocampal dentate gyrus of male mice remained unchanged (Greetfeld, Schmidt et al. 2009). This points to a stress-dependent regulation of CRHR1 in different neuronal populations of specific regions. In contrast to them we didn't find any stress-dependent changes in CRHR1 mRNA expression after stress in the hippocampus of male mice. A reason for this could be that in our study it was not possible to analyze subregions of the hippocampus separately. Only the total hippocampus could be dissected from the frozen tissue and therefore the mRNA of all subregions was pooled. The same was true for the cortex as in our study the total cortex was investigated and for this reason differences in subregions of the cortex were left out.

In the amygdala, we could detect a tendential decrease in CRHR1 mRNA expression in female *5-Htt* WT mice after stress. Additionally, CRHR1 expression was lower in male *5-Htt* KO mice than in female *5-Htt* KO mice – with and without stress. Interestingly, in the amygdala of female *5-Htt* WT mice also a tendential decrease in CRH expression could be found parallel to the decrease of the CRHR1 expression in this region. These results fit in with the findings of McEuen and associates who supposed that the profile of acute responses across behavioural stress tests clearly

marks gender and CRH receptor dysregulation as the two additive factors in stress sensitivity (McEuen, Semsar et al. 2009). They also suggest that gonadal hormones may have an influence on synaptic plasticity and neurotransmission which mediates these gender differences (McEuen, Semsar et al. 2009). It could be that in females, differences in responsivity may arise from rapid actions between estrogen receptors and glutamatergic signaling in limbic brain regions (Micevych and Mermelstein 2008), particularly within regions receiving stress-mediated 5-HT inputs such as the amygdala and the hippocampus.

4.1.10. Interactions of the 5-Htt and gene expression

Only tendential effects of *5-Htt* genotype could be found in the limbic regions of the brain (hypothalamus, hippocampus, amygdala) but not in the cortex and the raphe. In the hippocampus, CRH mRNA expression was higher in unstressed female *5-Htt* KO mice compared to *5-Htt* WT mice. In the hypothalamus of stressed male *5-Htt* KO mice, CRHR1 was expressed tendentially less than in that of stressed *5-Htt* WT mice. And in the amygdala, genotype effects concerning the expression of CRH (male control mice and female stressed mice) and the expression of CRHR1 (female stressed mice) could be detected. Interestingly, in the raphe, no differences in gene expression of CRH and its both receptors could be found comparing *5-Htt* WT mice and 5-Htt deficient mice. In all the investigated regions the expression of CRHR2 wasn't influenced by the 5-Htt genotype at all.

It is known that immobilization stress results in increased gene expression related to 5-HT production in the raphe (Chamas, Underwood et al. 2004). Moreover, alterations in excitatory and inhibitory influences on serotonergic raphe neurons could be found in previous studies (Kirby, Rice et al. 2000; Daugherty, Corley et al. 2001; Valentino, Liouterman et al. 2001; Kirby, Pernar et al. 2003; Roche, Commons et al. 2003; Keeney, Jessop et al. 2006). Sex hormones have been implicated as modulators of 5-HT transmission projection regions of the raphe (Fink, Sumner et al. 1996; Joffe and Cohen 1998) such as the prefrontal cortex, the amygdala and the hippocampus. McEuen and associates could show that gender affects 5-Htt levels in the dorsal and ventral hippocampus where females had overall lower 5-Htt levels than males (McEuen, Semsar et al. 2009). In the hippocampus we found tendentially lower CRHR1 mRNA levels in female *5-Htt* WT mice than in male *5-Htt* WT mice, whereas in *5-Htt* KO mice this effect was not to be found. This could be a hint that 5-Htt levels influence gender effects, at least in the hippocampus and in the amygdala. Here, CRHR1 was more strongly expressed in female than in male *5-Htt* KO mice, but not in *5-Htt* WT mice. We can say that the here detected influence of gender on gene expression is possibly caused by the deficiency of 5-Htt. In the same regions other studies revealed a sexually dimorphic pattern of 5-Htt expression in C57BL/6 WT mice. Female mice

had lower 5-Htt levels compared to males as reviewed by Koenig (Koenig 2009). In *CRHR2* KO mice, a sexually dimorphic pattern of 5-Htt expression was not observed (McEuen, Semsar et al. 2009). However, there was an almost complete loss of 5-Htt expression in the prefrontal cortex of the *CRHR2* KO mice (McEuen, Semsar et al. 2009). Our study showed that vice versa the loss of 5-Htt doesn't have an influence on CRHR2 expression in the cortical region as shown by the investigation of our *5-Htt* KO mice.

4.2. Three days of social stress as well as the *5-Htt* genotype have an influence on the morphology of pyramidal neurons in the brains of male C57BL6/6J mice

At the beginning of this study it was assumed that social stress during adulthood alone or in combination with the absence of the 5-Htt leads not only to changes in behaviour (Jansen et al. 2010) but also to morphological changes in the brain, as shown in a previous study of Wellman and associates who could demonstrate that the 5-Htt genotype had an influence on neuronal morphology in the murine brain (Wellman, Izquierdo et al. 2007). In the present study it could be shown that the presence of 5-Htt as well as social stress presumably have an impact on the dendritic and synaptic morphology and therefore on the synaptic plasticity of pyramidal cells in different brain regions associated with stress processing, fear and anxiety. In our study slight stress-dependent differences were detected regarding the dendritic length of pyramidal neurons in the IL of the *5-Htt* WT mice group. In IL, too, genotype effects in spine density occurred in the unstressed control group. Concerning genotypes we had only a look at *5-Htt* WT (+/+) and *5-Htt* KO (-/-) mice and omitted the investigation of *5-Htt* Het (+/-) mice, as Jansen and associates showed significant behavioural stress effects only between *5-Htt* WT and *5-Htt* KO (Jansen, Heiming et al. 2010).

4.2.1. Methodical considerations of the social stress study

The main problem in morphological analyses using Golgi-Cox stained neurons was the difficulty to detect very subtle differences or changes in the morphology of dendrites, as the stained dendrites were often covered by other stained dendrites or were even cut or broken in distal parts and therefore could not be reconstructed in their entire length. Moreover, only reasonably big changes could be quantified, as precisely the important distal or terminal parts of dendrites often became artificially shortened during the reconstruction procedure and these are considered to be the first area where morphological changes occur. This led to another problem of morphological analysis, namely the small amount of traceable neurons in some brain regions. In order to be exact, only a small number of really good samples was analyzed, as only those reconstructed neurons which looked complete were taken into account. Moreover, as some brain regions are quite small, only the best reconstructed neurons were evaluated.

As all investigated animals were sacrificed 10 days after social stress exposure, we can proceed from the assumption that the effects we saw were quite lasting. We cannot give information about how morphology of the neurons of our mice would change either immediately or some time after social stress exposure. Further studies showed that acute immobilization stress had an influence on the spine density in the amygdala 10 days after the stressful experience but not immediately after immobilization (Mitra, Jadhav et al. 2005). Also short social defeat stress produced changes in morphology of CA3 neurons in the hippocampus after 3 weeks of regeneration (Kole, Czeh et al. 2004). A shorter interval between the ending of the stressful period (3 x swim stress) and the removal of the brain 72 hour later also led to changes in the morphology of neurons in the prefrontal cortex (Izquierdo, Wellman et al. 2006). In the study of Wellman and associates (Wellman, Izquierdo et al. 2007) experimental animals were sacrificed directly after testing of extinction recall. Extinction recall was carried out 72 h after the ending of the stressful fear conditioning (Wellman, Izquierdo et al. 2007). In our study lasting effects of stress were evaluated while in the Wellman study more immediate morphological changes were investigated. Additionally the mice they used were not naïve mice as they had undergone the fear conditioning and the following extinction as well as extinction recall testing. In contrast to this, our control mice were completely unstressed animals.

There are other studies which indicate that at a morphological level, *5-Htt* WT loser mice correspond to unstressed *5-Htt* KO controls, e.g.in the lateral amygdala (La) and in the basolateral amygdala (BL), since there were no significant differences within spine densities between these two groups (Nietzer, Bonn et al. in preparation). However, in the IL, tendential differences between these two groups have been found too, especially concerning the dendritic length. These similarities could be substantiated in the present study as well. In the IL, differences in spine density were observed, even though there were no significant changes in dendritic length and/or spine number. But as the spine density is the quotient of the spine number and the length of dendritic material, it's perfectly possible that the arithmetic means respectively of the numerator (spine number) and of the denominator (length of dendritic material) are not significantly different between groups, whereas the arithmetic means of the quotient (spine number/length of dendritic material in µm) are significantly different between groups, seeing that the pairing of individual values of numerator and denominator plays an important role in constructing these quotients of spine density.

Additionally, the spine density is a morphological parameter, which is functionally relevant for electrophysiological reactions. Spines are important for the determination of a neuron's electrophysiological properties (for review: Spruston, 2008). Maybe the threshold value of neuronal excitability can be exceeded more quickly if the spine density is increased.

Branch order analysis of spine density per branch order was performed for pyramidal neurons of the IL, as it is one region which is associated with stress processing, fear and anxiety (Amat, Baratta et al.

2005). But this kind of analysis is critical in drawn-out cortical neurons such as in the IL, because the values of the same branching factors of different neurons are pooled. As the branching order is quite differently distributed in each reconstructed neuron even of the same animal, parts of neurons are pooled which lie in totally different layers of the cortex (Figure 4.3). To avoid this problem of pooling mixed data, we performed Sholl analysis for the evaluation of layer-based changes in morphological parameters.

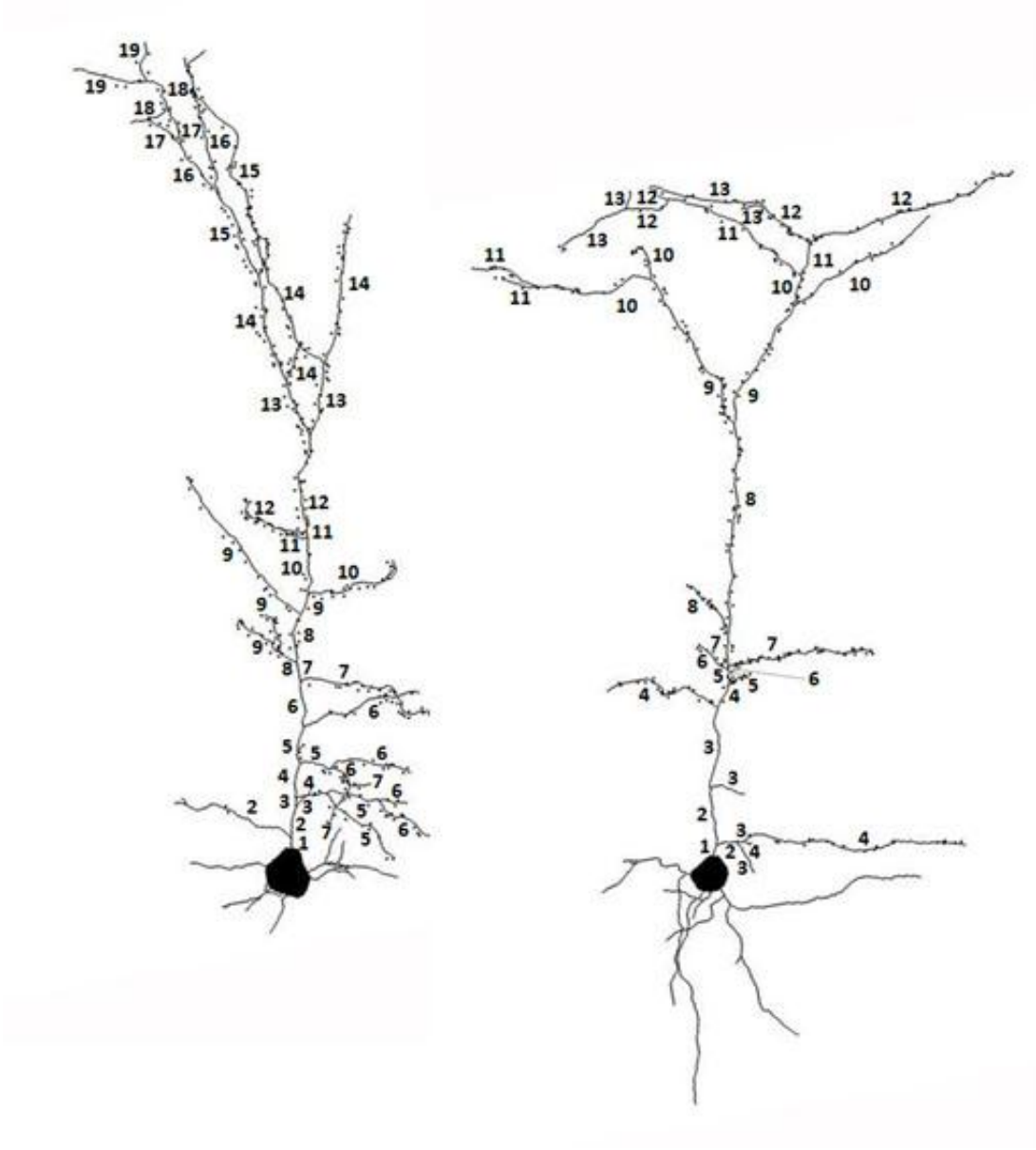


Figure 4.3: Branch Order Analysis. This analysis is questionable in drawn-out cortical neurons as the branching order is quite differently distributed in each reconstructed neuron even of the same animal. Parts of neurons are pooled which lie in totally different layers of the cortex. Both reconstructed neurons belong to the IL of the same animal, but the branch orders of each apical dendrite are distributed differently in space.

4.2.2. No influence of stress and 5-Htt genotype on CG and hippocampus

In contrast to previous studies (Radley, Sisti et al. 2004; Brown, Henning et al. 2005) where morphological changes in CG were found in rats after the animals had been chronically stressed by immobilization, we neither found stress-related morphological changes in this region of the mouse brain nor in the CA1 region of the hippocampus. One reason for this could be that rats differ from mice as shown in previous studies (Asan, Yilmazer-Hanke et al. 2005). Other studies revealed stress-induced changes in these CA1 neurons e.g. after prenatal stress (Martinez-Tellez, Hernandez-Torres et al. 2009) or after corticosterone treatment (Morales-Medina, Sanchez et al. 2009). But as these kinds of stress are chronic, it could be that the short social stress used here as well as the availability of 5-Htt have no influence on the general structure of pyramidal neurons in CG and hippocampus.

Furthermore, in the CA1 region of the hippocampus it was not possible to detect any differences in the morphological structure of pyramidal neurons. In this region, many silver-impregnated neurons were clustered together in columns, which made it very difficult to reconstruct them well. This clustering of CA1 neurons using Golgi-Cox staining is a common phenomenon but the reason for it is not known.

4.2.3. Differences in dendritic length and spine density - mainly in the IL

In the IL, it could be shown in this study that loser *5-Htt* WT mice had slightly more dendritic material in the proximal part of apical dendrites compared to control *5-Htt* WT mice while *5-Htt* KO mice showed no changes in IL morphology when they were stressed. This supports our hypothesis that *5-Htt* deficient mice show less stress-induced changes in neuronal morphology following social stress than *5-Htt* WT mice after stress.

Spines at different positions on a neuron in combination with different receptor subtypes can receive different inputs from various regions (Gulyas, Megias et al. 1999; Spruston 2008; Shansky, Hamo et al. 2009). There are also differences in morphological changes concerning neurons of different projection pathways. Shansky and colleagues showed that BL-projecting neurons in the IL showed no remodeling with stress and they suggested that this pathway might be particularly resilient against the effects of (immobilization) stress (Shansky, Hamo et al. 2009).

Wellman and colleagues showed that there were morphological differences between *5-Htt* WT mice and *5-Htt* KO mice concerning the morphology of IL pyramidal neurons: they found out that pyramidal neurons in the IL of *5-Htt* KO mice had slightly more dendritic material compared to pyramidal neurons in IL of *5-Htt* WT mice (Wellman, Izquierdo et al. 2007). These results are in contrast to our results, as we didn't find any differences between *5-Htt* KO control mice and *5-Htt* WT control mice. But as Wellman and colleagues investigated pyramidal neurons of all cortical layers and the neurons reconstructed in our study were only neurons in a depth of about 400 µm distance from the surface of the brain which can have different connections (Shansky, Hamo et al. 2009). Our

results do not contradict the results of Wellman and associates. Furthermore, they compared mice which had undergone fear conditioning and extinction as well as extinction recall. Hence, they were only comparable to our loser mice, as the control mice we used in our study were completely unstressed. Additionally, the time between stress and brain dissection was different in both studies and then it is not clear how comparable these two different kinds of stress are (fear conditioning and extinction vs. resident-intruder paradigm). Therefore this is the first study looking into morphological differences of the brain of really naïve 5-Htt deficient mice and *5-Htt* WT mice. It is well-known that *5-Htt* KO mice show some differences to *5-Htt* WT mice in consequence of their development without any 5-Htt (Esaki, Cook et al. 2005; Hariri and Holmes 2006; Murphy and Lesch 2008). Pavlovian fear conditioning as well as fear extinction was the same in *5-Htt* WT mice and *5-Htt* KO mice in the study of Wellman and associates (Wellman, Izquierdo et al. 2007). However, they could show that *5-Htt* KO mice displayed deficits in extinction recall.

In the IL, pyramidal neurons in stressed *5-Htt* WT mice exhibited slightly more dendritic material in the proximal part of the apical dendrite compared to pyramidal neurons in unstressed *5-Htt* WT mice. *5-Htt* KO mice showed no changes in the morphology of pyramidal neurons in the IL when they were stressed. In general, it seems to be that 5-Htt deficient mice show less stress-induced changes in neuronal morphology following social stress than *5-Htt* WT mice after stress. However, it is remarkable that in the IL, the stress effects in the control group were opposite to the results in the La and in the BL, which could mean that in unstressed *5-Htt* KO mice social stress results in lower spine density of apical dendrites compared to *5-Htt* WT controls (Nietzer, Bonn et al. in preparation). A possible model for this could be that a lower spine density in the IL leads to a disinhibition of the amygdala. Additionally, a higher spine density in pyramidal neurons of the La and the BL could lead to the intensification of signal processing within the amygdala. This would lead to a higher output and therefore to an increased anxiety-like behaviour (Nietzer, Bonn et al. in preparation).

4.2.4. Correlation of behaviour and morphological changes

In the elevated-plus maze no behavioural differences have been found between *5-Htt* WT and *5-Htt* KO control mice. However, after suffering a loser experience, *5-Htt* KO mice displayed significantly increased anxiety-like behaviour. In the IL, spine density was shown to be lower in the more distal parts of pyramidal apical dendrites of *5-Htt* KO control mice compared to *5-Htt* WT control mice. In *5-Htt* KO loser mice it was increased within one sphere compared to *5-Htt* WT loser mice. During a lifetime without any social stress, these differences in spine density may possibly have no impact on anxiety-like behaviour. However, after social stress, *5-Htt* KO mice are not able to compensate these stressful life events. Additionally, as the IL is connected with the amygdala and as it has an inhibitory effect on the activity of the amygdala (Milad and Quirk 2002; Rosenkranz and Grace 2003; Rosenkranz, Moore et al. 2003; Sotres-Bayon, Bush et al. 2004; Amat, Baratta et al. 2005; Cryan and

Holmes 2005), a decreased spine density in the IL could lead to a disinhibition of amygdala activity and therefore to a hyperactivation of the amygdala. This could lead to a higher amygdala output and hence to an increased anxiety-like behaviour. So the here described morphological differences between *5-Htt* genotypes could describe morphological correlates to the behavioural differences between these genotypes which become apparent only after stress.

Furthermore, after social stress has been manifested in loser experiences, *5-Htt* KO mice spent significantly less time in the open regions of the elevated-plus maze than *5-Htt* WT mice did after this stressful loser experience. In the present study, no big differences have been found concerning IL pyramidal neurons of mice with different *5-Htt* genotypes when they were stressed. There might be differences in other brain regions such as in the amygdala (Nietzer, Bonn et al. in preparation) or e.g. adaptations in the neurochemistry of the brain or in gene expression. For example, Mitra and associates could show that rats which were well-adapted to predator stress displayed alterations in dendritic length and also in the expression of ARC mRNA in the CA1 region of the hippocampus compared to mal-adapted animals (Mitra, Adamec et al. 2009).

It seems to be that *5-Htt* KO control mice as well as *5-Htt* KO loser mice show behavioural and neuromorphological similarities to stressed *5-Htt* WT mice as regards the morphology of neurons in brain regions of fear- and anxiety-processing circuits. After social stress, the morphology in *5-Htt* WT mice changes in order to compensate the stress-evoked physiological changes whereas in 5-Htt deficient mice it seems that no further compensation is possible. This phenomenon can be seen in the amygdala (Nietzer, Bonn et al. in preparation) as well as in the IL (this study).

The results of Pavlovian fear conditioning as well as fear extinction were not different between *5-Htt* WT mice and *5-Htt* KO mice in the study of Wellman and associates (Wellman, Izquierdo et al. 2007). However, they could show that *5-Htt* KO mice displayed deficits in extinction recall. A possible explanation for these results could be that 5-Htt deficient mice were not as easily able to forget negative experiences as *5-Htt* WT mice and hence showed more anxiety-related behaviour in consequence to negative experiences. So the loss of the 5-Htt seems to play a greater role in negative experiences like losing and not in positive experiences (winning), although both situations are stressful for all mice involved as shown by analyzing blood plasma corticosterone levels (Jansen, Heiming et al. 2010).

Although there are no differences between *5-Htt* WT and *5-Htt* KO after Pavlovian fear conditioning and fear extinction (Wellman, Izquierdo et al. 2007), *5-Htt* KO mice displayed increased freezing response compared to *5-Htt* WT mice. That means that these mice did not have the ability to keep extinction memory “in mind”. Certain aspects of the resident intruder paradigm, e.g. spontaneous attacks of the resident, could be comparable to a spontaneous electric shock like it is used in the Wellman study (Wellman, Izquierdo et al. 2007). Maybe *5-Htt* KO mice are not able to delete such

negative experiences and therefore show increased anxiety-like behaviour in comparison to *5-Htt* WT mice. Problems in acquiring or retaining extinction memory also occur in anxiety disorders such as PTSD (American Psychiatric Association, 1994). We conclude from the present data that the plasticity of fear circuits in the brain proceeds during adulthood and that this neuronal plasticity is influenced by the *5-Htt*/*5-HTT* genotype as well as by social experience. In this process the reaction to stressful life experience is influenced by the *5-Htt* genotype.

4.2.5. Short summary and outlook

The kind of social stress used in the present study (resident-intruder paradigm) was shown to increase anxiety-like behaviour (Buwalda, Kole et al. 2005; Jansen, Heiming et al. 2010) and it can be seen as a kind of stress which is comparable to social stress in humans. It has far-reaching effects on further behaviour. Social anxiety or depression is for example often developed after negative social experience.

As there are parallels to human genomics concerning the amount of 5-Htt/5-HTT based on length polymorphism in the SLC6A4, our animal model can in part be applied to the human situation. It is well known that social stress leads to various disorders in humans e.g. depression. How far morphological neuronal changes are involved in the human development of stress-related disorders has not been investigated yet.

The possibility of drawing parallels between neuronal morphological changes in mice and men has to be investigated further. Another problem of experimental design is that certain processes are observed in many different animals and then evaluated as if they had occurred in a single organism.

But these animals are very different individually, although genetically they may be very similar. This means that their reaction to stress may vary very much according to their disposition. If you really wanted to find out what happens after different types of stress, you would have to look into a single animal. This is not possible with the described methods. At least they can suggest how single neurons of the brain are influenced by stress. Another goal would be the investigation of how morphology and electrophysiology are related. Studies already exist in which these two latter fields of research are combined. It was shown that CA1 pyramidal neurons of young male *5-Htt* KO mice have increased conductance compared to young male *5-Htt* WT mice and that this increased conductance reduces the excitability of neurons in the hippocampus of *5-Htt* KO mice as shown by Araragi et al. (poster: Araragi, Nietzer et al. 2010). The morphological analysis of neurons which have been characterized electrophysiologically will bring new insights into the functioning of the morphological features of the different neurons in the brain.

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Danke!

Mein Dank geht an **Prof. Dr. Jürgen Deckert**, der mir als Leiter der Klinik das Anfertigen meiner Dissertation in den Laboren des Instituts ermöglichte.

Des Weiteren möchte ich mich bei **Prof. Dr. Klaus-Peter Lesch** für die Finanzierungsmöglichkeiten und die Betreuung meiner Projekte bedanken.

Herrn **Prof. Dr. Thomas Dandekar** danke ich sehr herzlich dafür, dass es sich als Zweitgutachter dieser Arbeit zur Verfügung stellte!

Prof. Dr. Paul Pauli und dem Graduiertenkolleg (GK) RTG 1253/1 „Emotions“ danke ich für die Finanzierung diverser Kongressaufenthalte sowie für die zahlreichen Veranstaltungen, die im Rahmen des GKs ermöglicht wurden.

Ganz herzlich möchte ich mich bei **Dr. Angelika Schmitt** für die Betreuung meiner Arbeit, die seelische und moralische Unterstützung während meiner gesamten Zeit im Labor in der Füchsleinstraße und für die jahrelange gute Zusammenarbeit bedanken!

Prof. Dr. Esther Asan und **Maria Bonn** möchte ich für alle Mühe im Bereich des Morphologie-Projektes bedanken. Ebenso **Rebecca Heiming**, **Friederike Jansen** und **Prof. Dr. Norbert Sachser** für die gute Zusammenarbeit im Rahmen des SFB-TRR 58.

Mein Dank auch an **Dr. Reinhard Braul** von Microbrightfield, der mir bei jedem Problem bezüglich des Neurolucida-Systems mit Rat und Tat beistand.

I’d like to thank **Dr. Jose Sulon** for conducting the cortisol analyses at the Université de Liège.

Herzlichen Dank an **Marion Winnig** für die ausgezeichneten Gehirnpräparate und die gute Zusammenarbeit bei der Gehirn-Aufbereitung auch an Feiertagen und Wochenenden! Ebenso an **Gabriela Ortega** für die Hilfe bei der qRT-PCR, **Sissi Jakob** und **Dr. Claudia Kriegebaum** für die Unterstützung bei den Stress-Versuchen und der Firma **BioRad** für das Zur-Verfügung-Stellen des CFX384.

Natürlich möchte ich mich auch sehr bei meinen Eltern **Kurt Nietzer** und **Jutta Habedanck-Nietzer** bedanken, die mir das alles ermöglichten und immer hinter mir standen.

Ein ganz herzliches Danke auch an meinen Bruder **Samuel Nietzer** für emotionalen Rückhalt (und für’s Fische-Füttern ☺), an **Michael Ok** für die Hilfe beim Bewältigen von Computer- und anderen technischen Problemen, an **Andreas Staab** für die R- und Grafik-Unterstützung und an **René Ciak** für die mathematische Hilfestellung bei der Neuronen-Auswertung.

Danke an alle Mitarbeiter/innen aus dem Labor der Psychatrie von Würzburg!

Es war eine sehr schöne Zeit mit euch!

5-HIAA	**5-hydroxyindoleacetic acid**
5-HT	**serotonin**
5-HTT	**Serotonin transporter**
ACTH	**adrenocorticotropic hormone**
ADHD	**attention-deficit hyperactivity disorder**
AP	**activator protein**
BL	**basolateral amygdala**
c-Fos	**cellular FBJ murine osteosarcoma**
CG	**cingulate cortex**
CNS	**central nervous system**
Cplx	**complexin**
CRH	**corticotropin releasing hormone**
CRH-BP	**CRH-binding protein**
CRHR1	**CRH receptor 1**
CRHR2	**CRH receptor 2**
CSF	**cerebrospinal fluid**
Ct	**threshold cycle**
DAT	**dopamine transporter**
dd	**doubly distilled**
DIG	**digoxigenin**
EGR1	**early growth response factor 1**
Fra-2	**Fos-related antigen 2**
GABA	**γ-aminobutyric acid**
GnRH	**gonadotropin releasing hormone**
HET	**heterozygous**
qRT-PCR	**quantitative real-time polymerase chain reaction**
HPA	**hypothalamic-pituitary-adrenal**
HPG	**hypothalamic-pituitary-gonadal**
IEG	**immediate early gene**
IL	**infralimbic cortex**
KO	**knockout**
La	**lateral amygdala**
LTP	**long-term potentiation**
MDMA	**3,4-Methylendioxy-N-methylamphetamin**
min	**minute**
mPFC	**medial prefrontal cortex**
NSF	**N-ethylmaleimide sensitive fusion protein**
PC12	**phaeochromocytoma 12**
PFA	**paraformaldehyde**
PTSD	**posttraumatic stress disorder**
RT	**room temperature**
SAM	**sympathetic adreno-medullary**
SNAPs	**soluble N-ethylmaleimide-sensitive factor attachment proteins**
SNAREs	**SNAP receptors**
SP	**synaptic protein**
SSC	**sodium saline citrate**
SSV	**small synaptic vesicles**
Stx	**syntaxin**
Syt	**synaptotagmin**
TBS	**Tris buffered saline**
TCA	**tricyclic antidepressant**
TNF- α	**tumor necrosis factor α**
Ucn	**urocortin**
WT	**wild-type**

Figures:

Tables:

Curriculum Vitae

Name:	Sarah Louise Nietzer
Personal Data:	born on 17.10.1981 in Marktheidenfeld

School and Studying:

01/2007 – 12/2009	Writing the thesis within the doctorate program GRK 1253/1 "Processing of affective stimuli: from the molecular basis to the emotional experience" (“Emotions”) (In cooperation with Prof. Dr. Norbert Sachser of the Westphalian Wilhelms-University of Münster in the context of the project A1 of the SFB-TRR 58 „Fear, Anxiety, Anxiety disorders“)
01/2006 – 10/2006	Diploma thesis „Untersuchungen zur Expression synaptischer Proteine in Serotonin-Transporter-Knockout-Mäusen, mit und ohne akuter Stressexposition“ (Department of Psychiatry, Psychosomatics and Psychotherapy)
10/2001 – 10/2006	Studying Biology at the Julius-Maximilians-University of Würzburg (neurobiology, genetics and bioinformatics)
09/1998 - 06/2001	Matthias-Grünewald-Gymnasium (97941 Tauberbischofsheim)
09/1991 – 06/1998	Dietrich-Bonhoeffer-Gymnasium (97877 Wertheim)
09/1988 – 06/1991	elementary school

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(Place, Date) (Sarah Nietzer)

Affidavit

(Eidesstattliche Erklärung)

I hereby declare that my thesis entitled “Gene and environment interactions in serotonin transporter knockout mice – how stress influences gene expression and neuronal morphology” is the result of my own work. I did not receive any help or support from commercial consultants. All sources an/or material applied are listed and specified in the thesis.

Furthermore, I verify that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg_____

Date Signature