Dissertation

Behavioral and physiologic consequences of inducible inactivation of the *Tryptophan hydroxylase* 2 gene in interaction with early-life adversity

Verhaltens und physiologische Konsequenzen einer induzierbaren Inaktivierung des *Tryptophan hydroxylase* 2-Gens Interaktion mit frühkindlichen Stresses

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Summary

Disruptions in brain serotonin (5-hydroxytryptamine, 5-HT) signaling pathways have been associated with etiology and pathogenesis of various neuropsychiatric disorders, but specific neural mechanisms of 5-HT function are yet to be fully elucidated. Tryptophan hydroxylase 2 (TPH2) is the rate-limiting enzyme for brain 5-HT synthesis. Therefore, in this study a tamoxifen (Tam)-inducible cre-mediated conditional gene (*Tph2*) knockout in adult mouse brain (*Tph2icKO*) has been established to decipher the specific role of brain 5-HT in the regulation of behavior in adulthood.

Immunohistochemistry and high-performance liquid chromatography (HPLC) were used first to test the efficacy of Tam-inducible inactivation of *Tph2* and consequential reduction of 5-HT in adult mouse brain. Tam treatment resulted in ≥90% reduction in the number of 5-HT immuno-reactive cells in the anterior raphe nuclei. HPLC revealed a significant reduction in concentration of 5-HT and its metabolite 5-hydroxyindole acetic acid (5-HIAA) in selected brain regions of *Tph2icKO*, indicating the effectiveness of the protocol used.

Second, standard behavioral tests were used to assess whether reduced brain 5-HT concentrations could alter anxiety-, fear- and depressive-like behavior in mice. No altered anxiety- and depressive-like behaviors were observed in *Tph2icKO* compared to control mice (*Tph2CON*) in all indices measured, but *Tph2icKO* mice exhibited intense and sustained freezing during context-dependent fear memory retrieval. *Tph2icKO* mice also exhibited locomotor hyperactivity in the aversive environments, such as the open field, and consumed more food and fluid than *Tph2CON* mice.

Lastly, the combined effect of maternal separation (MS) stress and adult brain 5-HT depletion on behavior was assessed in male and female mice. Here, MS stress, 5-HT depletion and their interaction elicited anxiety-like behavior in a sex-dependent manner. MS reduced exploratory behavior in both male and female mice. Reduced 5-HT enhanced anxiety in female, but not in male mice.

Furthermore, expression of genes related to the 5-HT system and emotionality (*Tph2, Htr1a, Htr2a, Maoa* and *Avpr1a*) was assessed by performing a quantitative real-time PCR. In *Tph2icKO* mice there was a reduction in expression of *Tph2* in the raphe nuclei of both male and female mice. Interaction between MS stress and 5-HT deficiency was detected showing increased *Htr2a and Maoa* expression in raphe and

hippocampus respectively of female mice. In male mice, MS stress and 5-HT depletion interaction effects reduced *Avpr1a* expression in raphe, while the expression of *Htr1a, Htr2a* and *Maoa* was differentially altered by 5-HT depletion and MS in various brain regions.

Key words: Anxiety, Depression, Serotonin, Tamoxifen, Tryptophan hydroxylase

Zusammenfassung

Unterbrechungen der Serotonin-Stoffwechselwege (5-Hydroxytryptamin, 5-HT) im Gehirn wurden mit der Ätiologie und der Pathogenese von verschiedenen neuropsychiatrischen Erkrankungen assoziiert, wobei die neuronalen Mechanismen der 5-HT Funktionen noch vollständig entschlüsselt werden müssen. Die Tryptophan-Hydroxylase 2 (TPH2) ist das limitierende Enzym für die 5-HT Synthese im Gehirn, weshalb der durch Tamoxifen (Tam) induzierbare, cre-vermittelte *Tph2* Gen-Knockout (*Tph2icKO*) im adulten Mausgehirn möglicherweise helfen könnte die spezifische Rolle von 5-HT im Gehirn in der Regulation von adultem Verhalten zu entschlüsseln. Zuerst wurden Hochleistungsflüssigkeitschromatographie (HPLC) und Immunhistochemische Analysen durchgeführt um die Effizienz der Tam induzierten Inaktivierung des *Tph2* und die daraus folgende Reduktion von 5-HT im Gehirn zu überprüfen. Die Behandlung mit Tam resultierte in einer ≥86% Reduktion der Anzahl von 5-HT immunoreaktiven Zellen in der anterioren Raphe im Gehirn. Die HPLC zeigte eine signifikante Reduktion der 5-HT Konzentration und dessen Stoffwechselprodukts 5-Hydroxyindolylessigsäure (5-HIAA) in ausgewählten Gehirn regionen von *Tph2icKO,* was auf die Effektivität des benutzten Protokolls hindeutet.

Danach wurden standarisierte Verhaltens tests durchgeführt um festzustellen, ob eine reduzierte 5-HT Konzentrationen im Gehirn zu einer Veränderung in der Angstreaktion, Depression und im Furchtverhalten der Mäuse führt. Bei allen Tests konnte sowohl in den *Tph2icKO*-Mäusen als auch in den Kontrolltieren kein offensichtliches angstbezogenes und depressionsähnliches Verhalten festgestellt werden, wobei die *Tph2icKO*-Mäuse intensive und anhaltende Furcht im Kontext „dependent fear retrieval" zeigten. *Tph2icKO*-Mäuse zeigten zudem lokomotorische Hyperaktivität und konsumierten mehr Futter und Flüssigkeit als die Kontrolltiere.

Zuletzt wurde der kombinierte Effekt von Stress durch mütterliche Trennung (MS) und adulter 5-HT Reduktion im Gehirn auf das Verhalten von männlichen und weiblichen Mäusen untersucht. Wieder rief nicht der depressionsähnliche Phänotyp, sondernder Stress durch die mütterliche Trennung (MS) und 5-HT Verarmung und deren Interaktion ein angstähnliches Verhalten in Abhängigkeit vom Geschlecht hervor. Reduziertes 5-HT vergrößerte die Angst in weiblichen, aber nicht in männlichen Mäusen. Stress durch mütterliche Trennung (MS) reduzierte das explorative Verhalten sowohl in Männchen als auch in Weibchen. Die Expression von

Genen, welche im Bezug zum 5-HT System stehen (*Tph2, Htr1a, Htr2a, Maoa* und *Avpr1a*) wurden mit Hilfe von quantitativer Real-Time PCR untersucht. Die Tam Behandlung reduzierte das*Tph2* Level in der Raphe bei beiden Geschlechtern signifikant. In weiblichen Mäusen steigertedie Interaktion zwischen Stress durch mütterliche Trennung (MS) und 5-HT Verarmung das *Htr2a* und *Maoa* Expressions level in der Raphe und im Hippokampus. In männlichen Mäusen reduzierte die Interaktion von Stress durch mütterliche Trennung (MS) und 5-HT Reduktion die *Avpr1a* Expression in der Raphe. Die Expression von *Htr1a, Htr2a* und *Maoa* wurde in verschiedenen Gehirn regionen unterschiedlich von Tam und Mütterliche Trennung MS verändert. In der Amygdala wurde nur ein MS Effekt auf die *Tph2* Expression in den Mäusen sichtbar.

Schlagwörter: Angst, Depression, Serotonin, Tamoxifen, Tryptophan hydroxylase

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1 Introduction

1.1 Discovery of serotonin

Serotonin (5-hydroxytryptamine, 5-HT) was discovered in the late 1930s by Dr. Erspamer as an indole-like substance in the enterochromaffin cells of the gut, which caused contraction of smooth muscles and was named Enteramine. About 20 years later, the work of Irvine Page, Maurice Rapport and Arda Green in trying to identify the vasoconstriction agent in blood which was responsible for hypertension led to the isolation and characterization of serum bound substance which was eventually named “serotonin” (literally meaning “serum-tonus”). Just at the same time Betty Twarog, while working with Irvine Page, made a stunning discovery of 5-HT as a neurotransmitter in the brain of vertebrates, and what was left was identifying its role(s) in mental health. When the structural similarities between 5-HT and lysergic acid diethylamide (LSD; psychedelic and potential psychotherapeutic agent) became apparent (Figure 1-1 A&B), it was hypothesized for the first time that 5-HT may function in regulation of behavior and as a risk factor for psychiatric disorders as reviewed by (Whitaker-Azmitia 1999; Twarog 1988; Erspamer and Asero 1952; Williams and Erickson 2000). Current understanding on 5-HT show that it controls many important functions of brain, which include modulation of mood states, hunger, sex, sleep, memory, emotion, anxiety, endocrine effects, and many others (Nichols and Nichols 2008). Besides vertebrates, 5-HT is present in plants and invertebrates as well (Whitaker-Azmitia 1999). Although 5-HT was discovered by serendipity, it marks the time of birth of neuroscience.

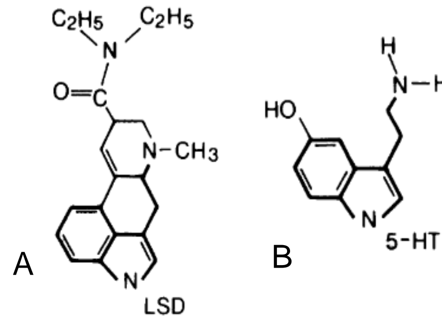

Figure 1-1: The molecular structure of (A) Lysergic acid diethylamide (LSD) and (B) 5-hydroxytryptamine (5-HT).The thick dark lines indicate similarities in their molecular structures. Modified from: (Berridge and Prince 1974).

1.2 The dual central 5-HT system

5-HT is a monoamine neurotransmitter which exerts influence on a variety of central and peripheral body physiology. In the brain, 5-HT controls sleep-wake cycle, food intake, sexual behavior, anxiety and depression (Walther and Bader 2003; Alenina and Klempin 2015). In the periphery, it is involved in gut motility, basic homeostasis, vascular tone and cell mediated immunity (Walther et al. 2003). The brainstem raphe region (medulla oblongata, pons and midbrain) contains serotonergic and non-serotonergic nuclear clusters whose axonal projections form an extensive monoaminergic fiber arbor innervating several areas in vertebrate brain (Aznar, Qian, and Knudsen 2004).

Along the rostro-caudal midline extension of the raphe is a cluster of serotonergic nuclei embedded within the reticular formation. These nuclei have been categorized into 9 groups (B1-B9) (Dahlstrom and Fuxe 1964; Dahlstroem and Fuxe 1964). The B1-B4 nuclei; namely raphe pallidus (B1 and B4), raphe obscurus (B2) and raphe magnus (B3) are located in the caudal portion of the brainstem (Jacobs and Azmitia 1992) from where they send axonal projections to spinal cord and cerebellum to control pain sensation and autonomic nervous activities. The rostral part of the raphe is further divided into; caudal linear nucleus (CLN: B8), most rostral and stretches from red nucleus to the anterior border of the superior cerebellar decussation (SCD); dorsal raphe nucleus (DRN: B6, B7), located in the ventral part of periaqueductal grey; and median raphe nucleus (MRN: B9, B8 and B5), inferior and caudal to SCD up to the level of trigeminal motor nucleus and their serotonergic fiber projections target frontal, parietal, and occipital cortices where 5-HT controls cognition and

personal perception (Hornung 2003; Jacobs and Azmitia 1992; Lesch and Waider 2012).

Median raphe (MR) serotonergic projections reach the hippocampus via two paths; (i) infracallosal domain from rostral directions through fimbria and dorsal fornix; and (ii) supracallosal domain through the cingulate bundle. Dorsal raphe (DR) serotonergic projections reach the hippocampus via supracallosal route or follow ventral route through amygdala and entorhinal cortex (Gulyas, Acsady, and Freund 1999; Kosofsky and Molliver 1987). Retrograde tracing has revealed topographical organization of DR in the rostro-caudal plane such that, rostrally organized serotonergic neurons send projection exclusively to specific forebrain sites such as the caudate putamen, substantia nigra, and virtually all neocortical regions, whereas caudally located serotonergic neurons project to the forebrain limbic regions. However, some individual serotonergic fibers send collateral projections to central nucleus of amygdala (CeA) and the paraventricular nucleus of the hypothalamus, which are involved in central autonomic control, anxiety, and conditioned fear (Pollak Dorocic et al. 2014; Abrams et al. 2004).

Studies have proved that 5-HT fibers arising from MR (M fibers) and DR (D fibers) differ, in addition to origin, in their morphology and response to pharmacological agents, especially p-chloroamphetamine which degenerates D-fibers but not M-fibers. Type D- fibers have been reported to contain serotonin transporter (5-HTT) which is absent in type M-fibers and this could account for their differences in response to the neurotoxin p-chloroamphetamine (Brown and Molliver 2000; Mamounas et al. 1991; Mamounas and Molliver 1988; Asan, Steinke, and Lesch 2013). MR projecting serotonergic fibers display variable fiber thickness with large irregular spaced spherical varicosities. However, DR fibers are very fine in thickness and have small evenly spaced varicosities, which are granular or fusiform in shape (Hensler 2006; Kosofsky and Molliver 1987). MR projecting serotonergic fibers make classical synaptic contacts with GABAergic interneurons in hippocampus, thereby potentially providing a fast neuromodulation of networks in the hippocampal formation (Varga et al. 2009).

1.3 5-HT biosynthesis, neurotransmission and metabolism

5-HT synthesis in the brain occurs in the serotonergic cellular cluster located in the raphe. Outside the central nervous system (CNS), 5-HT is synthesized in the pineal gland and in enterochromaffin cells of gut. The biosynthesis of 5-HT occurs in two basic steps. The process begins with the ring hydroxylation of the essential amino acid, tryptophan (Trp), into 5-hydroxytryptophan (5-HTP) by the rate limiting enzyme, tryptophan hydroxylase (TPH: EC 1.14.16.4). This initial step requires Fe^{2+} as co-factor and tetrahydrobiopterin (BH4) and molecular oxygen (O_2) as co-substrates (Walther and Bader 2003; Roberts and Fitzpatrick 2013; Moran et al. 2000; Pavon and Fitzpatrick 2006). So far two isoforms of TPH have been identified; TPH1 and TPH2 are the rate limiting enzymes responsible for initiation of 5-HT synthesis in the periphery and brain respectively (Walther and Bader 2003). The second step is side chain decarboxylation of 5-HTP to form 5-HT by the enzyme, aromatic L-amino acid decarboxylase (AADC) which is also involved in the synthesis of adrenaline, noradrenaline, dopamine and histamines. Intracellular packaging of 5-HT as well as catecholamines and histamine is achieved via action of vesicular monoamine transporters (VMATs) enzymes. There exist two closely related VMATs isoforms; VMAT1 is expressed in neuroendocrine cells and VMAT2 is chiefly expressed in the CNS (Peter et al. 1995; Wimalasena 2011; Yaffe, Forrest, and Schuldiner 2018).
When an action potential travelling along the axon reaches the axonal bulb, it causes depolarization of the membrane, Ca^{2+} channels open and there is an influx of Ca^{2+}, which activates presynaptic vesicles to fuse with the membrane and release 5-HT into the synaptic cleft. 5-HT spreads across the synapse and bind to specific receptor sites on the postsynaptic neuron to generate either an excitatory post synaptic potential (EPSP) or inhibitory post synaptic potential (IPSP).

The 5-HT receptors comprise a large family of G-protein coupled (GPCR) neurotransmitter receptors of 13 distinct genes encoding for receptors of the G-protein coupled seven-transmembrane class of the type A family and one ligand-gated ion channel, the 5-HT_3 receptor of the Cys loop family which constitutes heteropentamers (Nichols and Nichols 2008; Hannon and Hoyer 2008; Baez et al. 1995). The seven classes of 5-HT receptor (5-HT_{1-7}) have been grouped into at least 14 subtypes (5-HT_{1A-F}, 5-HT_{2A-C}, 5-HT_3, 5-HT_4, 5-HT_{5A-B}, 5-HT_6 and 5-HT_7). The 5-HT_1 consists of four members (5-$HT_{1A, B, D, \& E}$) because 5-HT_{1C} is now known as 5-

HT_{2C} based on its transductional properties and molecular structure (Hannon and Hoyer 2008; Humphrey, Hartig, and Hoyer 1993; Shih et al. 1991; Hartig et al. 1992; Tanaka, Samuels, and Hen 2012).

Firing of raphe 5-HT neurons is controlled by inhibitory somatodendritic autoreceptors such as 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D}. Activation of these autoreceptors localized in the membrane of the presynaptic neurons induces the opening of potassium channels that hyperpolarize the neuron thereby inhibiting cell firing in a negative feedback loop (Andrade et al. 2015; Gothert 1990; Araragi et al. 2013). When 5-HT binds to an excitatory post synaptic heteroreceptor, ion channel opens permitting Na^+ or both Na^+ and K^+ into the cell. This leads to depolarization of the postsynaptic plasma membrane, promoting generation of an action potential. In contrast, binding of a 5-HT to an inhibitory receptor on the postsynaptic cell causes opening of K^+ or Cl^- channels which results in membrane hyperpolarization and inhibits generation of an action potential in the postsynaptic cell (Lodish H, Berk A, and Zipursky SL 2000). 5-HT re-uptake, removal of 5-HT after release, determines the extent, duration, and degree of widespread receptor activation. 5-HT re-uptakes achieved by the action of 5-HTT which removes 5-HT out of the extracellular space back into the presynaptic neuron and VMAT2 repackages the 5-HT into vesicles (Vergo et al. 2007; Torres, Gainetdinov, and Caron 2003).

Excess 5-HT in axon bulb is oxidized by the enzyme monoamine oxidase (MAO), an outer mitochondrial membrane protein. There exist two isoforms (MAO-A and MAO-B) which share about 70% amino acid identity and require flavin adenine dinucleotide (FAD) binding domains. Both MAO-A and MAO-B metabolize 5-HT, norepinephrine, and dopamine, but MAO-B metabolizes 5-HT only slowly (Gaweska and Fitzpatrick 2011; Chen and Shih 1998). The FAD catalyzed monoamine oxidase A (MAO-A) oxidation of 5-HT results in formation of 5-hydroxyindole-3-acetaldehyde (5-HIAL) which is subsequently oxidized by aldehyde dehydrogenase (ALDH) to form 5-hydroxyindolacetic acid (5-HIAA; Figure 1-2) (Beck et al. 1995; Wang et al. 2002). 5-HT is also converted to melatonin in pineal gland and to melanin in retina (Figure 1-3). Cocaine and alcohol seeking behavior in mice is controlled by exposure to ALDH inhibitor (Koppaka et al. 2012; Huang 2010) indicating that many genes of the 5-HT system control several behaviors.

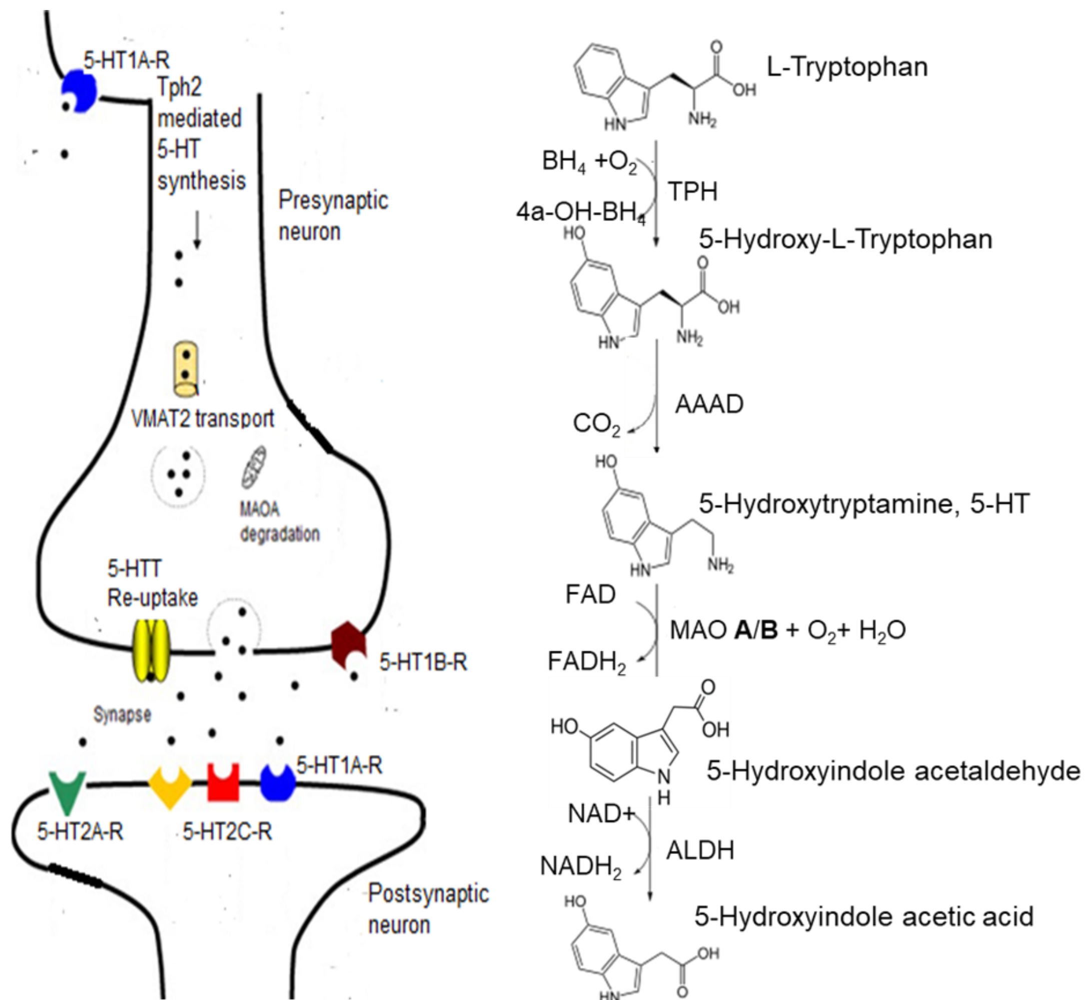

Figure 1-2: Biochemical pathway for the synthesis and metabolism of 5-HT. ***Left***: Schematic illustration of a serotonergic neuron with synapse. ***Right***: Molecular formulae of the 5-HT metabolism. The amino acid L-tryptophan is hydroxylated into 5-hydroxy-L-tryptophan by the enzyme tryptophan hydroxylase (TPH). 5-hydroxy-L-tryptophan is then converted into 5-HT by the aromatic L-amino acid decarboxylase (AAAD) enzyme and packaged into vesicles by vesicular monoamine transporter 2 (VMAT2). When an action potential reaches the axon terminal the presynaptic 5-HTergic neuron becomes depolarized thereby inducing Ca^{2+} influx. 5-HT filled vesicles bind to presynaptic membrane and release 5-HT into synaptic cleft. The 5-HT bind to specific postsynaptic receptors which generates excitatory postsynaptic potential and nerve impulse. 5-HT is actively transported back into the presynaptic terminals by the 5-HT transporter (5-HTT).The mitochondrial membrane enzyme monoamine oxidase (MAO-A/B) deaminates 5-HT to 5-hydroxyindol acetaldehyde which is oxidized by the aldehyde dehydrogenase (ALDH) into 5-hydroxyindol acetic acid. Modified from (Wang et al. 2002; Holmes 2008)

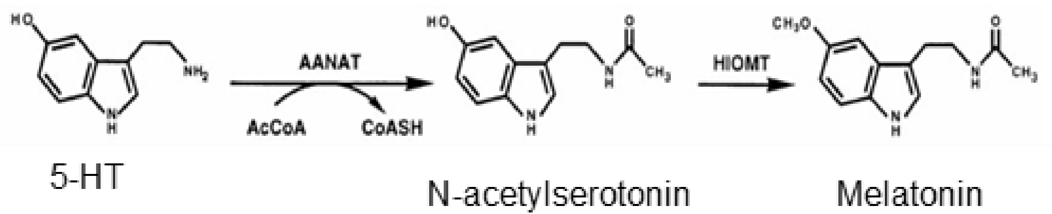

Figure 1-3: Biochemical pathway for the synthesis of melatonin.5-HT is first converted to N-acetylserotonin by serotonin N-acetyltransferase (AANAT).This step requires acetyl coenzyme A (AcCoA). In the second step, N-acetylserotonin is converted to melatonin by hydroxy-indole-O-methyl transferase (HIOMT). Adopted from (Hickman, Klein, and Dyda 1999).

1.4 The tryptophan hydroxylase enzymes

TPH is the enzyme for the catalytic hydroxylation of L-tryptophan (L-Trp) into 5-hydroxy-L-tryptophan, from which 5-HT is synthesized. TPH also hydroxylates L-phenylalanine (L-Phe) (Windahl et al. 2008), but not L-tyrosine (L-Tyr) (McKinney et al. 2001). For many years only one form of TPH was known and it was believed that it was responsible for the synthesis of both central and peripheral 5-HT. However, the fact that the first generation of TPH- specific antibodies failed to detect TPH in the brain, but in the gut raised the speculation that another isoform of TPH exists (Hasegawa et al. 1987). To augment this claim, staining with monoclonal and polyclonal TPH-specific antibodies showed differences in the relative intensities of TPH immuno-labeling across tissues. The monoclonal antibodies preferentially labeled TPH from raphe nuclei (Haycock et al. 2002; Chung et al. 2001), but the polyclonal antibodies selectively labeled TPH from human pineal gland (Haycock et al. 2002). These observed differences were attributed to post-translational modifications of TPH in the raphe and pineal gland. Successful genetic manipulation of the *Tph* (*Tph1*) resulting in absence of 5-HT in blood and other tissues, but not in the brain, brought to light that a second *Tph* isoform (*Tph2*) indeed exists in the brain. TPH1 deficient mice (*Tph1*$^{-/-}$) mice were generated by (Walther et al. 2003; Cote et al. 2003) and these mice had only a slight reduction in raphe 5-HT levels and exhibited no deficit in anxiety behavior, although they lacked 5-HT in gut, blood and pineal gland, suggesting the existence of another *Tph* isoform (*Tph2),* which functions independently from *Tph1*.

1.4.1 Regulation of TPH enzymes

Just like other aromatic amino acid hydroxylase (AAAH) family of enzymes, TPH1 and TPH2 are tetrameric proteins that possess three structural domains; a homologous catalytic, divergent regulatory N-terminal and a C-terminal tetramerization domain (Fitzpatrick 2015; Carkaci-Salli et al. 2006). TPH2 bears an extended N-terminal domain comprising 41 amino acid sequences which is lacking in TPH1 (Murphy et al. 2008; Zhang, Hinck, and Fitzpatrick 2016), which indicates TPH1 and TPH2 are regulated differently. The regulatory mechanisms of TPH, unlike phenylalanine hydroxylase (PAH) and tyrosine hydroxylase (TH), is poorly understood because it is difficult to obtain a stable recombinant TPH (Zhang, Hinck, and Fitzpatrick 2016). In humans, TPH1 and TPH2 share 71% homology in amino acid sequence and their functional activity is regulated through phosphorylation by protein kinase A (PKA) and calcium/calmodulin-dependent protein kinase II (CAMKII) (Waloen et al. 2017; Walther and Bader 2003; Kuhn, Arthur, and States 1997; Johansen et al. 1995).

Phosphorylation of amino acid residue in the enzyme activates the TPH, while dephosphorylation reduces activity of TPH (Voronova and Kulikov 1991). Earlier studies predicted PKA phosphorylation of TPH1 and TPH2 at serine-58 site (Darmon et al. 1988; Kuhn, Arthur, and States 1997) and CAMKII phosphorylation at serine-260 and serine-443 sites of the amino acid sequence of the TPH enzyme (Darmon et al. 1988). PKA also phosphorylates TPH2 at serine 104 site within the regulatory domain of the enzyme (Winge et al. 2008) while the serine-19 site of TPH2 is phosphorylated by both CaMKII and PKA. The serine-19 of TPH2 is homologous to serine-19 of TH but serine-19 site of TPH1 is not phosphorylated by both CaMKII and PKA (Waloen et al. 2017). Post phosphorylation regulation of TPHs involves interaction of the phosphorylated serine with 14-3-3 family of proteins, which prevents dephosphorylation and stabilizes the protein. The 14-3-3 family of proteins essentially regulates TH and TPH activities and modulates biosynthesis of catecholamine and 5-HT (Kleppe et al. 2011; Kleppe, Toska, and Haavik 2001). Phosphorylated amino acid residues of both TPH isoforms are stabilized by 14-3-3 protein in order to prolonging the enzymatic activity during the synthesis of monoamines (Winge et al. 2008). Protein of the 14-3-3 family are believed to inhibit degradation of TPH1 after it has been phosphorylated by CaMKII (Walther and Bader 2003).

1.4.2 Properties of TPH enzymes

Differences exist in the kinetic properties and substrate specificity between human TPH1 and TPH2. McKinney and his Colleagues (McKinney, Knappskog, and Haavik 2005) expressed both isoforms of human TPH in *Escherichia coli* and reported a lower Km values for BH4 cofactor for TPH2 than for TPH1 but a higher Km values for Trp for TPH2 than for TPH1. The Km for Trp is about fourfold higher for TPH2 than for TPH1. In their earlier study, the authors (McKinney et al. 2004) found higher Vmax values of TPH1 than TPH2, which was attributed to differences in catalytic efficiency or stability of TPH isoforms. There appears to be a higher substrate preference for Trp of TPH2 compared to TPH1. In the nutshell, TPH2 is more soluble, and differs significantly from TPH1 in terms of molecular weight and kinetic properties, including its catalytic efficiency towards phenylalanine (McKinney, Knappskog, and Haavik 2005).

1.4.3 Tissue specific distribution of TPH isoforms

The genes encoding TPH1 and TPH2 are located on chromosome 7 and 10 in the mouse and on chromosome 11 and 12 respectively in humans. Although distinct genes code for the two TPH isoforms, the high degree of sequence homology makes it difficult for regional characterization, as many antibodies or mRNA probes detect both isoforms (Yu et al. 1999). Sakowski *and coworkers* (Sakowski et al. 2006) developed antibodies which revealed molecular weight of TPH1 as 51kDa and that of TPH2 as 56kDa on western blot. Analysis of mouse tissues revealed exclusive expression of TPH1 in pineal gland and in P815 mastocytoma cells, and TPH2 predominantly in mesencephalic tegmentum, striatum, and hippocampus of brain.

In situ hybridization survey for relative mRNA levels of Tph1 and Tph2 in brain of rats showed that Tph2 mRNA is expressed almost exclusively in raphe while Tph1 is detected largely in pineal gland with no substantial overlap in their expression pattern. Tph2 mRNA level in the raphe was approximately 2.5-fold greater than the level of Tph1 mRNA expression in pineal gland (Patel, Pontrello, and Burke 2004). Tph2 mRNA levels in the rat dorsal and median raphe is influenced by circadian rhythm. Constant darkness maintains Tph2 mRNA levels at same variation while at the light phase, Tph2 mRNA levels present daily variation within both median and dorsal raphe nuclei (Malek et al. 2005; Malek et al. 2007). Even though TPH2 is

expressed predominantly in neurons of the raphe nuclei (Zill, Buttner, Eisenmenger, Bondy, et al. 2004), immuno-labelling has revealed presence of TPH2 in myenteric plexus of rodents (Cote et al. 2003) and enterochromaffin cells in humans (Gutknecht et al. 2009).

Other than the pineal gland, non-neuronal TPH1 is found in the small intestines (Gutknecht et al. 2009; Cote et al. 2003; Fiorica-Howells, Maroteaux, and Gershon 2000), mast cells (Finocchiaro et al. 1988) and embryonic stem cells (Walther and Bader 1999). TPH is also present in photoreceptors of the eye (Green, Cahill, and Besharse 1995). In rats, both Tph1 and Tph2 mRNA are expressed in the eye but Tph1 is predominant, and both isoforms exhibit robust diurnal rhythms of abundance (Liang et al. 2004). This indicates that Tph1 is the enzyme responsible for melanin production in the eye (Lambrus et al. 2015). In a study of human postmortem brain, expression of TPH2 mRNA in the raphe nuclei was approximately 4-fold greater than that of TPH1, but TPH1 and TPH2 mRNA levels in other brain regions (cortex, thalamus, hippocampus, cerebellum) were comparatively similar and in an unexpected conditions TPH1 mRNA level in hypothalamus and amygdala was significantly high than TPH2 mRNA level (Zill et al. 2007). This observation may indicate that fiber projections from pineal gland may influence 5-HT neurotransmission in forebrain regions (Aldegunde, Miguez, and Veira 1985). In summary, Tph1 is the predominant isoform in the periphery and the fact that only Tph1 transcripts are detected in the brain indicated that the Tph1 mRNA arise from projection fibers from pinealocytes to the brain. TPH2 is presumably the only enzyme responsible for CNS 5-HT synthesis.

1.5 Genetic manipulations of genes of the central serotonergic systems

1.5.1 *Tph2*-deficient mice

Since the discovery of a second TPH isoform (TPH2) in the brain, several laboratories (Alenina and Klempin 2015; Migliarini et al. 2013; Yadav et al. 2009; Liu et al. 2014), including ours (Gutknecht et al. 2008), have generated rodents that genetically lack the brain TPH2 enzyme. As a result, these mice lack the ability to synthesize brain 5-HT from embryonic to adult stages of development. There were no observable morphological abnormalities of 5-HT neurons (Gutknecht et al. 2008), but

altered levels of norepinephrine and dopamine in several brain regions was reported in these mice (Gutknecht et al. 2012), indicating that brain 5-HT may not be a prerequisite for neuronal survival. However, this phenotype is characterized by smaller body size, delayed growth and lower body weight compared with wildtype (WT) litter mate in early post-natal life (Alenina and Klempin 2015; Gutknecht et al. 2008; Gutknecht et al. 2012; Migliarini et al. 2013). Interestingly, *Tph2*$^{-/-}$ mice gain weight and increase in body size such that they are indistinguishable from WT controls from 3 months of age (Alenina et al. 2009; Kane et al. 2012; Narboux-Neme et al. 2013). Although *Tph2*$^{-/-}$ mice are able to maintain normal body temperature (Solarewicz et al. 2015), they are unable to tolerate cold conditions (4ºC), exhibit altered heart rate and blood pressure (Alenina et al. 2009).
Genetically modified rodents have been used over the years to model psychiatric conditions in humans, and Tph2 KO mice provide insight into conditions related to emotions, mood and aggression. Interestingly, the first behavior studies in Tph2 KO mice yielded comparable outcome with WT control. Constitutive depletion of CNS 5-HT did not alter locomotor and anxiety-related behavior tested (Savelieva et al. 2008). That notwithstanding, extreme aggression in Tph2 deficient mice has been reported. In the residence-intruder test, Tph2 deficient mice were intolerant of intruder mice and aggressively attacked them. Their aggressive nature makes it difficult for Tph2 KO mice to be housed in groups or used for social interaction test (Kane et al. 2012; Beis et al. 2015; Beis et al. 2016). Aggression towards pups is reflected in poor maternal care (Alenina et al. 2009). These aggressive mice are less anxious in aversive arenas as they explored and spent more time in the potentially life threatening center of open-field arena or the light compartment of light-dark-box (Angoa-Perez et al. 2012; Lesch et al. 2012; Mosienko et al. 2012), however, a recent study reported panic-escape and agoraphobia phenotype characterized by increased jumping in open field (Waider et al. 2017).

Depression-like behavior has been reported in Tph2 KO mice such that genetic depletion of brain 5-HT predisposes mice to depression in adult life. The forced swimming test (FST) and tail suspension test (TST) in mice usually produced opposite results in depression test. Tph2 deficient mice exposed to the two tests showed increased depression-like behavior in FST but not in TST (Mosienko et al. 2012). Depression-like behavior in FST is aggravated by prior exposure to chronic

mild stress in mice (Gutknecht et al. 2015). Tph2 deficient mice give up struggling early when put in water and remain immobile for longer time than WT control mice.

Exaggerated fear responses to foot shock stress has been observed in Tph2 deficient mice. Fear in mice is described as freezing without moving any body part other than breathing (Gutknecht et al. 2015). Tph2 deficient mice display increased freezing associated with increased number of neuronal activation marker, *cfos*, in the amygdala which indicates extreme activation of neural circuits in amygdala (Waider et al. 2017). For detailed review of behavior in Tph2 KO mice, refer to (Lesch et al. 2012; Lesch and Waider 2012; Mosienko et al. 2015). Although, mice behavior has been used to explain human conditions, caution must be taken as striking differences in brain anatomy and neural connectivity between humans and mice exist. Again human emotions and self-evaluation (self-esteem) or suicide behavior are not possible to be depicted in these animals.

1.5.2 Inducible inactivation Tph2 in the adult brain

Inducible conditional *Tph2* silencing techniques are transgenic tools that permit spatio-temporal inactivation of the gene. Time-specific manipulation of *Tph2* helps to decipher anatomical and physiological mechanisms that regulate 5-HT neurotransmission in adult brain. *Tph2* functions as WT gene until appropriate treatment is given to the animal to induce conditional gene knockout (icKO). The cre-loxP system permits spatio-temporal study of *Tph2* functions. The loxP site is a sequence of nucleotide base pairs consisting of nonpalindromic 8 base pairs sequence (GCATACAT or ATGTATGC), sandwiched between two inverted 13 base pair sequence (ATAACTTCGTATA) (Sharma and Zhu 2014). Any DNA introduced between two loxP sites is termed floxed DNA (flanked by loxP). The cre (cyclization recombination) recombinase is a 38kDa gene product of the P1 bacteriophage which recognizes two specific loxP sites and mediates site specific crossover of the loxP sites leading to deletion of targeted (*Tph2*) genes.

A more stringent inducible model was introduced by conjugation of cre recombinase with a mutated ligand binding domain of the estrogen receptor (ERT), which permits conditional inactivation of genes (Metzger et al. 1995; Sinha et al. 2010; Feil et al. 1996). The mutated ligand ensures that the cre is not activated by endogenous ligand (estrogen, glucocorticoids or progesterone) but rather an exogenous ligand (Tam).

The conjugated creERT which is synthesized in the cytoplasm does not have the required conformational shape to enter into the nucleus to cause gene deletion until it is selectively activated by Tam or its derivative 4-hydroxytamoxifen (Brocard et al. 1997; Feil et al. 1996). The activated Tam bound creERT is quickly transported into the nucleoplasm, binds to loxP sequence in the genome and induces temporal *in vivo* gene inactivation (Figure 1-5).

Tph2$^{flox/flox}$ mouse line which expresses Cre conjugated with mutated ligand binding domain of human estrogen receptor (creERT2), under *Tph2* specific regulatory elements in all serotonergic neurons, and having genomic exon 5 flanked by loxP sequences was generated. Excision of exon 5 of *Tph2* creates a shift in the reading frame resulting in synthesis of a truncated non-functional TPH2 protein by (Gutknecht et al. 2008a). Effective Tam induced Cre-loxP mediated recombination of floxed *Tph2* in serotonergic neurons in *Tph2*$^{Pet1\text{-}icre}$ mice was reported (Kriegebaum, Song, et al. 2010). Similarly a recent study which used *Tph2*$^{flox/null}$*::CMV-CreERT*^{2} mice expressing Cre under cytomegalovirus promoter (CMV) demonstrated a significant reduction in brain 5-HT ir cells within just seven days after last Tam treatment (Pelosi et al. 2015). In this study Tph2creERT2 mouse line was adopted and used to evaluate effect of acute inducible adult brain 5-HT depletion on emotions, mood and fear-related psychiatric conditions. Table 1 below illustrates established animal models of inducible inactivation of genes in serotonergic system.

Table 1- 1: Inducible deletion of genes in serotonergic system in adult rodent brain.

Induction approach	Target gene	Floxed Exon	% Reduction	Animal	References
AAV injection	*Tph2*	Exon 5	89%	Mouse $Tph2^{flox/flox}$	(Whitney et al. 2016)
Tamoxifen	*Tph2*	Exon 5	50% - 95%	Mouse $Tph2^{flox/flox}$	(Song et al. 2016)
Tamoxifen	*Tph2*	Exon 3	~100%	Mouse $Tph2^{fl/-::CMVcreERT}$	(Pelosi et al. 2015)
Tamoxifen	*GR*	Exon 5	80-90%	Mouse $GR^{fl/fl::TPH2-CreERT2}$	(Weber et al. 2009)
Tamoxifen	*Tph2*	Exon 5	80% Tph2-mRNA	Mouse Tph2Pet1-icre	(Kriegebaum, Song, et al. 2010)
Tamoxifen	*Tph2*			Rats $Tph2^{flox/flox}$	(Weber et al. 2011)
Tamoxifen	*Pet 1*	Exon 3	85%	Mouse $ePetCreER^{T2}$	(Liu et al. 2010)
Tamoxifen	*Lmx 1b*		60%	*Mouse* $Lmx1b^{flox/flox}$	(Song et al. 2011)
DOX	*Htr1a*		30%	*Mouse* $Htr1a^{tetO/tetO}$	(Richardson-Jones et al. 2010)
DOX	*Htr1a*		80%	*Mouse* $Htr1a^{tetO/tetO}$	(Richardson-Jones et al. 2011)

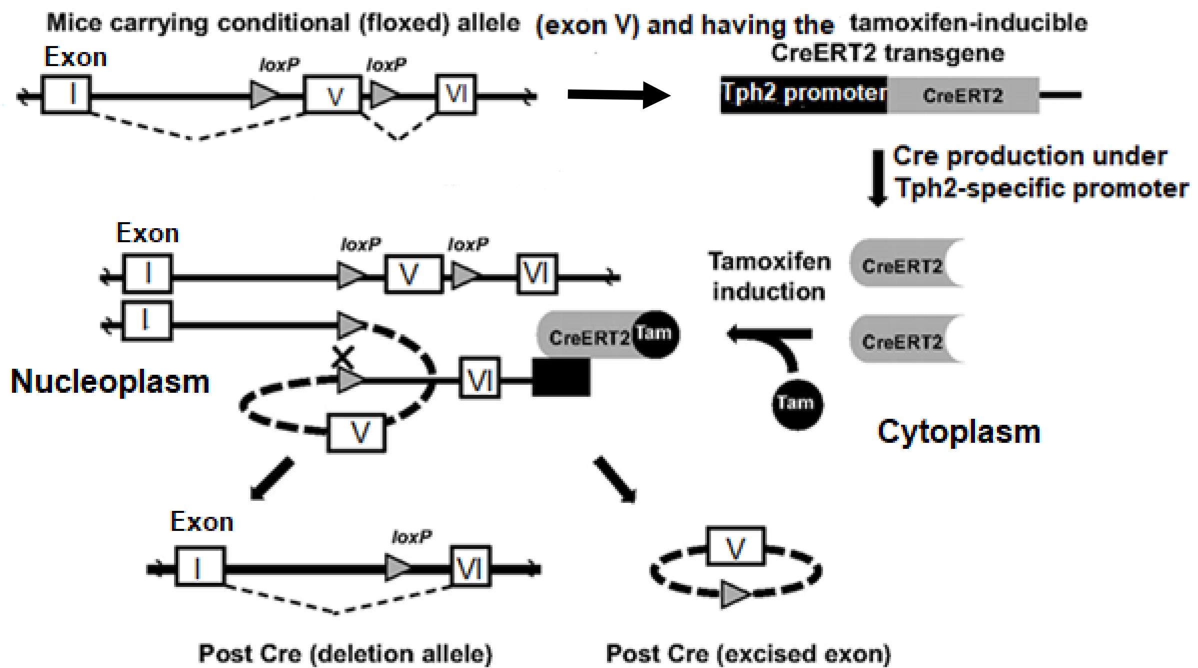

Figure 1-4: Inducible conditional Tph2 targeting. Tamoxifen binds to the CreERT2 which results in translocation of the Cre recombinase into the nucleus where it binds to and deletes floxed exon V of Tph2. Modified from (Doetschman and Azhar 2012).

1.5.3 Mouse model of altered formation of 5-HT neurons and 5-HT metabolism

***Lmx 1b* Knockout mice:** The LIM homeodomain transcription factor 1 beta (Lmx1b) controls differentiation and maintenance of 5-HT neurons as well as other neurons in the CNS (Dai, Hu, et al. 2008; Cheng et al. 2003). *Lmx-1b* deficient mice do not express 5-HT, 5-Htt and *Pet-1* (Ding et al. 2003) and suffer developmental abnormalities of the limbs (Feenstra et al. 2012), eyes, brain, and kidneys (Burghardt et al. 2013), indicating that *Lmx1b*-knockout is lethal to the embryo (Endele et al. 2007). Inducible Cre activity in $Lmx1b^{Pet1-icre}$ mice revealed drastic reduction in brain 5-HT concentration and altered expression of *Tph2*, *5-Htt* and *Vmat2* (Song et al. 2011). This indicates that *Lmx1b* plays an important role in 5-HT biosynthesis in adult mouse brain and in regulation of genes related to serotonergic system.

***Pet-1* knockout mice:** The rodent *Pet-1*ETS is an E26-specific (ETS) transcription factor gene whose human orthologue is Fifth Ewing Variant (*FEV*) (Deneris 2011; Kriegebaum, Gutknecht, et al. 2010). *Pet-1* expression is restricted to brain 5-HT neurons and is essentially involved in the cascade of enzymatic pathway responsible for 5-HT synthesis in prenatal post-mitotic precursors (Liu et al. 2010; Hendricks et al.

1999; Hendricks et al. 2003). In mice lacking *Pet-1,* the majority of 5-HT neurons fail to differentiate and remaining differentiated neurons had 80% deficiency of brain 5-HT and lacked expression of genes of 5-HT system (Hendricks et al. 2003). Although expression of *Pet-1* is regulated by *Lmx-1b* during embryogenesis (Deneris and Wyler 2012), *Pet-1*$^{-/-}$ mice are viable and grow to adult stage of development and show extreme anxiety-like and aggressive behavior in adults (Hendricks et al. 2003). Similar behavior was seen in Tam inducible Pet-1 deficiency (*Pet-1*^{Pet1aCKO}) mice. These mice have reduction in brain 5-HT and 5-HIAA concentrations and altered expression of genes related to serotonergic system (Liu et al. 2010).

5-HTT transporter knockout mice: The 5-HTT controls the extent of action 5-HT by periodically removing released 5-HT from extracellular space. In humans, polymorphism in the transcriptional control region of the 5-HTT gene (*SLC6A4*) which results in altered expression of human 5-HTT gene is reported to renders individuals more susceptible to adverse early life experience and hence to the development of psychiatric diseases (Lesch et al. 1996; Caspi et al. 2003; Hariri et al. 2002). *5-Htt*$^{-/-}$ mice are viable and grow to adulthood, however there is deficit in synaptic 5-HT clearance, leading to accumulation of 5-HT in the brain, detected by microdialysis (Montanez et al. 2003; Holmes, Murphy, and Crawley 2003; Kim et al. 2005; Mathews et al. 2004). On the other hand, reduced extracellular 5-HT concentration in brain homogenates of hippocampus, frontal cortex and the striatum has been reported (Bengel et al. 1998), which may indicate a compensatory response to inadequate re-uptake and/or recycling extracellular 5-HT. 5-HTTdeficient mice show altered behavior characterized by increased anxiety-like, depression-related and exaggerated stress responses (Kastner et al. 2015; Holmes, Murphy, and Crawley 2003). In humans, carriers of the s allele show increased amygdala response during negative emotion (Kobiella et al. 2011), and suffer from major depressive disorder (MDD) (Nautiyal and Hen 2017) while the l/l genotype is associated with development of attention deficit hyperactivity disorder (ADHD) (Kane et al. 2012).

***Vmat2* knockout mice:** Vesicular monoamine transporters 2 (VMAT2) enzyme is responsible for packaging of neuronal 5-HT into storage vesicles at the axon terminal (Vergo et al. 2007). In *Vmat2*$^{-/-}$ mice, monoamine cell groups and fiber projections are indistinguishable from WT mice, but *Vmat2*$^{-/-}$ mice have severe impairment in

monoamine storage and vesicular release (Alvarez et al. 2002). These mice are less mobile, feed poorly and often die within a few days after birth (Wang et al. 1997).

Monoamine oxidase knockout mice: Monoamine oxidases (MAO) enzymes degrade biogenic amines and deactivate brain monoamine neurotransmitters. MAO-A KO mice exhibit elevated level of 5-HT, NE and DA, while MAO-B have increased Phe concentration in the brain (Shih and Chen 1999). MAO-A/B mutants are viable and grow to adulthood. MAO-A mutant mice exhibit hypo-locomotor phenotype in novel open field (Shih and Chen 1999) and are very aggressive to intruders (McDermott et al. 2009; Scott et al. 2008), but MAO-B KO mice are less aggressive (Shih and Chen 1999). Depression, Parkinson's disease and other neurodegenerative diseases are usually treated with monoamine oxidase inhibitors (Gaweska and Fitzpatrick 2011).

5-HT receptor knockout mice: The 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors are among the G-protein coupled receptor proteins which have been of much interest because they serve therapeutic target for antipsychotics drugs (Rioux et al. 1999). Conflicting reports on effects of 5-HT$_{1A}$ deficiency on brain 5-HT levels have been reported. Extracellular levels of 5-HT in striatum and hippocampus of 5-HT$_{1A}$ KO mice (SW and 129sv background) did not differ from WT mice (Knobelman, Kung, and Lucki 2000; Knobelman et al. 2001; Toth 2003), however significantly higher extracellular levels of 5-HT was reported in 5-HT$_{1A}$ mutants on a C57Bl6J background (Parsons, Kerr, and Tecott 2001). Obviously, mouse strain background, but also methodological differences and sample size could account for the observed differences. There is an interplay between 5-HT$_{1A}$ and 5-HT$_{2A}$ in regulating neuronal activity in the brain (Guiard and Di Giovanni 2015). While 5-HT$_{1A}$ KO mice exhibit increased anxiety-like behavior in open field and elevated plus maze tests (Olivier et al. 2001; Julius 1998; Lesch and Mossner 1999), 5-HT$_{2A}$ KO mice exhibit reduced baseline anxiety-like behavior in open field. In humans, reduced postsynaptic 5-HT$_{1A}$ receptor in forebrain is associated with depression (Sargent et al. 2000; Shrestha et al. 2012; Nautiyal and Hen 2017).

1.6 TPH2 polymorphism in human psychopathology

Dysregulation in brain 5-HT neurotransmission has been implicated in the etiology of many human psychiatric disorders such as depression, anxiety, panic, schizophrenia, impulse control disorders, autism and aggression (Marazziti 2017; Lin, Lee, and Yang 2014). Patients who suffer from depression have low cerebrospinal fluid levels of 5-HIAA and drugs such as selective 5-HT re-uptake inhibitors (SSRIs) improve mood of depressed individuals. Genetic studies on single nucleotide polymorphisms (SNPs) of *TPH2* have been linked to bipolar disorder (BP) (Bicalho et al. 2006; Gao et al. 2016; Lin et al. 2007; Alves et al. 2012) and the SNPs rs4760820 and rs11178998 are associated with BP (Gao et al. 2016). In one study, the *TPH2* SNP (rs11178997) was linked to unipolar disorder (UP) while rs4131348 polymorphism was linked strongly to both UP and BP (Van den Bogaert et al. 2006). Again suicide behavior has been linked with SNPs of *TPH2* gene (Ackenheil et al. 2004; Fudalej et al. 2010; Lopez-Narvaez et al. 2015; Zill, Buttner, Eisenmenger, Moller, et al. 2004; Stefulj and Bordukalo-Niksic 2010). The rs7305115 polymorphism of *TPH2* was found to be associated with suicidal attempts, but lack of association between *TPH2* polymorphisms and suicide behavior have also been reported (Gonzalez-Castro et al. 2014; De Luca et al. 2004). The functional (G1463A) SNP in the TPH2 gene, in which arginine residue at position 441 is substituted by a histidine (Arg441His) was linked to unipolar major depression (Zhang et al. 2005). All later studies that attempted to replicate this study in larger populations failed to find association between the G1463A and depression (Glatt et al. 2005; Zhou et al. 2005; Van Den Bogaert et al. 2005). Some other studies also failed to establish a direct association between SNP in *TPH2* and depression or suicide (Garriock et al. 2005; Gizatullin et al. 2008), therefore this rare polymorphism may not be a major determinant risk for depression. These discrepancies have been attributed to false negative/positive results, ethnicity of study participants, age and gender differences (Lopez-Narvaez et al. 2015). However, meta-analysis of several studies is needed to confirm these observations.

1.7 Gene-environment (GxE) interaction

The phenotypic make-up of an individual is likely the result of interplay between genes and the influences of the environment (GxE) during growth and development.

This hypothesis has been tested in twin studies. For example, anxiety disorders were tested in both monozygotic and dizygotic twins and the outcome revealed that approximately 30-40% of the incidence was attributable to genetic variations, confirming environmental influence (Gross and Hen 2004). However, some resilience factors inherent in the genes reduce vulnerability independent of levels of environmental stress. It implies that certain genes confer environmentally sensitive plasticity such that some individuals are rendered vulnerable under adverse environmental conditions, while the same gene variant provides benefit to other individuals under similar conditions (Nugent et al. 2011). There are only few genetic variations associated with anxiety and depression in humans, and polymorphism in the promoter region of *5-HTT* (*5-HTTLPR)* has gained much attention (Lesch et al. 1996; Caspi et al. 2003). The 5-HTTLPR consists of variable lengths of repetitive sequence of GC-rich elements in the promoter region of the gene. A mutation (deletion/insertion) in the promoter region creates a short(s) allele and a long (l) allele with the l-allele having up to threefold higher mRNA expression than the s-allele, therefore the s-allele has lower 5-HT uptake activity than the l variant (Nakamura et al. 2000; Plieger et al. 2014). Personality inventory questionnaire revealed that individuals carrying s/s homozygous allele are less agreeable and have higher neuroticism scores compared with the s/l and l/l individuals (Blom et al. 2011; Greenberg et al. 2000; Mazzanti et al. 1998; Sen, Burmeister, and Ghosh 2004). Childhood temperament and anxiety-related behavior in infants indicated increased susceptibility of the s/s *5-HTTLPR* homozygous than s/l and l/l individuals (Lakatos et al. 2003; Auerbach et al. 1999). Homozygous s/s individuals who suffer adverse life experience such as war, famine, sickness etc. are also susceptible to depression (Caspi et al. 2003; Lesch et al. 1996; Daniele et al. 2011) and posttraumatic stress disorder (PTSD) (Lee et al. 2005; Zhao et al. 2017; Gressier et al. 2013; Walsh et al. 2014). On the contrary, one study found no association between *5-HTTLPR* polymorphism and affective disorders among Europeans (Mendlewicz et al. 2004). Another study also reported no GxE interaction in depressive symptoms in the Taiwanese population, although traumatic experience was shown to be associated with depressive symptoms (Goldman et al. 2010). Diverse arguments on interplay between genes and environment as susceptibility factors to mood and affective disorders have been reviewed (Dreimuller et al. 2012; Margoob and Mushtaq 2011). These discrepancies may be attributed to several reasons but the bottom line is that

the neural and molecular basis by which genes, environment and their interactions constitute disease risk in humans, remain to be completely understood.
Preclinical studies (GxE experimental models) for understanding the pathogenesis of psychiatric illness, involves subjecting animals to prenatal stress, maternal separation (MS) stress in early-life or adult age chronic mild stress. MS stress has been linked to adult life alcoholism and substance abuse in mice (Cruz et al. 2008; Thomas et al. 2016). Genes associated with hypothalamic-pituitary-adrenal (HPA) axis coordinate neural, hormonal, and behavioral responses to environmental stressors. Exposure to early-life stressors (ELS) perturbs HPA axis functions and causes depression and anxiety disorders (e.g. PTSD) (Handwerger 2009; Gillespie et al. 2009; Marques, Silverman, and Sternberg 2009). Activation of the HPA axis due to ELS increases glucocorticoid levels which supposedly down-regulates brain-derived neurotrophic factor (BDNF). BDNF is a nerve growth factor that regulates neuronal survival and plasticity. Decreased BDNF has been proposed as a mechanism of neuronal atrophy and cell loss in the amygdala, prefrontal cortex, and hippocampus in rodents (de Kloet et al. 2005) an effect which has been associated with anxiety and depression.

Expression of *Tph2* is affected by exposure to various environmental stressors. For example, increased *Tph2* expression in dorsal raphe has been reported in adult male mice, which experienced chronic mild stress which correlates with reduced anxiety-related behavior (McEuen, Beck, and Bale 2008; Hale, Shekhar, and Lowry 2011). Conversely, early-life stress and later-life social defeat stress did not alter brain tryptophan hydroxylase expression in rats, rather it revealed topographical activation (*cfos* expression) of 5-HT synthesizing neurons in the raphe which was associated with submissive behavior in ELS exposure (Gardner et al. 2005). This indicates that different regions of the raphe are activated in response to different stress stimuli (Grahn et al. 1999; Gardner et al. 2005; McEuen, Beck, and Bale 2008). In effect inputs from the environment can selectively and consistently modify gene responses which ultimately define the phenotypic features of an individual, and such genetic modifications can be passed on to unborn generations.

1.8 Aims of the study

In this study, I took advantage of the CreERT2-loxP system, which permits inducible conditional manipulation of *Tph2* in spatio-temporal manner in adult murine brain, and investigated its effect on brain neurochemistry and behavior. The study sought to address the following research questions:

1. What are the consequences of induced conditional *Tph2* knockout (icKO) on anxiety, depression and fear-related behaviour independent of adaptive mechanisms during development?

The mice (10-12 weeks old) were treated with Tam to generate icKO mice and the efficacy of this method was assessed by performing immunohistochemistry and HPLC at different time points to measure the concentration of 5-HT and its metabolite 5-HIAA as well as dopamine (DA) and norepinephrine (NE) in the brain. Behavioural responses to adult brain 5-HT depletion were also assessed starting from 4 weeks after Tam treatment.

2. Would MS stress followed by adult stage *Tph2*icKO impact susceptibility to disorders of emotion and mood?

Some mice were subjected to MS, 3 h daily, for two weeks starting from postnatal day 2 while control mice were left in home cage undisturbed. Both MS exposed and control mice were either treated with Tam or vehicle. First we assessed whether MS independently alter anxiety and depression-related behaviours in mice. We then assessed whether MS followed by 5-HT depletion altered anxiety and depression-related behavior and differential expression of selected genes; *Tph2, Htr1a, Htr2a, Maoa* and *Avpr1a* of the central 5-HT system was also assessed in sex specific manner.

This inducible conditional approach sheds light of physiological and molecular mechanisms that control and regulate 5-HT neurotransmission in adult brain and distinguish the developmental need of brain 5-HT from its regulatory roles in adulthood.

2 Materials and Methods

2.1 Materials

2.1.1 Reagents

Table 2- 1: Reagents used for the study

Name	Company, location
Normal Horse serum	Vector, Peterborough, United Kingdom
DAPI	Sigma, Steinheim, Germany
Triton X-100	Sigma, Steinheim, Germany
Tris HCl	Roth, Karlsruhe, Germany
Paraformaldehyde	Roth, Karlsruhe, Germany
Fluoro-Gel	Electron microscopy sciences, Hatfield, PA
HCl	AppliChem,Damstadt, Germany
NaOH	AppliChem, Damstadt, Germany
Heparin	Ratiopharm, Ulm, Germany
D-Sucrose	AppliChem, Damstadt, Germany
TissueTek	Sukura Finetek Europe bv, Alphen aan den Rijn, The Netherlands
Ethanol (absolute)	Sigma, Steinheim, Germany
2-Mercaptoethanol	AppliChem, Damstadt, Germany
Trichloromethane	Carl Roth GmbH, Karlsruhe, Germany
Ethanol (absolute) for molecular study	AppliChem, Damstadt, Germany
Water for Chromatography	Millipore, Darmstadt, Germany
EDTA	Sigma, Steinheim, Germany
Agarose	Biozym, Viena, Austraia
Gel loading dye (6x)	BioLabs, New England
Isofluran	CP-Pharma, Burgdorf, Germany
Ethidiumbromide	AppliChem, Damstadt, Germany
Proteinase K	Qiagen, Germany
Tamoxifen	Sigma, Steinheim, Germany
H_3PO_4	Sigma, Steinheim, Germany
DTPA	Sigma, Steinheim, Germany

2.1.2 Equipment

Table 2- 2: Equipment and expandable material used for the study

Name	Company, location
Fusion FX Camera	Vilber Lourmat, Collégien, France
I.C. Capture 2.4 Camera	The Imaging Source Europe GmbH, Bremen, Germany
InfraMot TSE systems	TSE Systems Inc., Bad Homburg, Germany
VideoMot2	TSE Systems Inc., Bad Homburg, Germany
EthoVision XT 11.5	Noldus Information Technology bv, Wageningen, The Netherland
Avanti J-26s XP centrifuge	Beckman Coulter GmbH, Sinsheim Germany
Hettich Micko 200R Centrifuge	Merck KG, Damstadt, Germany
Cryostat (Leica 1950)	Leica Biosystems, Nussloch, Germany
Falcon tubes	Eppendorf, Hamburg, Germany
Fine balance	Mettler Toledo, Gießen, Germany
Electrochemical detector	Bio-Rad Lab. GmbH,München, Germany
Fluorescent microscope	Olympus, Hamburg, Germany
Nanodrop	PeQLab Biotech. GmbH, Erlangen, Germany
Electrophoresis Power Supply	Consort, Turnhout, Belgium
PCR tubes	SARSTEDT AG&Co.KG, Nuembrecht, Germany
Centrifuge tubes (75x12 mm) PP	SARSTEDT AG&Co.KG, Nuembrecht, Germany
pH Meter	Hanna Instruments Deutschland GmbH, Vöhringen, Germany
Pipettes	Eppendorf, Hamburg, Germany
Pipette tips	SARSTEDT AG&Co.KG, Nuembrecht, Germany
Tissue Lyzer	Qiagen, Hilden, Germany
Real time PCR machine (CFX384)	Bio-Rad Lab. GmbH,München, Germany
PCR machine	Biometra, Göttingen, Germany
Superfrost Plus slides	Paul Marienfeld GmbH & Co.KG, Lauda-Koenigshofen, Germany
Water bath	Grant Instruments ltd. Cambridge, UK
U-100 insulin syringes	Dispomed, Gelnhausen, Germany
Injection needles	BRAUN, Frankfurt, Germany

2.1.3 Kits and markers

Table 2- 3: Kits and Markers used for the study

Name	Company, location
AllPrep DNA/RNA/miRNA Universal Kit	Qiagen, Hilden, Germany
SYBR Green	Thermo Fisher Scientific, Waltham, MA USA
iScript™ kit	Bio-Rad, Munich, Germany

2.1.4 Antibodies

Table 2- 4: Antibodies used for detecting 5-HT immunoreative cells in raphe

Antibody	Dilution	Company, location
Primary Anti-5HT, rabbit	1:400	Immunostar; Hudson; USA
Secondary Anti-rabbit Cy3	1:1000	Jackson ImmunoResearch Europe Ltd. Cambridgeshire , UK

2.2 Methods

2.2.1 Animal husbandry and ethical considerations

In this study, mice (week 10-12) were used for two different series of experiments. In the first series, male mice (*Tph2*$^{+/+::Tph2CreERT2}$; *Tph2*$^{+/-::Tph2CreERT2}$; *Tph2*$^{fl/fl::Tph2CreERT2}$ and *Tph2*$^{fl/-::Tph2CreERT2}$) were treated with Tam to induce brain 5-HT depletion after which they were tested for effects of adult brain 5-HT depletion on anxiety, depression and fear-related behavior. In this study, mice are referred to as *Tph2*$^{+/+}$, *Tph2*$^{+/-}$, *Tph2*$^{fl/fl}$, *Tph2*$^{fl/-}$. Efficiency of brain 5-HT depletion was assessed by fluorescence 5-HT staining and high performance liquid chromatography (HPLC) ≥2 weeks after last injection (Figure 2-1 A).

In the second series, male and female mice (*Tph2*$^{fl/fl::Tph2CreERT2}$) were either subjected to MS for two weeks or left undisturbed (MS naive) after birth (Fig 2-1B). These mice were treated with Tam in adult life (10-12 week) to assess gene by environment (GxE) effects of early-life adversity and adult brain 5-HT depletion on anxiety and depression-like behavior starting from the 4th week after Tam injection. The brain samples from these mice were used to screen for expression patterns of candidate genes.

Materials and Methods

Experimental mice were housed individually in a controlled environment (12/12h light/dark cycle, 21±0.5°C room temperature, 50±5% humidity) with food and water *ad libitum*. Mice were acclimatized to the housing conditions for ≥3 weeks prior to the start of experiments. All experiments were performed in accordance with the European Parliament and Council Directive (2010/63/EU) and were approved by local authorities (Government of Lower Franconia, Wuerzburg: 55.2-2531.01- 57/12).

A

Tamoxifen

Tph2icKO Mice

Establishing and confirming icKO

Behaviour testing

HPLC/ICH

1st study

2nd study

LDB/OFT/FC

EPM/FST/SPT/FC

LDB/EPM/OFT/FST/SPT
Gene expression study:
Tph2, Htr1a, Htr2a, Avpr1a, Maoa

No MS stress

MS vs Non MS controls

B

P 2 | P 16 | P 23 | WK 0 | WK 2 | WK 4 | WK 5 | WK 6 | WK 8

MS began | MS ended | weaning | Injection | HPLC | HPLC/ICH/ LDB/EPM | OFT/SPT | HPLC/ICH/ FST/FC | GE

Figure 2-1: An overview of the study plan. ***A***: Effectiveness of Tam inducible creERT2 mediated *Tph2* gene deletion (icKO) was established using high performance liquid chromatography (HPLC) and immunohistochemistry (IHC). ***B***: After birth, mice were either left undisturbed in home cage (Non MS: 1st study) or exposed to maternal separation (MS: 2nd study). Mice were tested in light-dark-box (LDB), open field (OFT) and elevated plus maze (EPM) for anxiety; and in sucrose preference (SPT), forced swimming (FST) for depression; and fear conditioning (FC) and later gene expression (GE) study. Mice were injected with Tam for 5 days (marked as WK 0) at age (10-12 weeks) followed by behavior tests in subsequent weeks (WK). P 2 = Postnatal day 2; P 16 = Postnatal day 16, P 23 = Postnatal day 23.

2.2.2 Mice breeding and genotyping

Generation of Tam inducible Tph2-CreERT2 mice has been described (Weber et al. 2009). To generate mice that express CreERT^2 recombinase protein under *Tph2* specific promotor and having exon 5 of *Tph2* flanked by loxP sequence, heterozygous *Tph2*-deficient animals on C57Bl/6J background (Charles River, Germany) were crossed with Tph2-CreERT2 to generate *Tph2*$^{+/-::creERT2}$ mice. These mice were further crossed with *Tph2*$^{fl/fl}$ to generate hemizygote *Tph2*$^{fl/-::creERT2}$ mice (see Figure 2-2).

Genotyping of mice was done under guidance of Dr. J. Waider and N. Steigerwald by using the protocol described by our group (Gutknecht et al. 2008; Waider et al. 2013). When mice were weaned at age P21-24 days, biopsies were taken from the ear and kept in cold room (4 ^oC). The biopsies were incubated for 2 h in digest buffer (20 mM NaCl, 50 mM TrisHCl, 1 mM EDTA, 1% Sodium dodecyl sulfate (SDS), 1 mg/ml proteinase K), extracted with phenol/chloroform, precipitated with ethanol and suspended in 20 µl dH_2O. The DNA was stored at 4 ^oC. The *Tph2* locus was genotyped using a three-primer 38 cycle polymerase chain reaction (PCR) strategy to prevent false negative result (see Table 2-5 and Table 2-6 below).

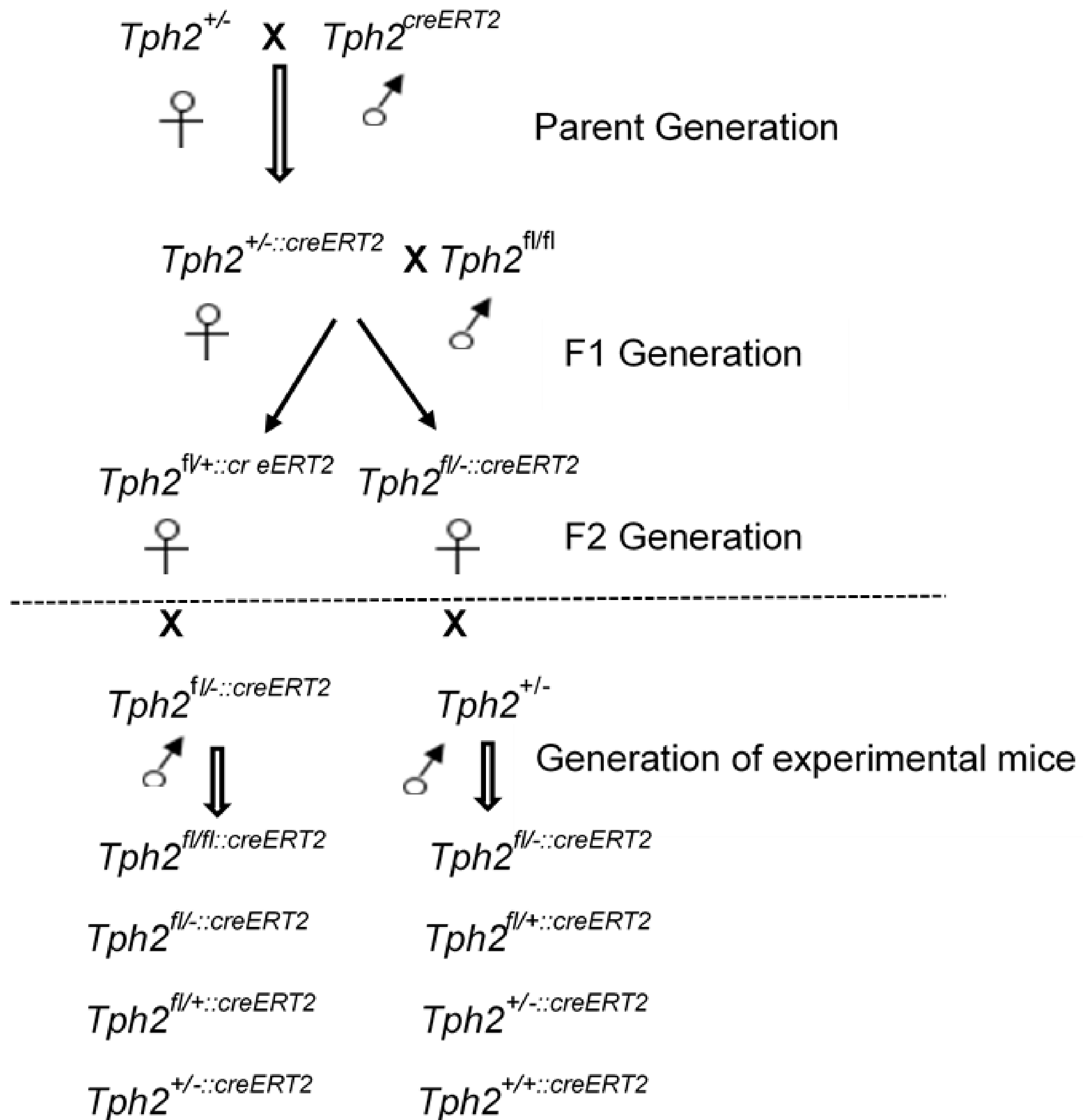

Figure 2-2: Breeding scheme used for obtaining *Tph2*$^{fl/-::creERT2}$ and *Tph2*$^{fl/fl::creERT2}$ mice. Mice heterozygotes for the (*Tph2*$^{+/-}$) were crossed with mice which express CreERT2 recombinant protein under Tph2 specific promoter. The F1 generations of mice (female) were further bred with male mice which have exon 5 of *Tph2* flanked by loxP sequence (floxed (fl) gene). The F2 generation has both loxP and CreERT2 genes incorporated in their genome and were used further breeding to obtain the experimental mice.

Table 2- 5: Primers used for genotyping mice

Primers	**Primer sequence**
3'Del mTph2 F	5'- tgg ggc atc tca gga cgt agt agt -3'
5'arm over const. Tph2 F	5'- cac ccc acc ttg cag aaa tgt tta -3'
3'over constr. Tph2 R	5'- tgg ggc ctg ccg ata gta aca c -3'

Table 2- 6: Reagents, enzymes and PCR condition used for genotyping mice

		PCR-Run	
Reagents	**[µl]**	**[°C]**	**[s]**
10x Goldstar	2.5	95	300
25 mM $MgCl_2$	1.5	95	40
2.5 mM dNTPs	1.0	64	40
3'Del F	1.0	72	50
5'over F	1.0	72	300
3'over R	1.0	4	300
DNA	1.0	10	….
Taq polymerase	1.0		
H_2O	15.0		
Total volume	25.0		

Product size

1. floxed 608 bp
2. WT 437 bp
3. KO 387 bp

2.2.3 Preparation of tamoxifen

Absolute ethanol (9 ml) was added to Tam crystals in a bottle. The bottle was well shaken until all crystals of Tam were dissolved. Afterwards, 1000 µl aliquots of the solution was pipetted into Eppendorf tubes and stored at 20 °C. The Tam aliquot (1000 µl) was dissolved in 9 ml of autoclaved corn oil. The Tam-corn oil mixture was sonicated on ice for 15 min in 15 ml Eppendorf tube to ensure dissolution of Tam crystals that might not have dissolved. The tube was tightly closed, wrapped with aluminum foil and placed on a rotator at about 150 rpm overnight. Tam is sensitive to light so the aluminum foil was used to protect it from bleaching.

2.2.4 Induction of TPH2 deficiency

The *Tph2icKO* mice (*Tph2*$^{fl/fl}$ and *Tph2*$^{fl/-}$) have exon 5 of *Tph2* flanked by loxP sequence (floxed (fl) gene) while the control (*Tph2CON*) mice (*Tph2*$^{+/+}$ and *Tph2*$^{+/-}$) lack loxP sequence. All mice express Tam inducible Tph2CreERT^2 recombinase protein under mouse *Tph2* specific promoter sequence (177kb). In order to generate *Tph2icKO*, mice aged 10-12 weeks were given intraperitoneal (i.p.) injection of 1 mg of Tam twice daily, 12 h interval (7:00pm -7:00am) for 5 consecutive days (Weber et al. 2009). Thin column U-100 insulin syringes and injection needles were used to inject the mice. The mice were carefully handled in order to minimize stress, avoid

injury to internal organs and eventual death. Mice were left in their home cage undisturbed until the start of subsequent experiments.

2.2.5 Assessment of effectiveness of *Tph2* deletion and subsequent brain 5-HT depletion

2.2.5.1 Immunohistochemistry

For immunohistochemical analysis, mice were placed in a desiccator containing cotton pad socked with Isofluran to render them unconscious. Transcardial perfusion of mice was done with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 10 min. Brains of mice were harvested and immersed in 4% PFA in PBS solution for 48 h. The brain samples were then washed in PBS and kept in 10% (w/v) sucrose solution overnight. The samples were again suspended in 20% (w/v) sucrose solution for 48 h after which they were frozen in Isopentane on dry ice and kept at -80 ^{o}C until later time.

In order to assess the effectiveness of brain 5-HT depletion, 30 μm sections of mice brains were cut in a cryostat and mounted on 6 series of Histobond microscope glass slide and stored at -80 ^{o}C. For the staining frozen sections were allowed to dry for 15-20 min followed by antigen retrieval in citrate buffer as previously described (Gutknecht et al. 2008). Sections were allowed to cool down to 40-50 °C and washed 3 x 5 min in Tris-buffered saline (TBS). Unspecific binding sites for the antibodies were blocked for 90 min at room temperature (RT) with blocking solution (BS; 5% Normal Horse Serum (NHS), 0.25% Triton-X100 in TBS. Sections were incubated with the primary antibody (1:400, goat-anti 5-HT) diluted in BS at 4 °C in a humid chamber overnight. Following three 5 min washing steps in TBS, sections were incubated with fluorescent secondary antibodies (goat anti rabbit Cy3) diluted in BS in the dark for 90 min. Sections were washed 3 x 5 min in TBS. For staining of the cell nuclei, sections were treated with 300 µM DAPI diluted 1:1000 in TBS for 5 min. With the aid of flurogel stain sections were covered with microscope glass coverslip and stored in a cool dry place.

2.2.6 Antigen retrieval

Frozen microscope slides were kept on bench for about 15 min. Water bath was heated to a temperature of 80 ^{o}C. The microscope slides were placed in beaker

containing Citrate buffer (10 mM Citric acid, without Tween 20, pH 6.0) and incubated in the water bath for 30 min. The beaker was kept on bench for about 20 min until temperature reached 40 ^{o}C.

2.2.7 Microscopy and cell counting

Pictures of anterior raphe (DRN and MRN) corresponding to Bregma -4.95 mm to -4.47 mm (Paxinos and Franklin 2007) were captured with fluorescence microscope (IX81, Olympus, Tokyo, Japan) using 20X objective lens. Four pictures per animal were selected and cells that were immuno-reactive (ir) for 5-HT were counted with the aid of Fiji (https://fiji.sc/) software. Four raphe sections per animal were selected and 5-HT ir cells counted. *Tph2CON* were vehicle-treated $Tph2^{fl/fl}$ and Tam-treated ($Tph2^{+/+}$ and $Tph2^{+/-}$) mice, while *Tph2icKO* were Tam-treated $Tph2^{fl/fl}$ and $Tph2^{fl/-}$ mice.

2.2.8 High-performance liquid chromatography (HPLC)

Mice were kept in a desiccator containing cotton pad socked with Isofluran to render them unconscious. Mice were transcardially perfused with heparinized PBS for 20 minutes to ensure adequate removal of blood. The brains were quickly harvested and kept frozen in isoflurane on dry ice, wrapped in aluminum foil and later stored at -80 ^{o}C. The brain regions for HPLC were quickly dissected from frozen brain under a stereomicroscope. For this, the brain was sliced on a cooling plate (~70 ^{o}C) with the aid of metallic matrix permitting sectioning at equal intervals. The total hippocampus, prefrontal cortex containing the frontal area, infralimbic, prelimbic as well as cingulate cortex, the whole dorsal raphe, and the complete amygdaloid complex were dissected out with preparation spatula and kept frozen at -80 ^{o}C until use. The brain homogenates were weighed and solubilized in 19 times the volume of the brain weight of transmitter buffer (150 mM H_3PO_4, 500 mM DTPA) by ultrasonifcation with a sonifier for ≤ 5 min on ice (10 pulses with 0.5 s length and 0.5 s pause, amplitude 15%) and centrifuged (19000 rpm, 20 min, 3-4 ^{o}C). For the detection of the neurotransmitters norepinephrine (NE), dopamine (DA), 5-HT and its metabolite (5-HIAA), supernatants (50 µl each) were injected into an HPLC system (Agilent) consisting of EC 250/4.6 Nucleosil 100-3-C18 reversed-phase columns (Machery-Nagel) and electrochemical detector (BioRad) which was adjusted to 0.75 V.

Composition of mobile phase was 90% 0.65 mM octanesulfonic acid, pH 3.51, 10% methanol, 0.5 mM triethylamine, 0.1 mM EDTA and 0.1 M NaH_2PO_4.

2.2.9 Data analysis

Data obtained from this study was assessed using GraphPad Prism 5.0. The total number of 5-HT ir cells in the anterior raphe of mice in the various groups was compared using One-way ANOVA (non-parametric). Kruskal-Wallis statistic (H) was computed and Dunn's multiple comparison test was used to compare means between groups. Outcome from HPLC was analyzed using Two-way ANOVA, with Tukey's multiple post-hoc test used to compare means. A probability value at $\alpha<0.05$ was set as level of significance.

2.2.10 Behavior

Mice were subjected to behavior testing 4 weeks after Tam injections. One group of mice were first tested for anxiety-like behavior in the light-dark-box test (LDB), followed by an open-field test (OFT) after which they were subjected to fear conditioning in automated fear conditioning chamber. A second group of mice were subjected to elevated plus maze test (EPM), sucrose preference test (SPT), Porsolt swim test (FST) and lastly full battery of fear conditioning and context-dependent memory testing. Videos were recorded with VideoMot2 (TSE Systems, Bad Homburg, Germany) and later analyzed with EthoVision XT 11.5, Noldus.

Light-dark box

The light-dark-box (LDB) consisted of a transparent Perspex 'light' compartment (40x40x27 cm) and a black opaque Perspex 'dark' compartment (40x20x27 cm). The dark chamber contained a small opening at floor level (5x5 cm) and was covered by a removable lid. The illumination in the dark compartment was between 0 and 10 lux, whereas illumination in the light compartment was approximately 100 lux. Mice were placed in the dark section of the box and behavior was automatically recorded with VideoMot2 (TSE Systems, Bad Homburg, Germany) for 10 min. The total distance travelled and the time spent in the lit compartment was measured to determine anxiety-like behavior.

Elevated-plus maze (EPM)

The plus-shaped maze was made of grey Perspex (TSE Systems Inc., Bad Homburg, Germany), raised to about 60 cm high above the floor. The apparatus comprises two open arms (30x5x0.25 cm) and two closed arms (30x5x15 cm), which intersect at a central platform (5x5 cm). Illumination intensity was 200 lux on the open arms, 120 lux on the central platform and 40 lux on the closed arms. Mice were gently positioned at the center, facing one of the open arms, and allowed to freely explore the maze for 10 min. The number of arm entries, the time spent in the open and closed arms and the total distance traveled were recorded using an automated video tracking system (VideoMot2, TSE Systems Inc., Bad Homburg, Germany).

Open-field test

The open-field (OF) consisted of a black quadratic box (50x50x40 cm) semi-permeable to infrared light. Illumination at floor level was between 50 and 100 lux (from the walls to the center of the arena). The arena was divided into a 30x30 cm center zone and the surrounding periphery. Mice were individually placed in one corner of the arena and automatically recorded for 30 min using VideoMot2 (TSE Systems, Bad Homburg, Germany). The distance traveled, and time spent moving were used to evaluate locomotor activity; frequency of entry into the center and the time spent in the center of the arena were considered as a measured of anxiety-like behavior.

Porsolt swim test

Mice were introduced to a transparent cylinder (height 20 cm, diameter 15 cm) filled with water (25 °C, height 12 cm) for 5 min. Parameters of floating behavior, referred to as immobility, defined as the absence of directed movements of animals' head and body were estimated. Latency to begin floating was scored as time between introduction of a mouse into the pool and the very first moment of complete immobility of the entire animal body, irrespective of the duration of the first floating episode. The total time spent floating was scored during the entire testing session. Included within the time spent immobile were short periods of slight activity where the animals made movements necessary to maintain their heads above water.

Materials and Methods

Fear conditioning and fear memory retrieval

Fear conditioning (FC) and fear memory retrieval were tested in an automated fear conditioning chamber (TSE Systems, Bad Homburg, Germany) using methods previously described (Sachs et al. 2013) with some modifications. After a 120 s habituation phase, mice received three pairings (60 s inter-trial interval) of the conditioned stimulus (CS) (20 s, 80 dB, 4 kHz tone) and the unconditioned stimulus (US) (2 s, 0.6 mA scrambled foot shock), which co-terminated with the CS. After a 120 s consolidation phase after the last CS - US pairing, mice were returned to their home cage. After 24 h, each mouse was place again in the fear conditioning chamber, and freezing behavior, characterized by complete lack of movement other than breathing, was recorded over a period of 15 min in the absence of tone or foot shock. Note: The TSE VMot3 animal tracking software failed to correctly track some mice in the context fear retention test so there is no data for these animals.

Sucrose preference test (SPT)

Baseline sucrose and water consumption was measured for 5 days in the fifth week after Tam injection. Therefore mice chose between two bottles, one with 2.5% sucrose solution and the other with tap water. The consumption of water, sucrose solution, and total intake of liquids was estimated by weighing the bottles every 24 h. The position of the bottles in the cage was switched every day. Quantity of solution consumed was calculated as the difference in volume of fluid measured between two consecutive days. Some mice constantly wasted solution and wetted their cages with water and sucrose solution so they were excluded from the study. Sucrose preference was calculated as a percentage of the consumed sucrose solution from the total amount of liquid drunk by the following formula:

$$\textit{Sucrose Preference} = \frac{\textit{Vol. (sucrose solution) x 100}}{\textit{Vol. (sucrose solution)+Vol.}\ (H_2O)}$$

Body weight, food and water intake

Body weight of mice was measured weekly for 7 weeks starting from the first day of injection. Body weight measurements were done form 10am to 11:00am. Two weeks after last injection, mice were single housed and were served pre-weighted food and water. The quantity of food consumed was weighed once weekly starting from two to six weeks after last injection.

2.2.11 Maternal separation (MS) stress

On the second day after birth, pups were separated from dams and kept in fresh cages for 3 h daily (between 10.00 and 13.00 h), for 14 consecutive days ($MS_{2\text{-}16}$). The pups were kept in a different room from dams in order that they would not be conditioned by the cry of the dams or the dams would not be conditioned by the distressed call of the pups. Warmth to pups was provided from red light which hanged about 70 cm above the cages and from body heat generated by cuddling behavior of the group. Stress naive control mice were not separated from the dams but were handled during routine cage change. The mice were weaned at about 25 days after birth and kept in groups of 2 to 5 mice per cage.

The protocol for injection (Tam and vehicle) of MS and Non MS control (Ctrl) has been described above. Out of the mice that were subjected to MS stressed, only male and female mice of the *Tph2*$^{fl/fl::Tph2CreERT2}$ genotype were used for the next stage of the experiments. Mice were tested for anxiety-related behavior in the EPM, LBD and OF followed by SPT and FST for depression related behavior. With the exception of OF which was done for 15 min, all other tests were done as described above.

2.2.12 Simultaneous purification of total RNA and miRNA

RNA extraction buffers (RPE, FRN, AW1, AW2) were mixed with ethanol/ isopropanol according to manufacturer's instructions and incubated at 37 ^{o}C until use. Buffer RLT Plus mix consists of Buffer RLT Plus, ß-mercaptoethanol (ß-ME) and DLX in proportions; 1x→ 10 µl ß-Me+1 ml RLT Plus+5.05 µl DLX.

For the isolation of granular RNA and miRNA, the raphe, whole hippocampus and amygdala (left and right) samples from male and female mice were pulled together. Tissue was homogenized in 600 µl Buffer RLT Plus mix and metal beads using

Tissue Lyzer set at 20 Hz for 90 s. Tubes containing homogenized lysate were briefly (about 15 s) centrifuged to reduce foam and kept at 37 °C for 1 min to dissolve caotrope salt. The homogenized lysate was mixed by pipetting 7-8x and transferred to an *AllPrep* DNA Mini spin column placed in a 2 ml collection tube. The spin column was centrifuged for 30 s at full speed (maximum speed of 14,000 xg). Maxtract tubes (2 ml) were briefly centrifuged and 150 µl H_2O added. The filtrate was added to the content of Maxtract tubes and the *AllPrep* DNA Mini spin column and residue stored at room temperature (15-25 °C) for DNA purification.

2.2.12.1 RNA extraction (including microRNA)

Chloroform (150 µl) was added to Maxtract tube, inverted several times (15 s) and centrifuged at 24 °C for 3 min at 12,000 xg to separate the phases. The aqueous phase was carefully transferred into a new 2 ml microcentrifuge tube (Biosphere). Proteinase K (80 µl) was added to sample and vortexed after which 350 µl absolute ethanol (96-100%) added and vortexed. Sample was incubated for 10 min at room temperature (20-30 °C). Next, absolute ethanol (750 µl) was added to sample and vortexed after which 700 µl of the sample transferred into RNeasy Mini spin column placed in a 2 ml collection tube and centrifuge for 15 s at full speed (14,000 xg). This step was repeated until the entire sample was passed through the RNeasy Mini spin column. Buffer RPE (500 µl) was added to the RNeasy Mini spin column and centrifuged for 23 s at full speed (14,000 xg). About 80 µl of DNase mix (DNAse + Buffer RDD) was placed directly onto the membrane of RNeasy Mini spin column and incubated for 15 min at room temperature.

Next, 500 µl Buffer FRN was added to the RNeasy Mini spin column and centrifuged for 23 s at full speed (14,000 xg). The RNeasy Mini spin column was placed in a new 2 ml collection tube and the flow-through (Buffer FRN) added to the spin column. Sample was centrifuged for 23 s at full speed (14,000 xg) and the flow-through discarded. Buffer RPE (500 µl) was applied to the RNeasy Mini spin column and centrifuged for 23 s at full speed (14,000 xg). This was followed by addition of 500 µl absolute ethanol (96-100%) to the RNeasy Mini spin column and again centrifuged for 2 min at full speed (14,000 xg) to wash the spin column membrane. The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution as residual ethanol may interfere with downstream reactions. The RNeasy Mini spin column was placed in a new 2 ml collection tube and

centrifuged at full speed for 2 min to eliminate any possible carryover of ethanol or to collect the residual flow-through that remains on the outside of the RNeasy Mini spin column. The RNeasy Mini spin column was placed in a new 1.5 ml collection tube (Biosphere) and 40-50 µl RNAse-free water directly to the spin column membrane. This was incubated at room temperature 1 min and centrifuged for 1 min at 14,000 xg to elute the RNA. RNA obtained was stored at -80 ^{o}C**.**

2.2.13 Synthesis of cDNA

The amount of RNA that was eluded from the above extraction methods was measure by using Nanodrop device. Two microliters (2 µl) of sample was utilized for quantification of RNA eluded. Synthesis of cDNA was performed by using of the iScript™ kit according to the manufacturer´s instructions. An Excel program was used to determine the ratio of RNA to water making a total of 15 µl for each sample. Then 1 µl of reverse transcriptase and 4 µl of iScript enzyme mix were added to sample to make a total of 20 µl reaction volumes. The sample was placed in a Thermocycler and run for about 45 min under the following PCR conditions (Table 2-7).

Table 2- 7: PCR conditions for synthesizing cDNA

Reaction steps	Temperature (^{o}C)	Time (min)
1	25.0	5
2	42.0	30
3	85.0	5
4	4.0	∞ hold

2.2.14 Gene expression studies

Quantification of relative gene expression was performed by quantitative (real-time) polymerase chain reaction (RT-qPCR). The reaction was run in triplicates using SYBR green dye according to manufacturer instructions. Reaction mixture comprised 6 µl (SYBR green + Primer (F+R)) mix and 4 µl cDNA making 10 µl reaction volume each. Mean efficiencies were calculated by LinReg (Ruijter et al. 2009) and Relative expression data were calculated by qbase+ (Biogazelle, Zwijnaarde, Belgium), with the normalization factors obtained from geNorm (geNorm M < 0.5) (Vandesompele et al. 2002). Reference genes: *glyceraldehyde 3-posphate dehydrogenase (GAPDH_3),*

beta-2 microglobulin (B2m_2), ubiquitin C (UBC_1) and ribosomal protein lateral stack subunit P0 (Rplpo). Target genes: *tryptophan hydroxylase 2 (Tph2), 5-HT receptor 1a (Htr1a), 5-HT receptor 2a (Htr2a), monoamine oxidase A (Maoa), arginine vasopressin 1a (Avpr1a)* (Table 2-8),

Table 2- 8: Primers used for PCR

Gene symbol	primer name	primer sequence
Tph2	mTph2_q_F mTph2_q_R	5´-TGGGGATTTGATGCCTAGAACC 5´-TGGGTTCTTTAGAGCATTTTTGTGT
Htr1a	m5-HTr1a_q_F m5-HTr1a_q_R	5´-AACCAGTTTTGTGTCCTCTCA 5´-AGCACCTAAATAATTTTCTTCTC
Htr2a	Mm5Htr2a_v1_F Mm5Htr2a_v1_R	5´-AACCCCATTCACCATAGCCG 5´-CCGAAGACTGGGATTGGCAT
Maoa	mMaoa_q_F mMaoa_q_R	5´-TCGGGAGAATTTTACCCAAACCA 5´-AACTCTATCCCGGGCTTCCA
Avpr1a	MmAVPR1a_q_F MmAVPR1a_q_R	5´-CAATTTCGTTTGGACCGATT 5´-GTAGATCCACGGGTTGCAG

2.2.15 Data analysis

Data obtained from this study was analyzed using GraphPad Prism 5.0. The results from mice that were tested for only effect of adult 5-HT depletion on anxiety- and depression-like behavior were tested using One-way ANOVA (assuming Gaussian distribution of data). Combined effects of MS and adult brain 5-HT depletion on behavior was tested (separately for male and female mice) using Two-way ANOVA, and Tukey’s multiple comparison post hoc analysis to test used for differences in mean between groups. Similarly, relative expression of genes relevant to the 5-HT system was analyzed using Two-way ANOVA, with Tukey’s multiple comparison post-hoc analysis. A probability value at $\alpha<0.05$ was set as level of significance.

3 Results

3.1 Time-specific disruption of brain 5-HT synthesis

3.1.1 Quantitative immunohistochemistry

In order to assess the efficacy of time specific *Tph2* depletion through intraperitoneal injection of Tam, fluorescence immunohistochemistry of the anterior raphe was performed on the 4th and 6th weeks after treatment.

Four weeks after Tam treatment, effective recombination characterized by fewer number of 5-HT immunoreactive (ir) cells, was observed in DRN (Figure 3-1: A-C) and MRN (Figure 3-2: J-L) of *Tph2icKO* (*Tph2*$^{fl/-}$ and *Tph2*$^{fl/fl}$) compared with *Tph2CON* (*Tph2*$^{+/+}$) mice. In DRN, One-way ANOVA (non-parametric) revealed that the average number of 5-HT ir cells in *Tph2*$^{fl/fl}$ and *Tph2*$^{fl/-}$ was significantly smaller than *Tph2*$^{+/+}$ (Kruskal-Wallis statistics (H) = 7.731; p = 0.0066). Dunn's multiple comparison test showed that number of 5-HT ir cells in *Tph2*$^{+/+}$ was significantly higher than *Tph2*$^{fl/-}$ (p = 0.0107) but only a trend between *Tph2*$^{fl/fl}$ mice (p = 0.0789). Quantitatively, about 95.07% and 97.64% reduction of 5-HT ir cells in *Tph2*$^{fl/fl}$ and *Tph2*$^{fl/-}$ mice respectively compared to *Tph2*$^{+/+}$ mice was detected. In the MRN, there was a significant reduction in the number of 5-HT ir cells in *Tph2*$^{fl/fl}$ and *Tph2*$^{fl/-}$ compared with *Tph2*$^{+/+}$ (H = 7.758; p = 0.0062). Dunn's multiple comparison test indicated that 5-HT ir cells in *Tph2*$^{+/+}$ was significantly higher than *Tph2*$^{fl/-}$ (p = 0.016) but only a trend with *Tph2*$^{fl/fl}$ mice (p = 0.0783).The efficiency of recombination was about 82.07% and 92.71% in *Tph2*$^{fl/fl}$ and *Tph2*$^{fl/-}$ respectively.

Six weeks after injection few scattered 5-HT ir cells were observed in *Tph2icKO* (*Tph2*$^{fl/-}$ and *Tph2*$^{fl/fl}$); DRN (Figure 3-1: E&F) and MRN (Figure 3-2: N&O) compared with the Tph2CON (*Tph2*$^{+/+}$ and *Tph2*$^{+/-}$); DRN (Figure 3-1: D,G-I) and MRN (Figure 3-2: M,P-R). As illustrated in Figure 3-3 e&f respectively, One-way ANOVA (non-parametric) revealed no significant difference in the number of 5-HT ir cells in DRN (H = 1.325; p = 0.5155) and MRN (H = 1.139; p = 0.5659) of all three *Tph2CON* (*Tph2*$^{fl/fl}$ Veh, and Tam treated *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$). However, *Tph2icKO* showed a significant reduction of brain 5-HT ir cells in DRN (Figure 3-3 c) compared with *Tph2CON* (H = 22.01; p < 0.0001). Dunn's multiple comparison test revealed that *Tph2*$^{fl/fl}$ recorded significantly fewer number of 5-HT ir cells than *Tph2*$^{+/+}$ (p = 0.0032)

and *Tph2*$^{+/-}$ (p = 0.0037). Again *Tph2*$^{fl/-}$ mice had significantly fewer number of 5-HT ir cells than *Tph2*$^{+/+}$ (p = 0.0086) and *Tph2*$^{+/-}$ (p = 0.0095). In percentagewise, quantitative analysis revealed 94.27% and 87.92% reduction of 5-HT ir in *Tph2*$^{fl/fl}$ and *Tph2*$^{fl/-}$ compared with *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ respectively. Similar observation was made with the number of 5-HT ir cells in the MRN (H = 22.39; p < 0.0001; Figure 3-3 d) which resulted in 86.38% and 85.93% reduction in 5-HT ir cells in *Tph2*$^{fl/fl}$ and *Tph2*$^{fl/-}$ respectively. Dunn's multiple comparison test showed that number of 5-HT ir cells in *Tph2*$^{fl/fl}$ mice was significantly fewer than *Tph2*$^{+/+}$ (p = 0.0259) and *Tph2*$^{+/-}$ (p = 0.0107). Again, in *Tph2*$^{fl/-}$ mice the number of 5-HT ir cells counted were significantly fewer than those counted in *Tph2*$^{+/+}$ (p = 0.0026) and *Tph2*$^{+/-}$ (p = 0.0012).

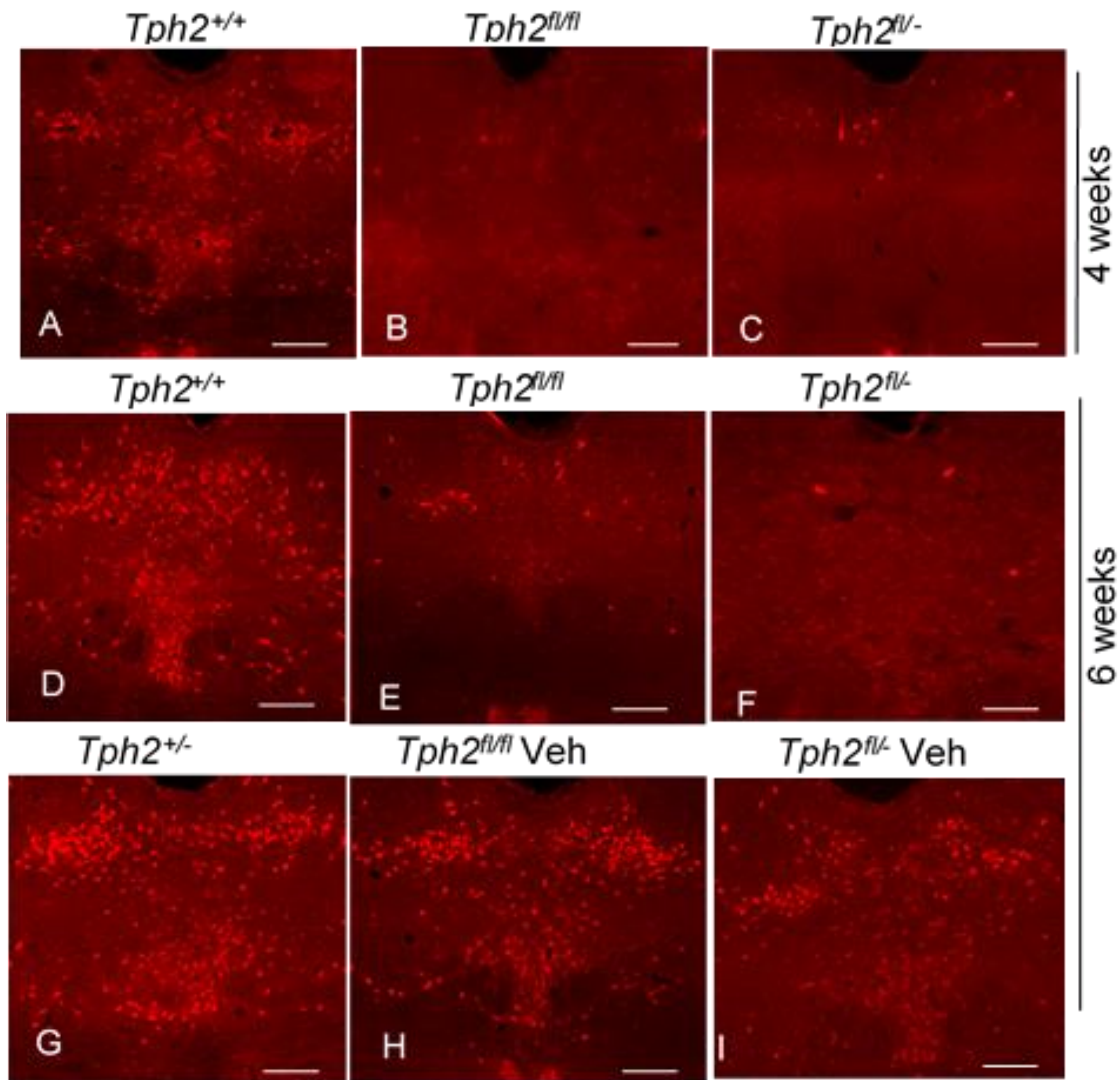

Figure 3-1: Immunohistochemistry of 5-HT ir cells in the dorsal raphe of mice. Fluorescence staining of 5-HT ir cells in the B7 DRN. Panel (A-C):- 4 weeks after Tam injection. Only few 5-HT ir cells are seen in *Tph2icKO* mice (B,C) compare with *Tph2CON* mice (A) which show strong 5-HT immunoreactivity. Panel (D-I):- 6 weeks after Tam injection. The number of 5-HT ir cells in *Tph2icKO* (E,F) remained significantly low in the 6th week compared with Tam-treated (D,G) and Veh-treated (H,I) *Tph2CON* mice. Scale bar is 200 μm.

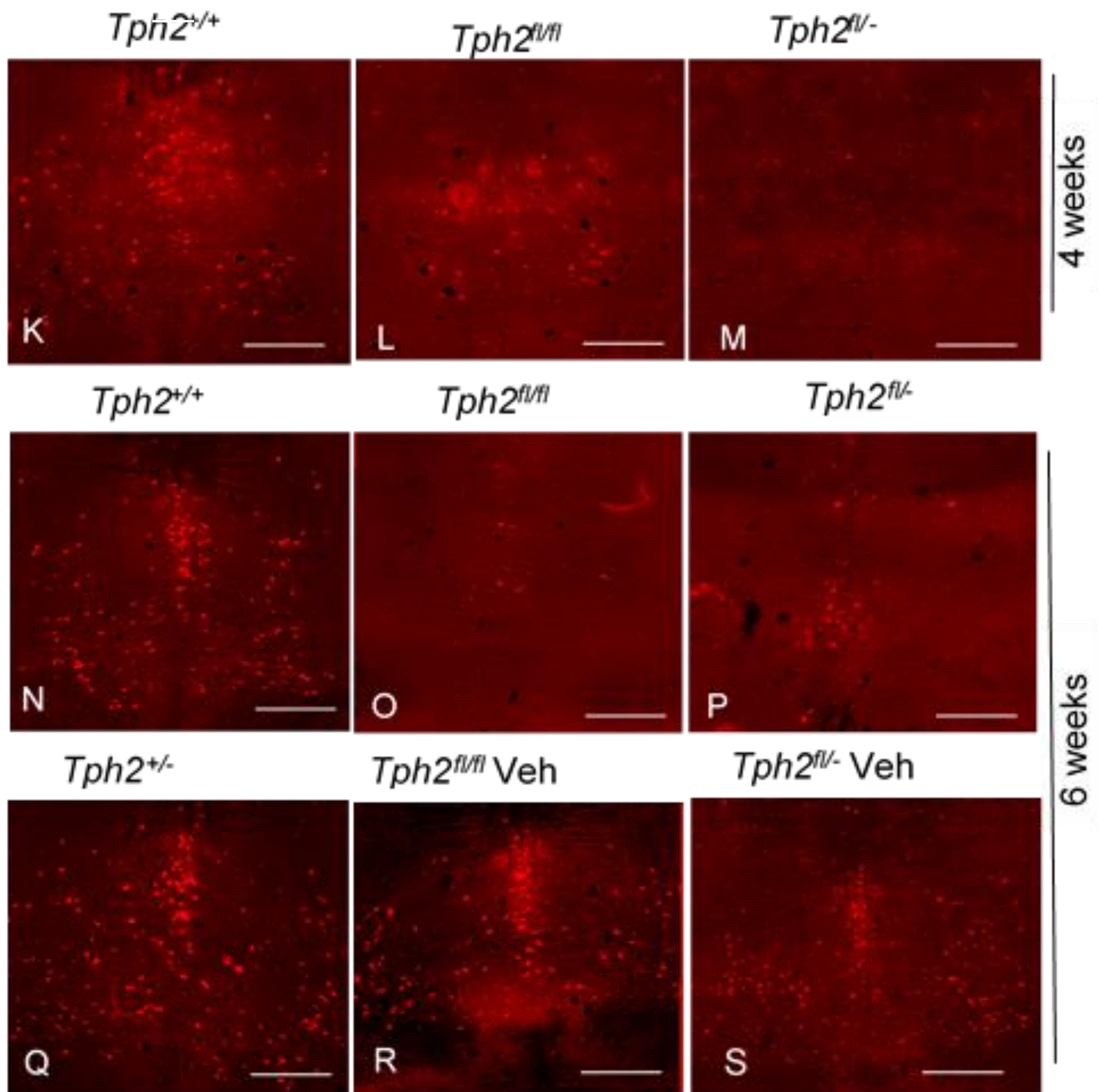

Figure 3-2: Immunohistochemistry of 5-HT ir cells in the median raphe of mice. Fluorescence staining of 5-HT ir cells in the B8, B9 MRN. Panel (K-M):- 4 weeks after Tam injection. Only few 5-HT ir cells are seen in *Tph2icKO* mice (L,M) compare with *Tph2CON* mice (K) which show strong 5-HT immunoreactivity. Panel (N-S):- 6 weeks after Tam injection. The number of 5-HT ir cells in *Tph2icKO* mice (O,P) remained significantly low in the 6th week compared with Tam-treated (N,Q) and Veh-treated (R,S) *Tph2CON* mice. Scale bar is 200 µm.

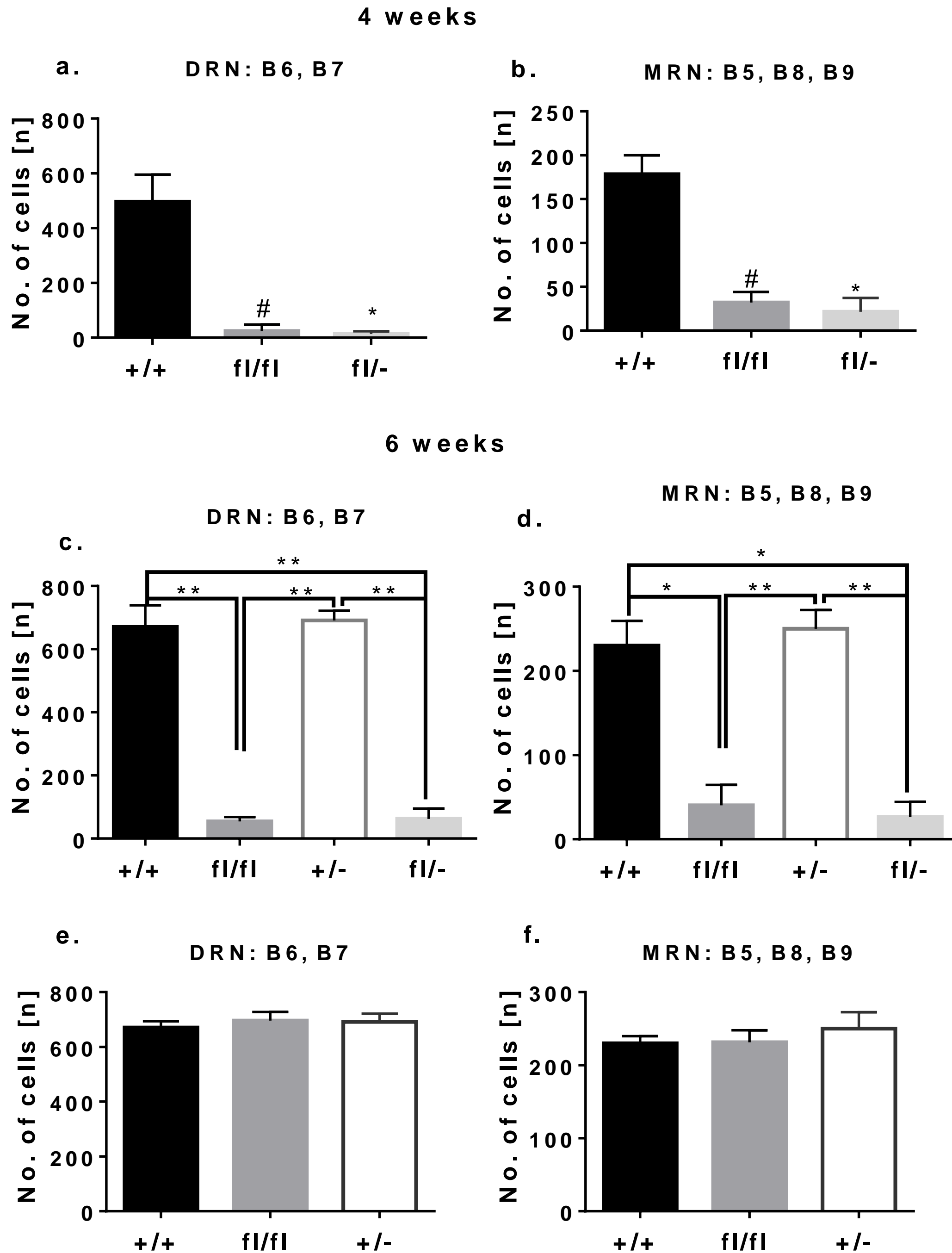

Figure 3-3: Tam induced cre-mediated depletion of adult brain 5-HT. 4 weeks (a&b): Number of 5-HT ir cells in, (a) DRN and (b) MRN. 6 weeks (c-f): Number of 5-HT ir cells in (c) DRN and (d) MRN of *Tph2icKO* and (e) DRN and (f) MRN of *Tph2CON* mice. Data represented as mean ± SEM. Number of mice per group; week 4, n = 4/group and Week 6: $Tph2^{+/+}$ = 9, $Tph2^{+/-}$ = 6, $Tph2^{fl/-}$ = 8, $Tph2^{fl/fl}$ (Tam) = 7, $Tph2^{fl/fl}$ (Veh) = 8. One-way ANOVA (*) indicate significant differences ($^{\#}p$:0.05≤p<0.1, *p<0.05, **p<0.01, ***p<0.001).

3.1.2 High-Performance Liquid Chromatography (HPLC)

Upon detecting that some few patches of 5-HT ir cells in the raphe, the levels of 5-HT and its metabolite, 5-HIAA, as well as NE and DA in the raphe and forebrain regions of heparinized PBS perfused brains was measured by performing HPLC at three different time points after Tam injection. The *Tph2icKO* were Tam-treated $Tph2^{fl/fl}$ and $Tph2^{fl/-}$ while $Tph2^{+/+}$ and $Tph2^{+/-}$ served as corresponding *Tph2CON* mice. In all the selected brain regions differences in levels of brain neurochemicals occurred largely in the 2nd and 6th week after Tam injection.

3.1.2.1 Levels of brain 5-HT

Raphe: In the raphe region, the levels of 5-HT in $Tph2^{fl/fl}$ and $Tph2^{fl/-}$ remained relatively stable at the three selected time points (Figure 3-4 a). A two-way ANOVA revealed significant main effects of genotype ($F_{(3,54)} = 5.05$; $p = 0.0037$) and a significant interaction between genotype and time ($F_{(6,54)} = 2.694$; $p = 0.0232$). Post-hoc analyses revealed no significant genotype differences in the level of 5-HT by 2nd week, however by week four, level 5-HT in $Tph2^{+/-}$ was higher that of $Tph2^{fl/-}$ mice ($p = 0.012$). Again, by week 6, the level of 5-HT in $Tph2^{+/+}$ was significantly higher than $Tph2^{+/-}$ and $Tph2^{fl/-}$ (both $p = 0.003$) and $Tph2^{fl/fl}$ ($p = 0.02$).

Frontal cortex: In the frontal cortex, a similar pattern of stability in levels of 5-HT in $Tph2^{fl/fl}$ and $Tph2^{fl/-}$ at the three selected time points was observed (Figure 3-4 b). A significant main effect of genotype ($F_{(3,54)} = 3.916$; $p = 0.013$) and a significant interaction between genotype and time ($F_{(6,54)} = 5.096$; $p = 0.0003$) occurred. Post hoc analyses revealed that by 2nd week the level of 5-HT in $Tph2^{fl/-}$ was significantly higher than in $Tph2^{+/-}$ ($p = 0.002$) and $Tph2^{+/+}$ ($p = 0.006$). $Tph2^{+/+}$ recorded higher 5-HT than $Tph2^{fl/-}$ ($p = 0.014$) in 4th week and $Tph2^{fl/fl}$ ($p = 0.024$) in 6th week.

Hippocampus: The outcome in the hippocampus deviates slightly from that of the raphe and frontal cortex (Figure 3-4 c). A significant main effect of genotype ($F_{(3,54)} = 5.353$; $p = 0.0027$) and a significant interaction between genotype and time ($F_{(6,54)} = 4.353$; $p = 0.0012$) were observed. In week 2, the amount of 5-HT detected in $Tph2^{fl/fl}$ was considerably higher than $Tph2^{+/+}$ ($p = 0.005$); $Tph2^{+/-}$ ($p = 0.0342$) and $Tph2^{fl/-}$ ($p = 0.0208$). In week 4, $Tph2^{fl/-}$ recorded significantly lower 5-HT than $Tph2^{+/+}$ (p =

0.0267) and *Tph2*$^{+/-}$ (p = 0.0183). In week 6, *Tph2*$^{+/+}$ recorded significantly higher 5-HT than *Tph2*$^{fl/-}$ (p = 0.0041) and *Tph2*$^{fl/fl}$ (p = 0.045).

Amygdala: In the amygdala, there were fluctuations and inconsistencies in the levels of 5-HT over time Figure 3-4 d. A significant main effect of time ($F_{(2,54)} = 4.151$; $p = 0.021$) and a significant interaction between genotype and time ($F_{(6,54)} = 8.781$; $p < 0.0001$) was observed. In week two, the levels of brain 5-HT in *Tph2*$^{+/+}$ was significantly lower than*Tph2*$^{fl/fl}$ (p = 0.0023) and *Tph2*$^{fl/-}$ (p = 0.0214). Again, a significantly lower 5-HT was recorded in *Tph2*$^{+/-}$ compared with *Tph2*$^{fl/fl}$ (p = 0.0115). This observation is not in concordance with the outcomes from the raphe and frontal cortex. In the 4th week, only a trend was observed between *Tph2*$^{fl/-}$ and *Tph2*$^{+/+}$ (p = 0.08) as well as *Tph2*$^{+/-}$ (p = 0.052). In week 6, the amount of 5-HT recorded in *Tph2*$^{+/+}$ was significantly higher than *Tph2*$^{fl/fl}$ (p = 0.0002); *Tph2*$^{fl/-}$ (p = 0.0032) as well as *Tph2*$^{+/-}$ (p < 0.0001). The above observations indicate that the rate of metabolism of 5-HT in brain of inducible knockout mice varies considerably in the various brain regions.

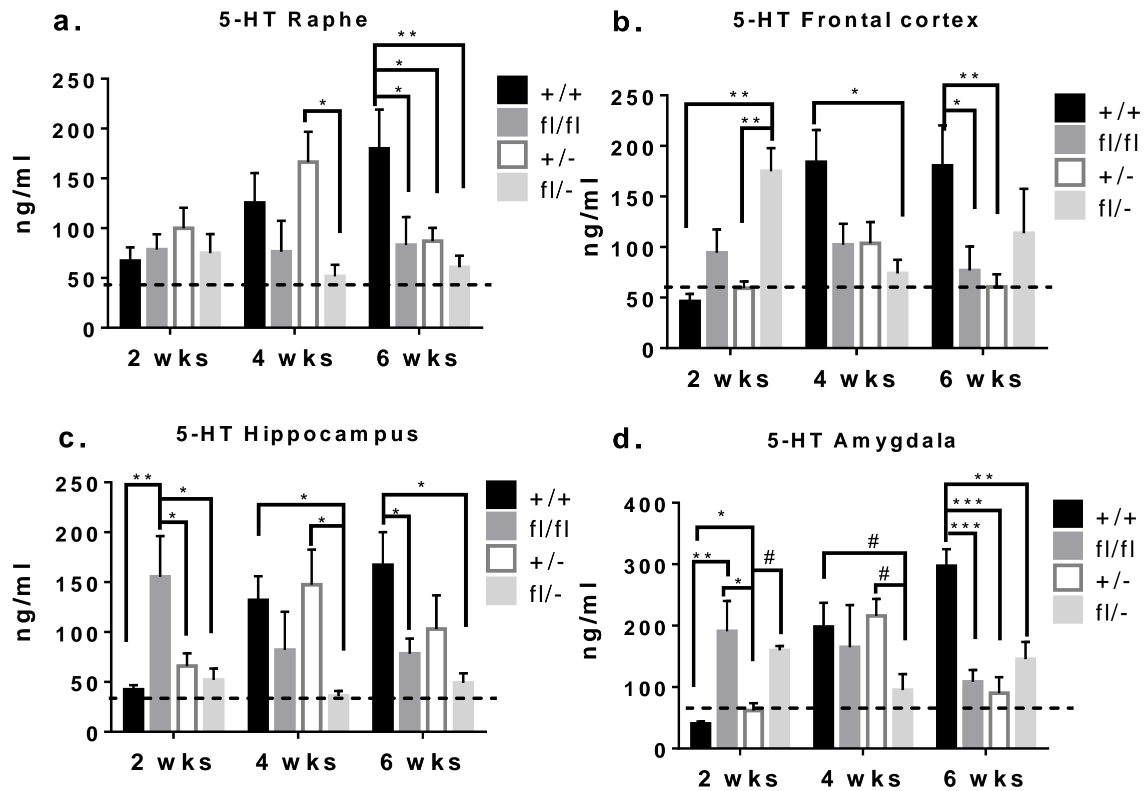

Figure 3-4: Quantitative estimation of 5-HT levels in different brain areas. HPLC analysis of 5-HT in, (a) raphe; (b) frontal cortex; (c) hippocampus and, (d) amygdala at week 2, week 4 and week 6. The *Th2icKO* (fl/fl and fl/-) were compared to respective *Tph2CON* (+/+ and +/-). Data are represented as mean ± SEM. Number of mice per group; week 2: $Tph2^{+/+}$ = 7, $Tph2^{fl/fl}$ = 5, $Tph2^{+/-}$ = 7, $Tph2^{fl/-}$ = 5; Week 4: $Tph2^{+/+}$ = 6, $Tph2^{fl/fl}$ = 4, $Tph2^{+/-}$ = 4, $Tph2^{fl/-}$ = 6; Week 6: $Tph2^{+/+}$ = 5, $Tph2^{fl/fl}$ = 6, $Tph2^{+/-}$ = 6, $Tph2^{fl/-}$ = 6. Two-way ANOVA (*) indicate significant differences (*p<0.05, **p<0.01 ***p<0.001, $^{\#}$p: 0.05≤p<0.1).

3.1.2.2 Levels of 5-hydroxyindolacetic acid (5-HIAA)

Raphe: The levels of 5-HIAA in $Tph2^{fl/fl}$ and $Tph2^{fl/-}$ was consistently low and invariable in the different brain regions throughout the experiment. Two-way ANOVA analysis of the amount of 5-HIAA revealed significant main effects of genotype ($F_{(3,54)}$ = 16.15; $p < 0.0001$); time($F_{(2,54)}$ = 15.70; $p < 0.0001$) and a significant interaction between genotype and time ($F_{(6, 54)}$ = 6.449; $p < 0.0001$). In week 2, a significantly higher level of 5-HIAA in $Tph2^{+/+}$ and $Tph2^{+/-}$ than $Tph2^{fl/fl}$ and $Tph2^{fl/-}$ (both $p < 0.0001$) was recorded. In week 6, only in $Tph2^{+/-}$ a significantly higher level of 5-HIAA compared to $Tph2^{fl/fl}$ ($p = 0.0274$) and $Tph2^{fl/-}$ ($p = 0.0224$) was recorded.

The level of brain 5-HIAA in *Tph2$^{fl/fl}$* and *Tph2$^{fl/-}$* remained relatively stable throughout the time line (Figure 3-5 a).

Frontal cortex: The level of 5-HIAA in the frontal cortex revealed significant main effects of genotype ($F_{(3,54)} = 21.25$; $p < 0.0001$); time($F_{(2,54)} = 13.56$; $p < 0.0001$) and a significant interaction between genotype and time ($F_{(6,54)} = 2.288$; $p = 0.0486$). In week 2, a significantly higher level of 5-HIAA in*Tph2$^{+/+}$* compared with the *Tph2icKO* mice (both $p < 0.0001$) was recorded (Figure 3-5 b). Again, a significantly higher amount was recorded in *Tph2$^{+/-}$* than *Tph2$^{fl/fl}$* ($p = 0.0003$) and *Tph2$^{fl/-}$* ($p = 0.0002$). In week 4, a significantly lower amount was recorded in *Tph2$^{fl/-}$* ($p = 0.0399$) compared with*Tph2$^{+/+}$*. In week 6, *Tph2$^{+/+}$* recorded significantly higher levels of 5-HIAA than *Tph2$^{fl/fl}$* ($p = 0.0444$) and *Tph2$^{fl/-}$* ($p = 0.0378$) whilst *Tph2$^{+/-}$* also significantly differed from *Tph2$^{fl/fl}$* ($p = 0.0003$) and *Tph2$^{fl/-}$* ($p = 0.0002$).

Hippocampus: The level of 5-HIAA recorded showed a significant main effect of genotype ($F_{(3,54)} = 27.85$; $p < 0.0001$); time ($F_{(2,54)} = 13.86$; $p < 0.0001$) and a significant interaction between genotype and time ($F_{(6,54)} = 3.434$; $p = 0.006$). In week 2, *Tph2$^{+/+}$* and *Tph2$^{+/-}$* recorded significantly higher levels of 5-HIAA than *Tph2$^{fl/fl}$* and *Tph2$^{fl/-}$* (all $p < 0.0001$). In week 6, only *Tph2$^{+/-}$* recorded significantly higher levels of 5HIAA than *Tph2$^{fl/fl}$* and *Tph2$^{fl/-}$* (both $p < 0.0001$) as well as *Tph2$^{+/+}$* ($p = 0.0256$; Figure 3-5 c).

Amygdala: The amount of 5-HIAA recorded showed a significant main effects of genotype ($F_{(3,54)} = 29.83$; $p < 0.0001$); time ($F_{(2,54)} = 17.12$; $p < 0.0001$) and a significant interaction between genotype and time ($F_{(6,54)} = 3.824$; $p = 0.003$). In week 2, *Tph2$^{+/+}$* and *Tph2$^{+/-}$* recorded significantly higher levels of 5-HIAA than *Tph2$^{fl/fl}$* and *Tph2$^{fl/-}$* (all $p<0.0001$). In week 6, only *Tph2$^{+/-}$* recorded significantly higher levels of 5-HIAA than *Tph2$^{fl/fl}$* and *Tph2$^{fl/-}$* (both $p < 0.0001$) as well as *Tph2$^{+/+}$* ($p = 0.0421$; Figure 3-5 d).

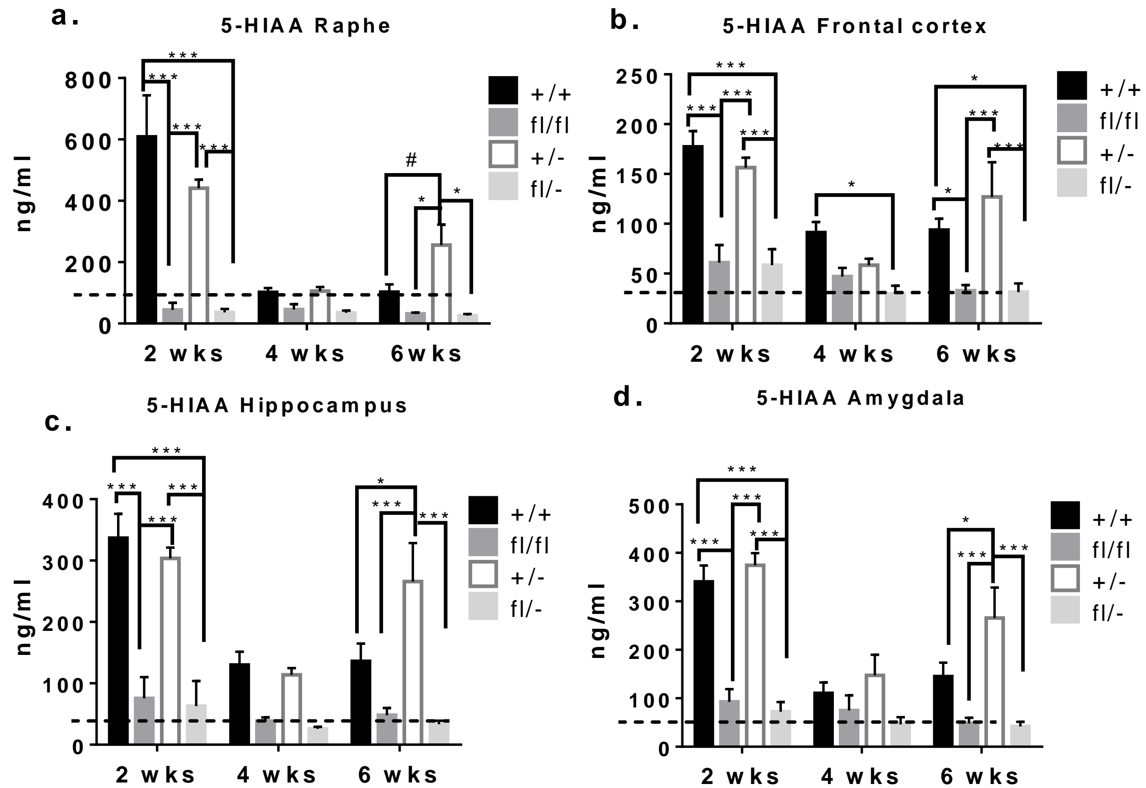

Figure 3-5: Quantitative estimation of 5-HIAA levels in different brain areas. HPLC analysis of 5-HIAA in, (a) raphe; (b) frontal cortex; (c) hippocampus and, (d) amygdala at week 2, week 4 and week 6. The *Tph2icKO* (fl/fl and fl/-) were compared to respective *Tph2CON* (+/+ and +/-). Data are represented as mean ± SEM. Number of mice per group; week 2: $Tph2^{+/+}$ = 7, $Tph2^{fl/fl}$ = 5, $Tph2^{+/-}$ = 7, $Tph2^{fl/-}$ = 5; Week 4: $Tph2^{+/+}$ = 6, $Tph2^{fl/fl}$ = 4, $Tph2^{+/-}$ = 4, $Tph2^{fl/-}$ = 6; Week 6: $Tph2^{+/+}$ = 5, $Tph2^{fl/fl}$ = 6, $Tph2^{+/-}$ = 6, $Tph2^{fl/-}$ = 6. Two-way ANOVA (*) indicate significant differences (*p<0.05, **p<0.01 ***p<0.001, $^{\#}$p: 0.05≤p<0.1).

3.1.2.3 5-HT turnover rate

Since we observed an unexpected high concentration of 5-HT in the forebrain regions of icKO mice specifically in week 2, 5HT turnover rates (5-HIAA/5-HT) was calculated. This is an index of neuronal activity in terms of 5-HT synthesis, release and re-uptake.

Raphe: Two-way ANOVA revealed significant main effects of genotype ($F_{(3,54)}$ = 5.256; p = 0.003); time ($F_{(2,54)}$ = 7982; p = 0.0009) and a significant interaction between genotype and time ($F_{(6, 54)}$ = 3.27; p = 0.0081). In week 2, turnover rate in

Tph2$^{+/+}$ was higher than *Tph2*$^{fl/fl}$ ($p = 0.0001$) and *Tph2*$^{fl/-}$ ($p = 0.0002$). Similarly, turnover rate in *Tph2*$^{+/-}$ was significantly higher compared to *Tph2*$^{fl/fl}$ ($p = 0.0019$) and *Tph2*$^{fl/-}$ ($p = 0.0024$; Figure 3-6 a).

Frontal cortex: The turnover rate in the frontal cortex revealed significant main effects of genotype ($F_{(3,54)} = 16.25$; $p < 0.0001$); time ($F_{(2,54)} = 19.06$; $p < 0.0001$) and a significant interaction between genotype and time ($F_{(6,54)} = 10.13$; $p < 0.0001$). In week 2, a significantly higher 5-HIAA/5-HT rate in *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ compared with the *Tph2*$^{fl/fl}$ and *Tph2*$^{fl/-}$ ($p < 0.0001$) was recorded (Figure 3-6 b). Again, a significantly higher rate was recorded in *Tph2*$^{+/+}$ than *Tph2*$^{+/-}$ ($p = 0.0232$) in the 2nd week. In week 6, *Tph2*$^{+/-}$ recorded significantly higher turnover rate than *Tph2*$^{fl/fl}$ ($p = 0.0024$), *Tph2*$^{fl/-}$ ($p = 0.0004$) and *Tph2*$^{+/+}$ ($p = 0.0016$).

Hippocampus: The turnover rate in the hippocampus revealed significant main effects of genotype ($F_{(3,54)} = 7.716$; $p = 0.0002$); time ($F_{(2,54)} = 10.13$; $p = 0.0002$) and a significant interaction between genotype and time ($F_{(6,54)} = 3.774$; $p = 0.0033$). In week 2, a significantly higher 5-HIAA/5-HT rate in *Tph2*$^{+/+}$ compared with the *Tph2*$^{fl/fl}$ and *Tph2*$^{fl/-}$ ($p < 0.0001$). Again, a higher turnover rate in *Tph2*$^{+/-}$ compared with *Tph2*$^{fl/fl}$ ($p = 0.0005$) and *Tph2*$^{fl/-}$ ($p = 0.0016$) was recorded in week 2 (Figure 3-6 c). In week 6, *Tph2*$^{+/-}$ recorded slightly higher turnover rate than *Tph2*$^{fl/fl}$ ($p = 0.089$).

Amygdala: The turnover rate in amygdala revealed significant main effects of genotype ($F_{(3,54)} = 14.98$; $p < 0.0001$); time ($F_{(2,54)} = 16.41$; $p < 0.0001$) and a significant interaction between genotype and time ($F_{(6,54)} = 6.752$; $p < 0.0001$; Figure 3-6 d). In week 2, a significantly higher 5-HIAA/5-HT rate in *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ compared with the *Tph2*$^{fl/fl}$ and *Tph2*$^{fl/-}$ ($p < 0.0001$) was recorded. In week 6, *Tph2*$^{+/-}$ recorded significantly higher turnover rate than *Tph2*$^{fl/fl}$ ($p = 0.0001$), *Tph2*$^{fl/-}$ ($p < 0.0001$) and *Tph2*$^{+/+}$ ($p = 0.0023$).The reduced 5-HT turnover rate in icKO mice indicates that, limited 5-HT synthesis or none at all occurred after Tam treatment. Remnants of 5-HT detected in the 2nd week might be homeostatic controls to retain 5-HT icKO mice.

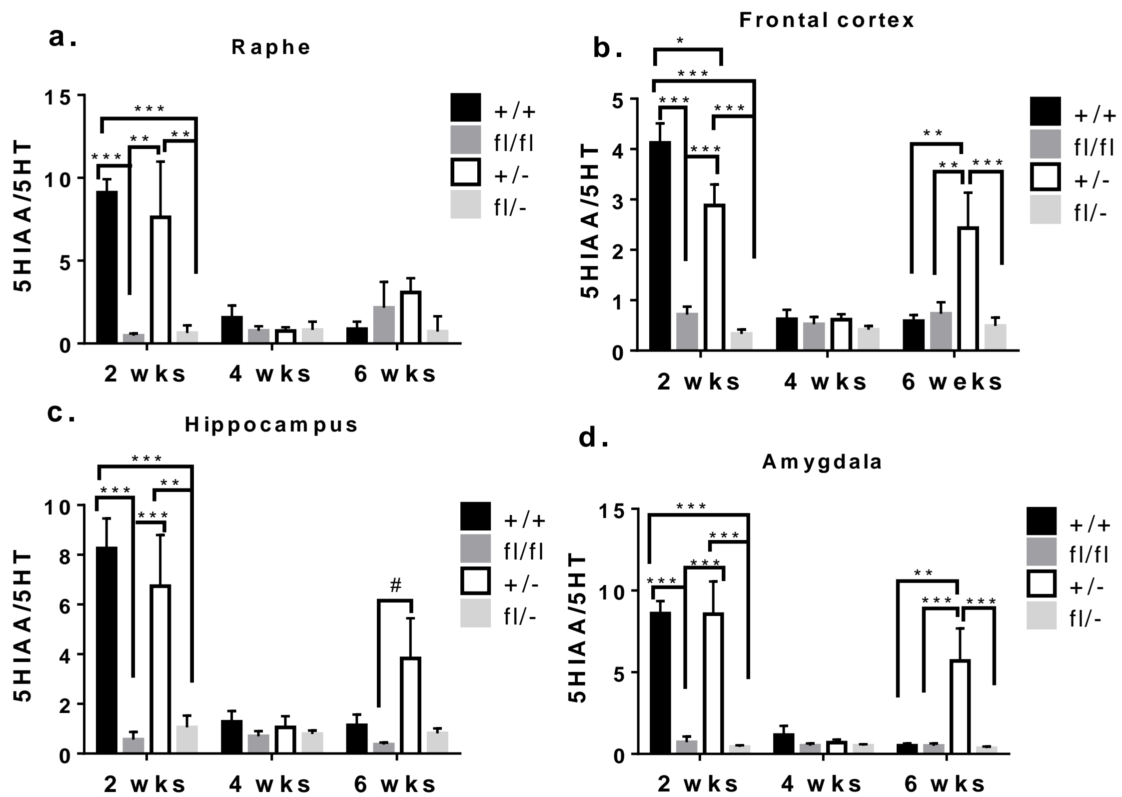

Figure 3-6: 5-HT turnover rate. Comparison of 5-HT turnover rate in, (a) raphe (b) frontal cortex; (c) hippocampus and (d) amygdala. Data represented as mean ± SEM. Number of mice per group; week 2: *Tph2*$^{+/+}$ = 7, *Tph2*$^{fl/fl}$ = 5, *Tph2*$^{+/-}$ = 7, *Tph2*$^{fl/-}$ = 5; week 4: *Tph2*$^{+/+}$ = 6, *Tph2*$^{fl/fl}$ = 4, *Tph2*$^{+/-}$ = 4, *Tph2*$^{fl/-}$ = 6; week 6: *Tph2*$^{+/+}$ = 5, *Tph2*$^{fl/fl}$ = 6, *Tph2*$^{+/-}$ = 6, *Tph2*$^{fl/-}$ = 6.One-way ANOVA (*) indicate significant differences (*p < 0.05, **p < 0.01, ***p < 0.001, $^{\#}$p: 0.05 ≤ p <0.1).

3.1.2.4 Dopamine and norepinephrine

The level of DA in the brain remained relatively the same in the raphe (Figure 3-7 a) and hippocampus (Figure 3-7 c) of mice with no significant differences recorded. In the frontal cortex (Figure 3-7 b), only a trend in genotype ($F_{(2,54)}$=3.045; p=0.06) factor. In the amygdala, there was a significant interaction between genotype and time ($F_{(6,54)}$ = 2.953; p = 0.0145). Tukey's multiple comparisons test shows significantly higher quantity in *Tph2*$^{fl/fl}$ than*Tph2*$^{+/+}$ (p = 0.0019) and *Tph2*$^{+/-}$ (p = 0.002) in the 2nd week (Figure 3-7 d).

Again, the level of NE in the *Tph2*$^{fl/fl}$ and *Tph2*$^{fl/-}$ remained stable in the raphe, amygdala and hippocampus throughout the experiment. However, some significant

differences in NE occurred in the second and sixth week after Tam treatment (Figure 3-8 a-d).

Raphe: Two-way ANOVA revealed significant main effects of genotype ($F_{(3,54)}$ = 11.42; p < 0.0001); time ($F_{(2,54)}$ = 25.17; p < 0.0001) and a significant interaction between genotype and time ($F_{(6, 54)}$ = 6.327; p < 0.0001). Post hoc analyses revealed that *Tph2CON* have a significantly lower level of NE than*Tph2icKO* (all p < 0.0001) in the 2nd week. However, by 6th week the level of NE in *Tph2*$^{+/-}$ *was* lower than *Tph2*$^{fl/fl}$ (p = 0.0015); *Tph2*$^{fl/-}$ mice (p = 0.0005) and *Tph2*$^{+/+}$ (p = 0.0056).

Frontal cortex: In the frontal cortex, there were significant main effects of genotype ($F_{(3,54)}$ = 8.545; p < 0.0001); time ($F_{(2,54)}$ = 5.134; p = 0.0091) and a significant interaction between genotype and time ($F_{(6,54)}$ = 3.849; p = 0.0029). Post hoc analyses showed that in the 2nd week the levels of NE in *Tph2*$^{fl/-}$ was higher than *Tph2*$^{fl/fl}$ (p = 0.0375); *Tph2*$^{+/-}$ and *Tph2*$^{+/+}$ (both p < 0.0001). In the 6th week the differences occurred between *Tph2*$^{+/+}$ and*Tph2*$^{+/-}$ (p = 0.025).

Hippocampus: The levels of NE recorded showed a significant main effect of genotype ($F_{(3,54)}$ = 10.96; p < 0.0001); time ($F_{(2,54)}$ = 10.08; p = 0.0001) and a significant interaction between genotype and time ($F_{(6,54)}$ = 6.656; p < 0.0001). In week 2, *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ recorded significantly reduced levels of NE than *Tph2*$^{fl/fl}$ and *Tph2*$^{fl/-}$ (all p < 0.0001). In week 6, only *Tph2*$^{+/-}$recorded significantly lower levels of NE than *Tph2*$^{+/+}$ (p = 0.0081) and *Tph2*$^{fl/-}$ (p = 0.0442). The above results show that reduction in adult brain 5-HT has no consequential effects on level of NE and DA levels.

Amygdala: In the amygdala, the level of NE recorded showed a significant main effect of genotype ($F_{(3,54)}$ = 11.42; p < 0.0001); time ($F_{(2,54)}$ = 13.90; p < 0.0001) and a significant interaction between genotype and NE levels ($F_{(6,54)}$ = 6.427; p < 0.0001). In week 2, both *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ recorded significantly reduced levels of NE than *Tph2*$^{fl/fl}$ and *Tph2*$^{fl/-}$ (all p < 0.0001). In week 6, level of NE *Tph2*$^{+/-}$ was significantly lower than *Tph2*$^{fl/fl}$ (p = 0.0081); *Tph2*$^{fl/-}$ (p = 0.0139) as well as *Tph2*$^{+/+}$ (p = 0.0046).

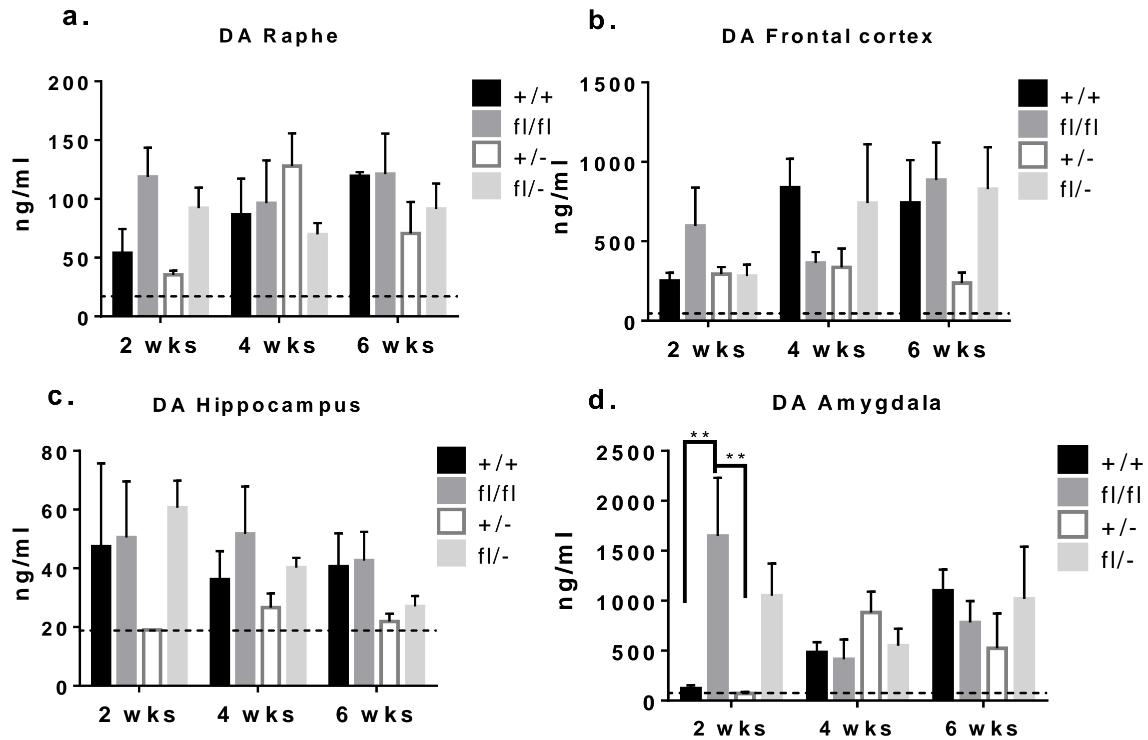

Figure 3-7: Quantitative estimation of levels of dopamine in different brain areas. HPLC analysis of dopamine in, (a) raphe; (b) frontal cortex; (c) hippocampus and(d)amygdala at week 2, week 4 and week 6. The *Tph2icKO* mice (fl/fl and fl/-) were compared to respective *Tph2CON* (+/+ and +/-). Data are represented as mean ± SEM. Number of mice per group; week 2: $Tph2^{+/+}$ = 7, $Tph2^{fl/fl}$ = 5, $Tph2^{+/-}$ = 7, $Tph2^{fl/-}$ = 5; week 4: $Tph2^{+/+}$ = 6, $Tph2^{fl/fl}$ = 4, $Tph2^{+/-}$ = 4, $Tph2^{fl/-}$ = 6; week 6: $Tph2^{+/+}$ = 5, $Tph2^{fl/fl}$ = 6, $Tph2^{+/-}$ = 6, $Tph2^{fl/-}$ = 6. Two-way ANOVA (*) indicate significant differences (*p<0.05, **p<0.01 ***p<0.001, #p: 0.05≤p<0.1).

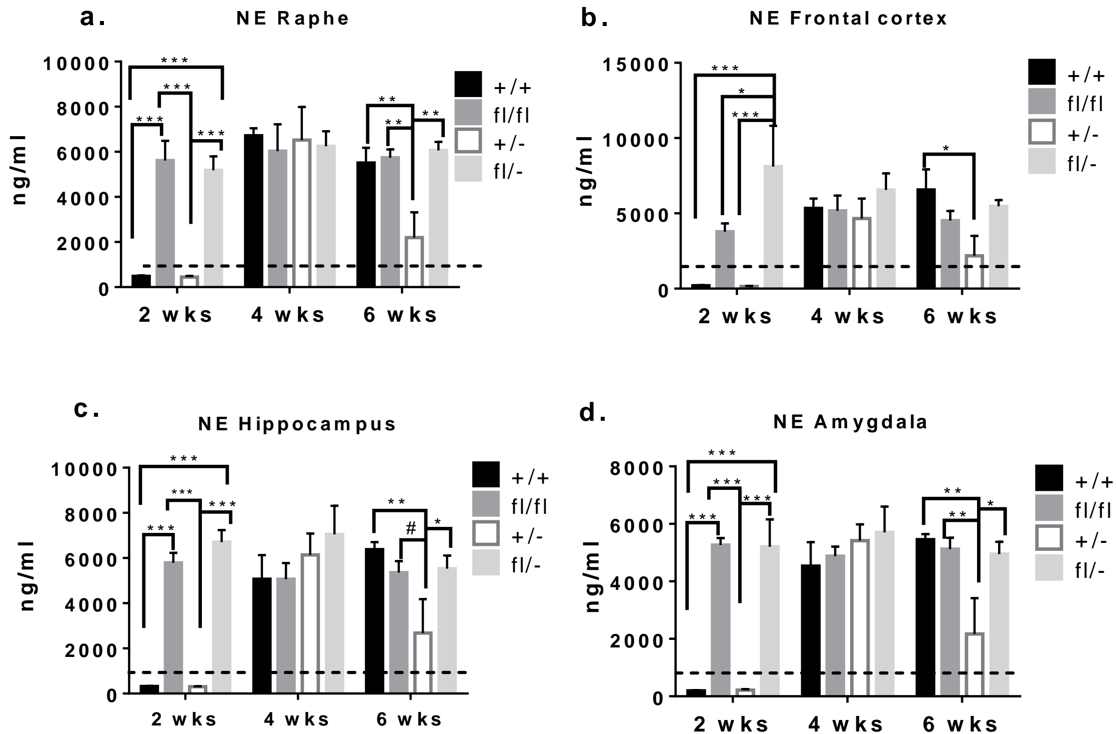

Figure 3-8: Quantitative estimation of levels of norepinephrine in different brain areas. HPLC analysis of NE in, (a) raphe; (b) frontal cortex; (c) hippocampus and (d) amygdala at week 2, week 4 and week 6. The *Tph2icKO* (fl/fl and fl/-) were compared to respective *Tph2CON* (+/+ and +/-). Data are represented as mean ± SEM. Number of mice per group; week 2; $Tph2^{+/+}$ = 7, $Tph2^{fl/fl}$ = 5, $Tph2^{+/-}$ = 7, $Tph2^{fl/-}$ = 5; week 4: $Tph2^{+/+}$ = 6, $Tph2^{fl/fl}$ = 4, $Tph2^{+/-}$ = 4, $Tph2^{fl/-}$ = 6; week 6: $Tph2^{+/+}$ = 5, $Tph2^{fl/fl}$ = 6, $Tph2^{+/-}$ = 6, $Tph2^{fl/-}$ = 6. Two-way ANOVA (*) indicate significant differences (*p<0.05, **p<0.01 ***p<0.001, $^{\#}$p: 0.05≤p<0.1).

3.2 Anxiety-related and exploratory behavior in icKO mice

Elevated-plus maze: The EPM functions on the principle that mice have aversion for height and the brightly lit aversive open arms. Therefore the frequency of entry and length of time spent in the open arms are inversely related to anxiety-like behavior. Behavioral outcome in the EPM is illustrated in. Figure 3-9 a-d. One-way ANOVA was performed to compare behavior of *Tph2icKO* ($Tph2^{fl/fl}$ and$Tph2^{fl/-}$) versus their respective *Tph2CON* ($Tph2^{+/-}$ and $Tph2^{+/+}$) mice. Compared with *Tph2CON*, the *Tph2icKO* mice did not show significant preference for open arms in terms frequency of visits ($F_{(3,32)}$ = 0.3565; p = 0.78; Figure 3-9 a) and cumulative time spent in open arms ($F_{(3,32)}$ = 0.9025; p = 0.45; Figure 3-9 b). Again, exploratory behavior assessed

by total arms entry (Figure 3-9 c) and total distance travelled (Figure 3-9 d), did not significantly vary between icKO and Ctrl mice.

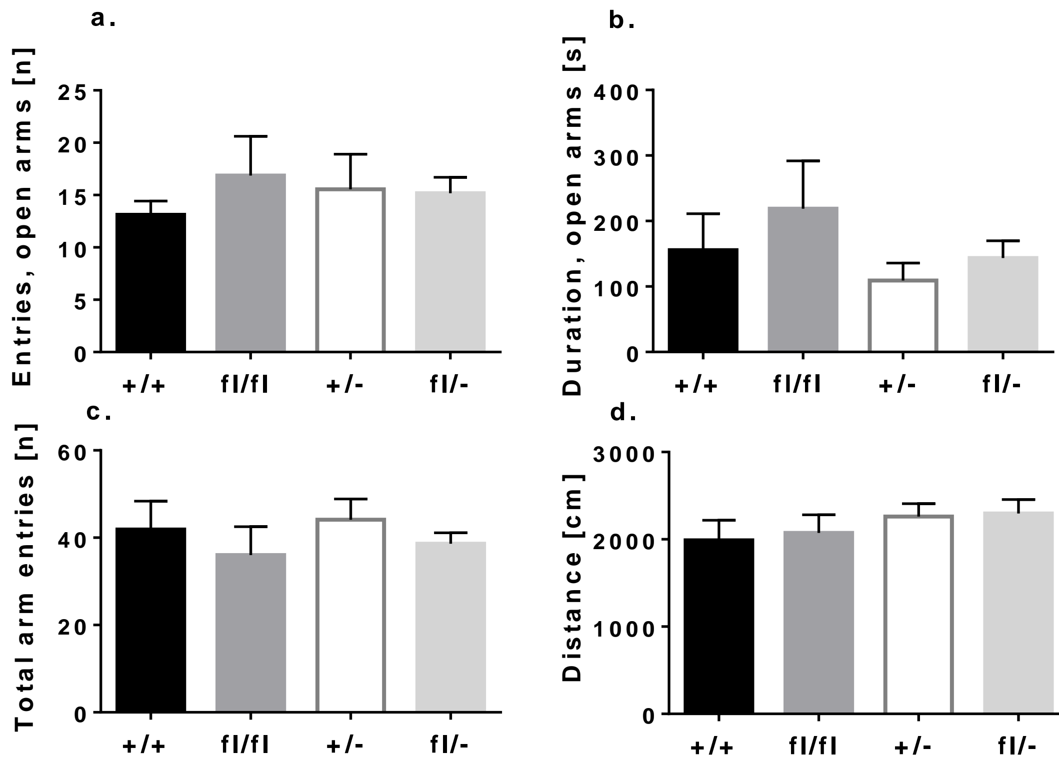

Figure 3-9: Exploratory and anxiety-related behavior of mice in EPM. No obvious anxiety in (a) number of visits to open arms, (b) total time spent in open arms, (c) total arms entries and (d) global distance travelled. Data represented as mean ± SEM; *Tph2*$^{+/+}$ = 9, *Tph2*$^{fl/fl}$ = 7, *Tph2*$^{+/-}$ = 9, *Tph2*$^{fl/-}$ = 11.

Light-dark-box: The LBD operates on the fact that mice show strong avoidance of the brightly lit compartment of the set up. One-way ANOVA revealed no genotype differences in expression of anxiety in the potentially aversive light compartment. With respect to number of visits to the light compartment (Figure 3-10 a), all the groups of mice did not differ significantly ($F_{(3,51)}$ = 1.85; p = 0.015). Comparatively, mice spent similar amount of time (Figure 3-10 b) in the open aversive compartment ($F_{(3,51)}$ = 0.4591; p = 0.71). Examination of exploratory behavior showed no genotype differences in total distance travelled ($F_{(3,51)}$ = 1.183; p = 0.3256) in the box (Figure 3-10 d). The frequency of rearing which is also an exploratory behavior varied among

the mice. *Tph2*$^{+/-}$ and *Tph2*$^{fl/-}$ mice showed increased exploration compared with homozygous ($F_{(3,51)}$ = 11.92; $p < 0.0001$; Figure 3-10 c). Tukey's multiple comparisons test showed that *Tph2*$^{+/-}$ mice explored the light box significantly more than *Tph2*$^{fl/fl}$ ($p < 0.0001$) and *Tph2*$^{+/+}$ ($p = 0.0002$). Also *Tph2*$^{fl/-}$ exhibited higher exploration than *Tph2*$^{fl/fl}$ ($p = 0.0039$).

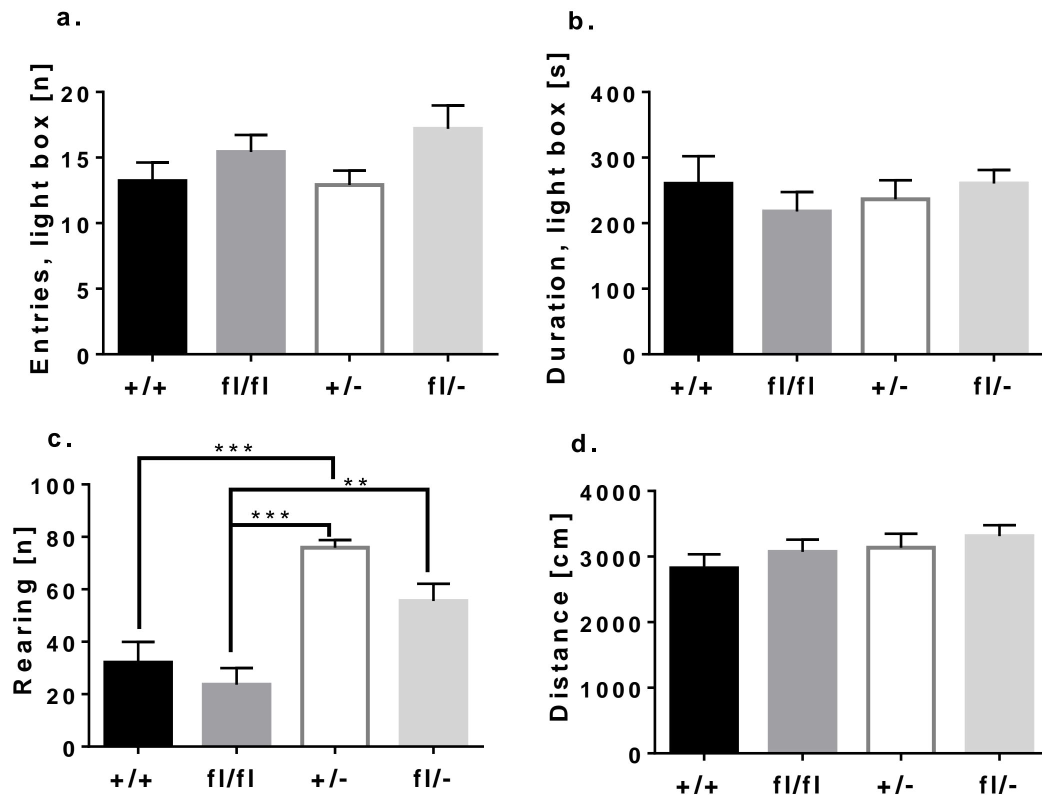

Figure 3-10: Exploratory and anxiety-related behavior of mice in LDB. No obvious anxiety index in mice in, (a) number of visits to light box, (b) total time spent in light box, (c) rearing and, (d)global distance travelled. Data represented as mean ± SEM; *Tph2*$^{+/+}$ = 14, *Tph2*$^{fl/fl}$ = 14, *Tph2*$^{+/-}$ = 11, *Tph2*$^{fl/-}$ = 16. One-way ANOVA (*) indicate significant differences (**p<0.01, ***p<0.001).

Open-field test: In this study, frequency of visits to aversive center as well as cumulative time spent at the center was used as a measure of anxiety like behavior. It was observed that the mice did not exhibit any significant measure of anxiety in the OFT in terms of number visits to center ($F_{(3,49)}$ = 1.943; $p = 0.13$; Figure 3-11 a) and cumulative center time ($F_{(3,49)}$ = 0.8365; $p = 0.48$; Figure 3-11 b). However, some

mice showed potential panic-escape ($F_{(3,49)}$=2.532; p=0.0678) characterized by jumping (Figure 3-11 c). Striking genotype differences were observed with respect to exploratory behavior (Figure 3-11 d). The total distance travelled by *Tph2*$^{fl/-}$ mice during the 30 minutes duration was significantly higher than all the other genotypes ($F_{(3,48)}$ = 6.929; p = 0.0006). Tukey's multiple comparison revealed that *Tph2*$^{+/-}$(p = 0.001) and *Tph2*$^{+/+}$ (p = 0.004) and *Tph2*$^{fl/fl}$ (p = 0.0269) were less active in the arena compared to *Tph2*$^{fl/-}$ mice.

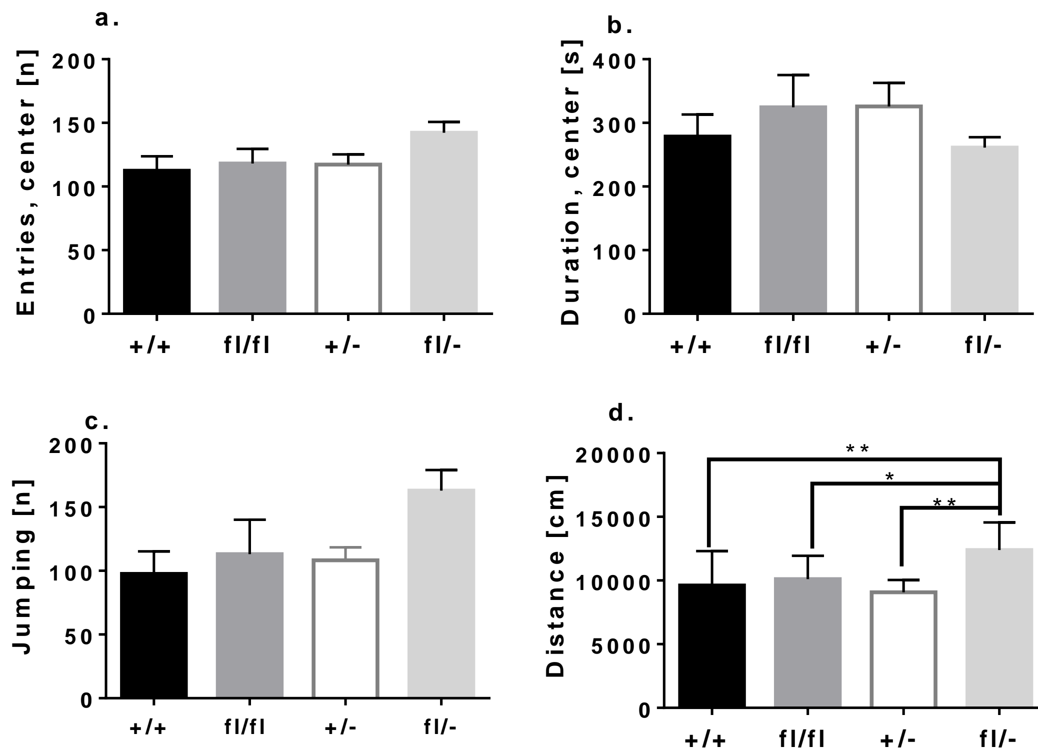

Figure 3-11: Exploratory and anxiety-related behavior of mice in OFT. No obvious anxiety index in mice in, (a) number of visits to aversive center, (b) total time spent in center (c) hopping and, (d) global distance travelled. Data represented as mean ± SEM. Number of mice per group; *Tph2*$^{+/+}$ = 14, *Tph2*$^{fl/fl}$ = 14, *Tph2*$^{+/-}$ = 11, *Tph2*$^{fl/-}$ = 16. One-way ANOVA (*) indicate significant differences (*p<0.05, **p<0.01, #p: 0.05≤p<0.1).

3.2.1 Fear-related behavior

Mice with constitutive deletion of *Tph2* exhibit intense freezing responses when exposed to mild foot shock. We tested freezing responses of *Tph2icKO* mice to mild foot shock. As indicated in Figure 3-12 a, freezing responses increased after each

tone-shock episode, however, both *Tph2icKO* and *Tph2CON* mice did not differ significantly at any phase of fear conditioning (day 1). In the context dependent fear retrieval test on the next day, Two-way ANOVA showed no significant genotype x time interaction ($F_{(39,364)} = 0.4494$; $p = 0.9984$; Figure 3-12 b), but significant effect of genotype ($F_{(3,364)} = 94.8$; $p < 0.0001$) and time ($F_{(13,364)} = 2.362$; $p = 0.004$) occurred. Fear responses to the potentially aversive context were very high in *Tph2icKO* than *Tph2CON* mice, indicating *Tph2icKO* mice are able to retain fear memory more than *Tph2CON* mice.

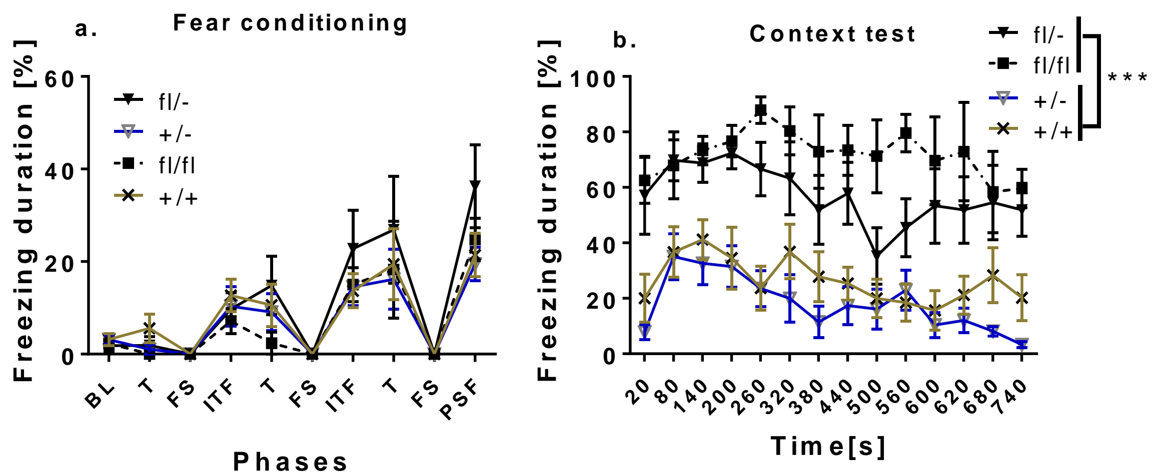

Figure 3-12: Cued fear conditioning (FC) and context dependent fear memory. (a) Duration of freezing during cued fear conditioning. No difference in base line (BL) freezing, tone induced freezing (T), foot shock (FS) reactivity, intertrial freezing (ITF) and post shock freezing (PSF) were observed. (b) Duration of freezing during context (same arena used for FC) dependent fear retention on 2nd day after FC. Data represented as mean ± SEM. Number of mice; FC: $Tph2^{+/+} = 10$, $Tph2^{fl/fl} = 7$, $Tph2^{+/-} = 11$, $Tph2^{fl/-} = 6$; Context: $Tph2^{+/+} = 10$, $Tph2^{fl/fl} = 4$, $Tph2^{+/-} = 9$, $Tph2^{fl/-} = 7$. Two-way ANOVA (*) indicate significant differences (*p<0.05, **p<0.01, ***p<0.001, $^{\#}$p:0.05 ≤ p < 0.1).

3.2.2 Altered depressive-like and anhedonic behavior

Because 5-HT deficiency has been associated with major depression, effects of adult brain 5-HT depletion on depressive like and anhedonia behavior were tested in the mice. Mice were subjected to FST for a period of 5 min and early latency at first (to) floating and prolonged duration of floating was used as direct measure of learned

helplessness and depressive-like behavior. One-Way ANOVA revealed no differences in latency to float ($F_{(3,33)} = 0.3713$; $p = 0.77$; Figure 3-13 a). Duration of floating significantly varied between genotypes ($F_{(3,33)} = 3.149$; $p = 0.0379$), but post hoc analysis indicated that *Tph2*$^{+/-}$mice differed only slightly from *Tph2*$^{fl/fl}$ ($p = 0.07$) and *Tph2*$^{fl/-}$ ($p = 0.06$) in the length of time the mice spent floating on surface of water (Figure 3-13 b). This indicates that adult brain 5-HT depletion alone may not impact depression in mice.

It has been reported that depressed individuals lose interest in pleasurable activities which they used to enjoy, a condition called anhedonia. Therefore, the quantity of sucrose solution and water consumed by *Tph2icKO* and their corresponding *Tph2CON*mice were measured daily for 5 days in the 5th week after last injection. One-way ANOVA showed genotype differences in total fluid consumed by the mice ($F_{(3,28)} = 6.481$; $p = 0.0018$). Tukey's multiple comparisons test revealed that *Tph2icKO* consumed a significantly higher quantity of fluid than *Tph2CON* mice (Figure 3-13 c). Again, *Tph2*$^{+/-}$ consumed significantly lower amount of fluid than *Tph2*$^{fl/fl}$ ($p = 0.014$) and *Tph2*$^{fl/-}$ ($p = 0.003$), however, only a trend was observed between *Tph2*$^{+/+}$ and *Tph2*$^{fl/-}$ ($p = 0.06$).

With respect to percentage sucrose consumption, genotype difference was observed ($F_{(3,28)} = 5.870$; $p = 0.003$; Figure 3-13 d). Tukey's multiple comparisons test indicated that *Tph2*$^{+/+}$ mice showed significantly less preference for sucrose solution in comparison with *Tph2*$^{fl/fl}$ ($p = 0.032$); *Tph2*$^{fl/-}$ ($p = 0.006$) and *Tph2*$^{+/-}$ ($p = 0.01$). The above outcome indicates that adult brain 5-HT depletion may not be a strong factor for anhedonia.

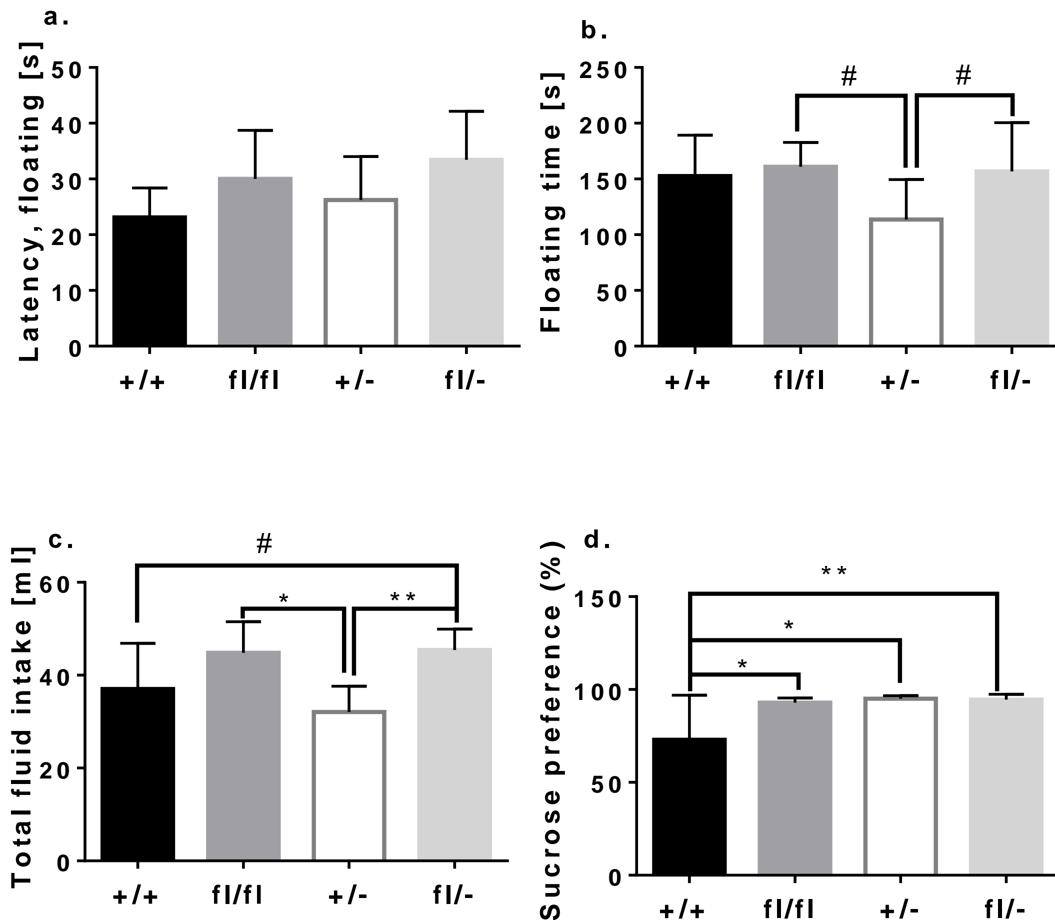

Figure 3-13: Depression and anhedonia related behavior. Force swimming test; (a) latency to floating, (b) floating time in icko mice. Sucrose preference test; (c). total fluid drunk by mice over a period of 6 days, (d) percentage sucrose solution consumed by mice. Data represented as mean ± SEM. Number of mice per group; FST: *Tph2*$^{+/+}$ = 10, *Tph2*$^{fl/fl}$ = 7, *Tph2*$^{+/-}$ = 9, *Tph2*$^{fl/-}$ = 11; SPT: *Tph2*$^{+/+}$ = 9, *Tph2*$^{fl/fl}$ = 6, *Tph2*$^{+/-}$ = 7, *Tph2*$^{fl/-}$ = 10. One-way ANOVA (*) indicate significant differences (*p<0.05, **p<0.01, $^{\#}$p:0.05≤p<0.1).

3.2.3 Body weight, food and water consumption

As previously shown lifelong lack of 5-HT synthesis in *Tph2 null* mutant mice results in reduced body weight but increased food and water intake (Gutknecht et al. 2012). In this study weight of *Tph2icKO* and *Tph2CON* mice was measure for seven continuous weeks starting from first day of injection. Conversely, body weight of mice remained comparatively similar throughout the period ($F_{(3,79)} = 0.261$; $p = 0.85$; Figure 3-14 a).

Two weeks after the last injection, mice were single-housed and the quantity of food and water consumed was measured for four continuous weeks. This time point was chosen because some studies have shown that there is substantial reduction in 5-HT within two weeks of Tam injection (Pelosi et al. 2015). The results displayed in Figure 3-14 b indicate genotype differences existing in the quantity of food consumed ($F_{(3,78)} = 14.01$; $p < 0.001$). Inter-group comparison showed that *Tph2*$^{fl/-}$ mice consumed much more food than *Tph2*$^{fl/fl}$ ($p < 0.0001$); *Tph2*$^{+/+}$ ($p < 0.0001$) and *Tph2*$^{+/-}$($p = 0.004$). In a similar fashion, differences in water consumption were observed between the groups ($F_{(3,78)} = 2.871$; $p = 0.042$; Figure 3-14c).However, multiple comparison revealed only a tendency towards significance between *Tph2*$^{fl/-}$ and *Tph2*$^{+/-}$ ($p = 0.08$).

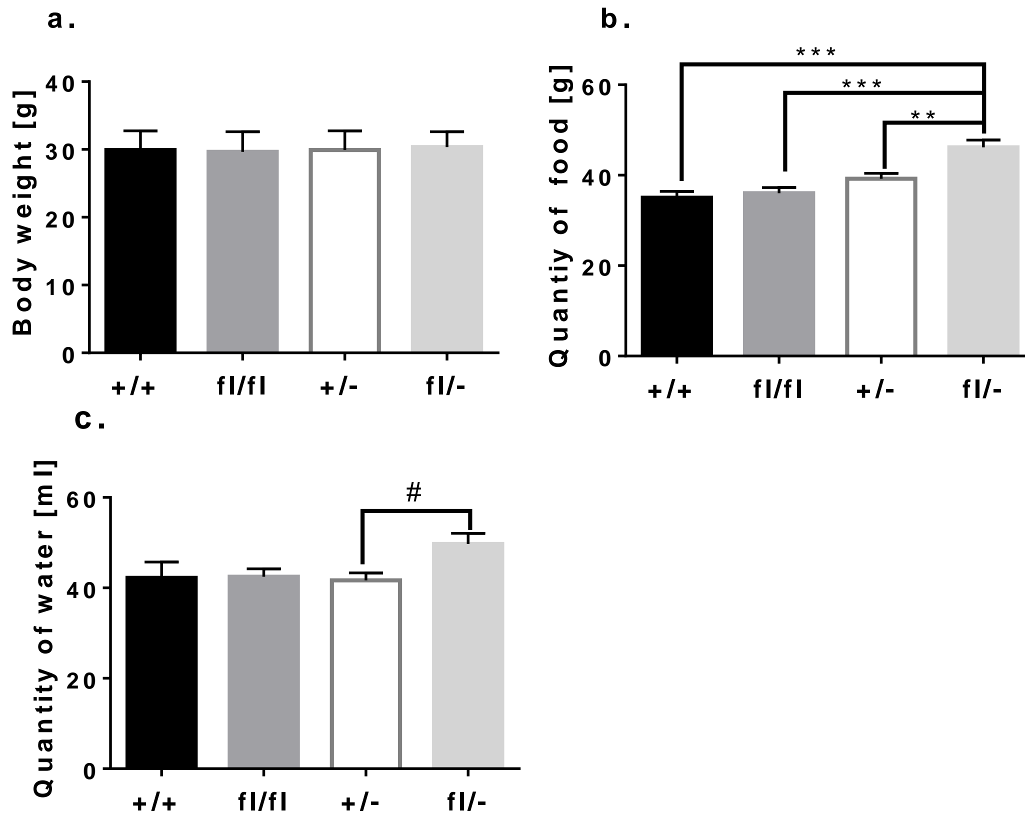

Figure 3-14: Effects of brain 5-HT reduction on metabolism. Change in (a) body weight over 7 weeks. Quantity of (b) food and (c) water consumption by mice. Data represented as mean ± SEM; Number of mice per group; *Tph2*$^{+/+}$ = 18, *Tph2*$^{fl/fl}$ = 21, *Tph2*$^{+/-}$ = 18, *Tph2*$^{fl/-}$ = 25. One-way ANOVA (*) indicate significant differences (**p<0.01, ***p<0.001, #p: 0.05≤p<0.1).

3.3 Effects of maternal separation and later life 5-HT depletion on anxiety-like and exploratory behavior

Elevated-plus maze: The effect of adult brain 5-HT depletion and MS on locomotor and anxiety like behavior was tested in Tam-treated *Tph2$^{fl/fl}$* (*Tph2icKO*) and Veh-treated *Tph2$^{fl/fl}$* (*Tph2CON*) male and female mice. Mice were tested first in the EPM. In male mice, Two-way ANOVA revealed no significant MS x treatment interaction effect in the overall distance covered ($F_{(1,32)}$ = 0.2576; p = 0.6153) however, mice which were subjected to MS cover less distance than Non MS ($F_{(1,32)}$ = 13.34; p = 0.0009) and *Tph2icKO* mice covered longer distance than *Tph2CON* mice ($F_{(1,32)}$ = 5.459; p = 0.0259), irrespective of early-life stress exposure (Figure 3-15 e). There was no observed differences in the number of times mice visited the open arms as well as time spent in the arms of the EPM (Figure 3-15 a&c).

Similarly, in female mice Two-way ANOVA indicated no significant interaction effect of MS x treatment in the overall distance covered ($F_{(1,30)}$ = 0.5191; p = 0.4768) however, MS mice cover less distance than Non MS ($F_{(1,30)}$ = 4.648; p = 0.03922) and *Tph2icKO* mice covered less distance compared with Veh-treated mice ($F_{(1,30)}$ = 4.499; p = 0.0423), irrespective of early-life stress exposure (Figure 3-15 f). With respect to the number of visits to the open arms of the EPM, no significant interaction effect of MS x treatment was observed ($F_{(1,30)}$ = 0.01847; p = 0.8928) but *Tph2icKO* mice ventured into the open arms less often than *Tph2CON* mice ($F_{(1,30)}$ = 5.708; p = 0.0234), irrespective of early-life stress exposure (Figure 3-15 b). Total time spent by female mice in the open arms (Figure 3-15 d) was comparatively similar ($F_{(1,30)}$ = 0.5838; p = 0.4506).

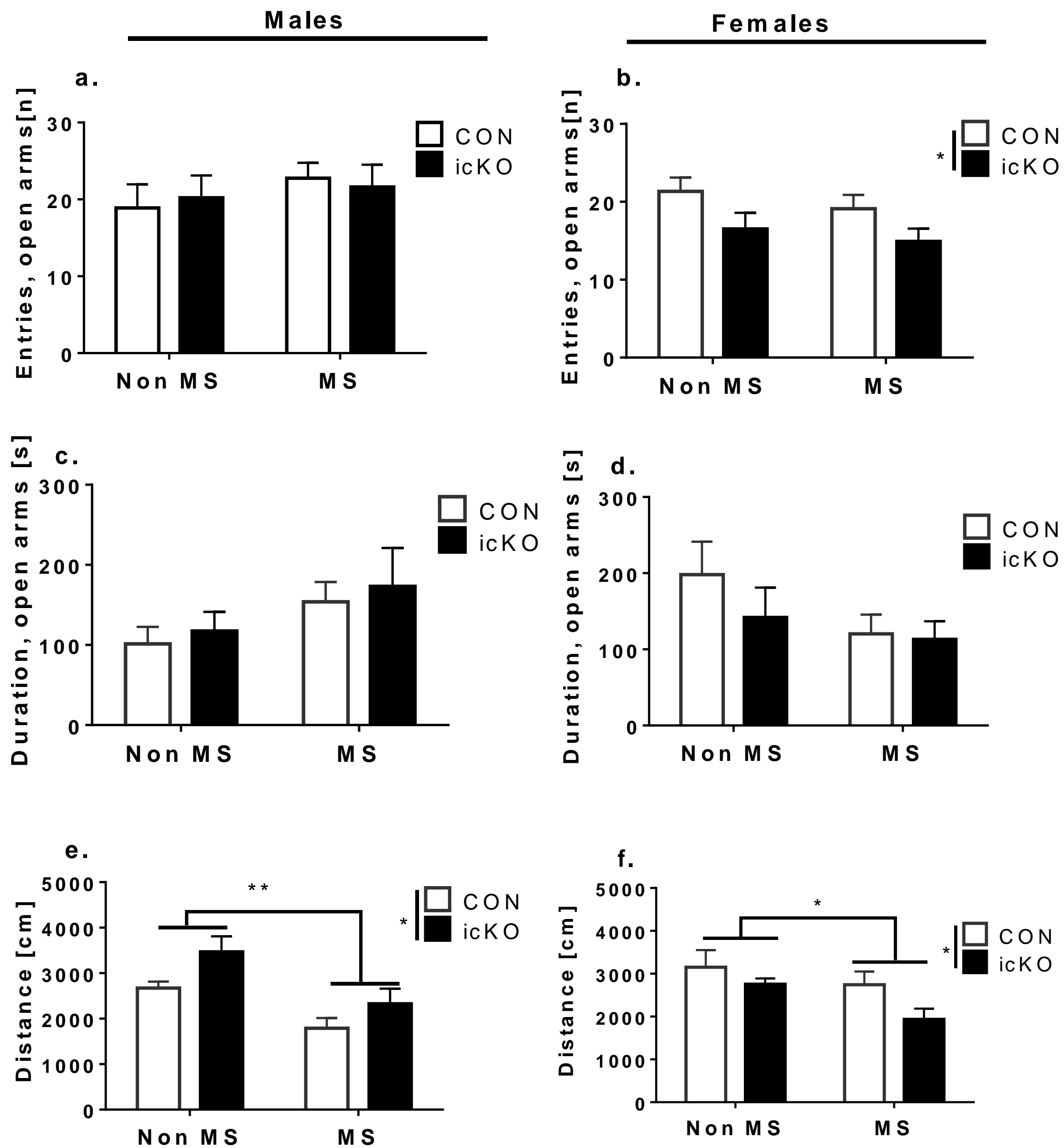

Figure 3-15: Exploratory and anxiety-related behavior of mice in EPM. Measures of anxiety in male (left column) and female (right column) mice, (a&b) number of visits to open arms and (c&d) cumulative time spent in open arms. Exploratory behavior (e&f) was low in MS exposed mice. Data represented as mean ± SEM. Number of mice per group; Male mice, **Non MS**: *Tph2icKO* = 9, *Tph2CON* = 9; **MS**: *Tph2icKO* = 10, *Tph2CON* = 8; Female mice, **Non MS**: *Tph2icKO* = 8, *Tph2CON* = 6; **MS**: *Tph2icKO* = 10, *Tph2CON* = 10. Two-Way ANOVA (*) indicate significant differences (*p<0.05, **p<0.01).

Light-dark-box: In the LDB, Two-way ANOVA showed no obvious significant MS x treatment interaction effect in overall distance $F_{(1,32)} = 0.6689$; $p = 0.4195$) in male mice, however, Non MS mice covered longer distance than MS exposed mice ($F_{(1,32)} = 12.49$; $p = 0.0013$) and *Tph2icKO* mice were more active than *Tph2CON* mice ($F_{(1,32)} = 4.672$; $p = 0.0382$), irrespective of early-life stress exposure (Figure 3-16 e). The number of visits and time spent in the aversive light compartment was comparable and no significant differences were observed between groups of mice (Figure 3-16 a&c).

Examination of activities of female mice in the LDB showed no significant interaction effect MS x treatment, but Non MS mice exhibited increased locomotor activity compared with MS mice ($F_{(1,29)} = 10.63$; $p = 0.0028$). Independent of early-life stress exposure, *Tph2CON* mice were very active and covered longer distance than *Tph2icKO* mice ($F_{(1,29)} = 47.309$; $p = 0.0114$; Figure 3-16 f). There was a significant main effect of treatment on the number visits to light box (Figure 3-16 b) and time spent there (Figure 3-16 d). *Tph2CON* mice among the Non MS and MS groups significantly visited ($F_{(1,29)} = 5.072$; $p = 0.0321$) and spent more time ($F_{(1,29)} = 7.618$; $p = 0.0099$) than *Tph2icKO* mice.

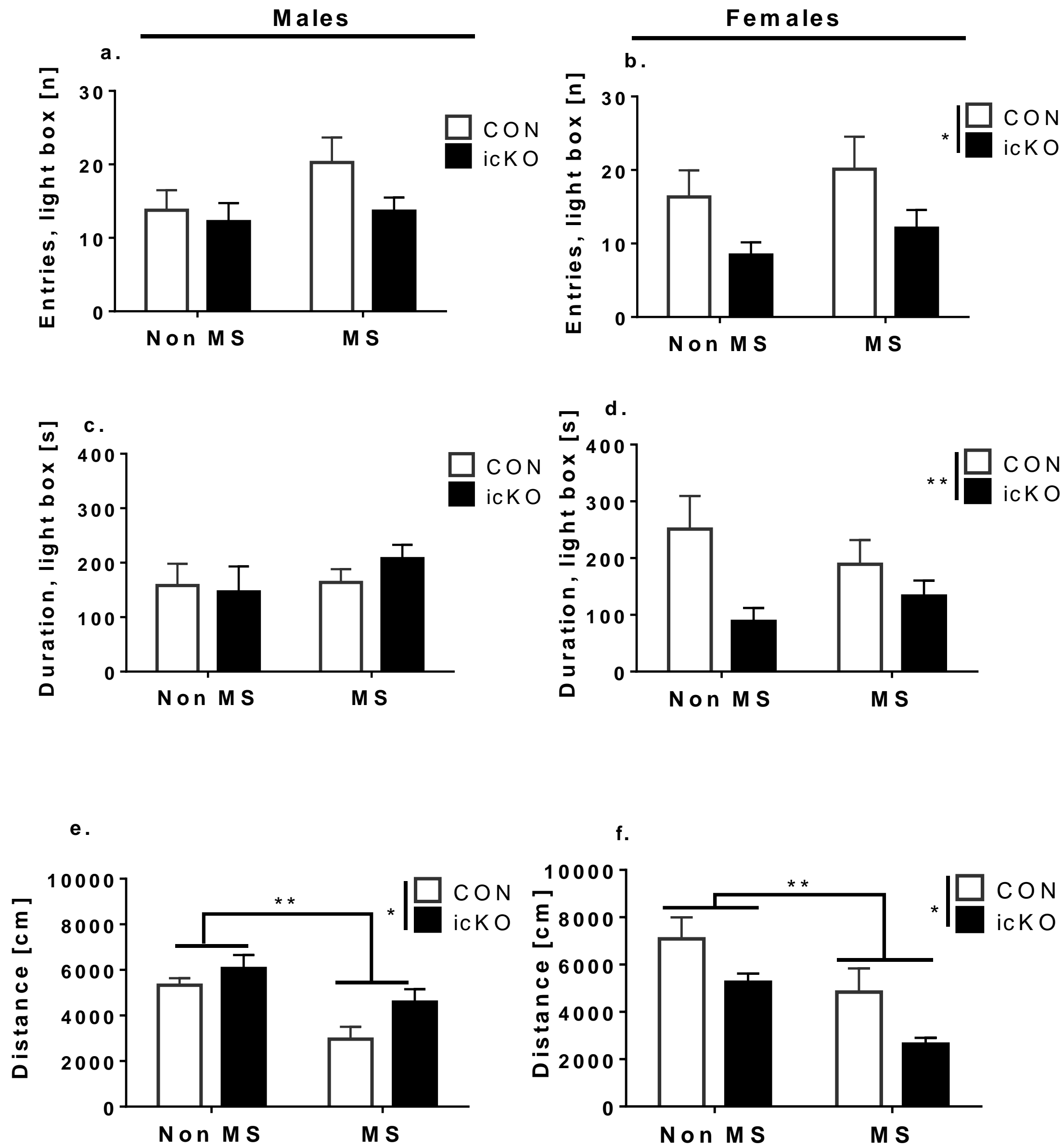

Figure 3-16: Exploratory and anxiety-related behavior of mice in LDB. Measures of anxiety in male (left column) and female (right column) mice, (a&b) number of visits to light compartment and (c&d) cumulative time spent in light compartment. Exploratory behavior (e&f) was low in MS exposed mice. Data represented as mean ± SEM. Number of mice per group; Male mice, **Non MS**: *Tph2icKO* = 9, *Tph2CON* = 9; **MS**: *Tph2icKO* = 10, *Tph2CON* = 8; Female mice, **Non MS:** *Tph2icKO* = 8, *Tph2CON* = 6; **MS**: *Tph2icKO* = 10, *Tph2CON* = 10. Two-way ANOVA (*) indicate significant differences (*$p<0.05$, **$p< 0.01$).

Open-field test: In the OFT, there was no significant interaction effect of MS x treatment on the overall locomotor activity in both male ($F_{(1,32)} = 1.200$; $p = 0.2816$) and female ($F_{(1,29)} = 0.2931$; $p = 0.5924$) mice. In males, there was no significant interaction of MS x treatment ($F_{(1,32)} = 1.387$; $p = 0.2476$) but a tendency in main effect of MS ($F_{(1,32)} = 3.039$; $p = 0.09$) on the activity in the center of the open field (Figure 3-17 e). Considering the total time spent in the center, a significant MS x treatment interaction effect ($F_{(1, 32)} = 6.562$; $p = 0.015$) was observed (Figure 3-17c), indicating that an icKO in males rescued the reduced time MS exposure mice spent in the center although the effect was very weak such that Tukey's multiple comparisons test indicated only a trend between Non MS *Tph2icKO* and MS *Tph2icKO* mice ($p = 0.08$).

In contrast to males, observation of total distance cover in the center of the open field in female mice revealed a significant interaction effect of MS x treatment in female mice ($F_{(1,29)} = 5.629$; $p = 0.0245$; Figure 3-17 f). MS exposure resulted in increased center activity (i.e. reduced anxiety) in *Tph2CON* mice compared with MS exposed *Tph2icKO* ($p = 0.0034$) as well as Non MS *Tph2CON* ($p = 0.0235$) and Non MS *Tph2icKO* ($p = 0.0083$). Thus inducible inactivation of *Tph2* blocked increased center activity in MS exposed female mice. Again, no significant interaction effect in the number of times female mice visited the center and time spent there was observed (Figure 3-17 b&d).

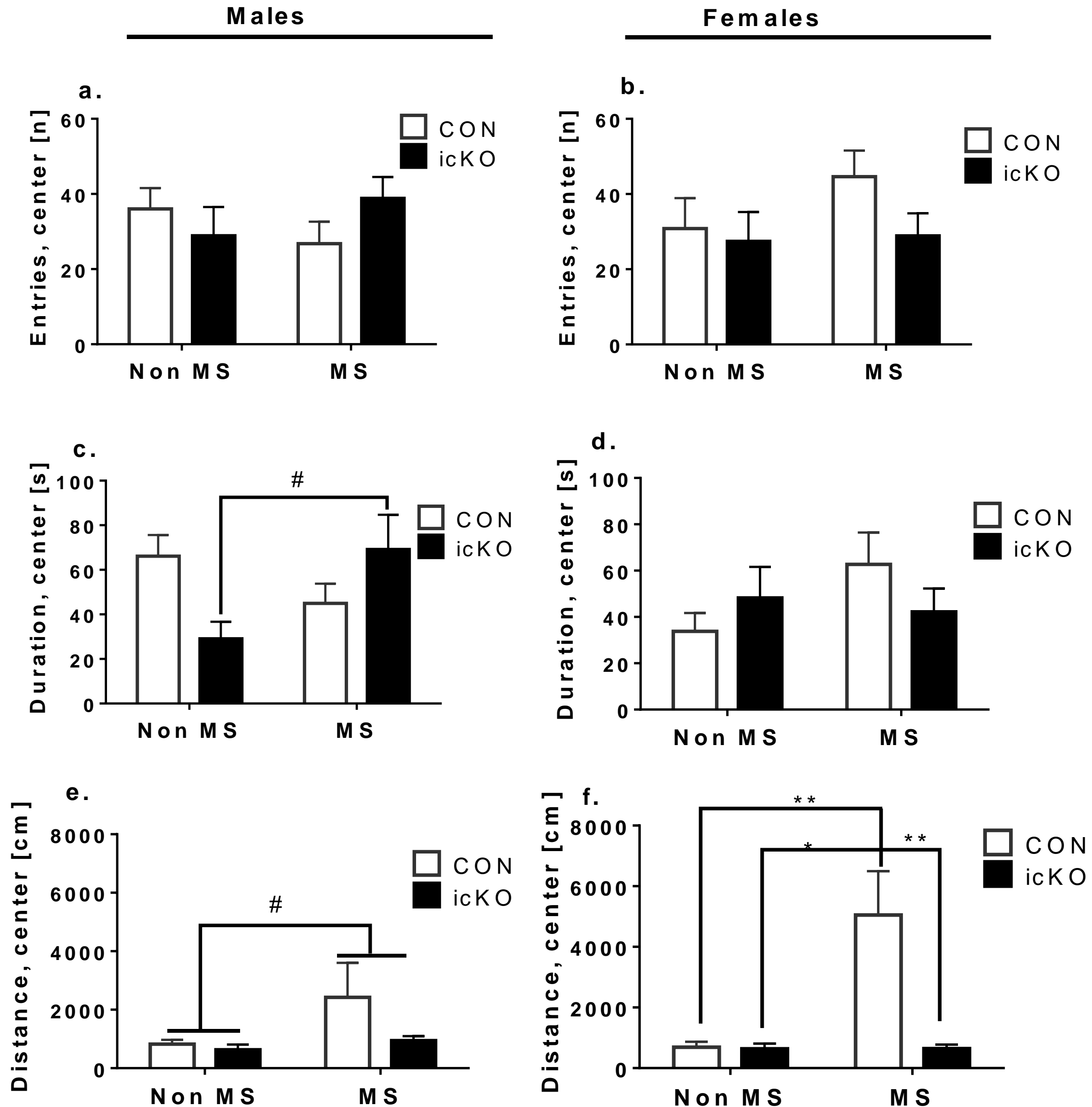

Figure 3-17: Exploratory and anxiety-related behavior of mice in OFT. Measures of anxiety in male (left column) mice and female (right column) mice, (a&b) number of visits to center and (c&d) cumulative time spent in center of OFT. Exploratory behavior (e&f) was high in MS exposed mice. Data represented as mean ± SEM. Number of mice per group; Male mice, **Non MS**: *Tph2icKO* = 9, *Tph2CON* = 9; **MS**: *Tph2icKO* = 10, *Tph2CON* = 8; Female mice, **Non MS:** *Tph2icKO* = 8, *Tph2CON* = 6; **MS**: *Tph2icKO* = 10, *Tph2CON* = 10. Two-way ANOVA (*) indicate significant differences (*$p<0.05$, **$p<0.01$, $^{\#}p$: $0.05 \leq p<0.1$).

3.3.1 Altered depression-like behavior and anhedonia in mice

Reduced brain 5-HT neurotransmission has been reported to predispose individuals to hedonic deficits and depression related disorders. In this study, mice were tested for preference for sucrose solution for 5 consecutive days. With respect to total fluid consumed, Two-way ANOVA indicated no significant effect of MS x treatment interactions, but, a significant treatment effect as *Tph2icKO* male ($F_{(1,31)} = 20.9$; $p < 0.0001$; Figure 3-18 a) and female ($F_{(1,28)} = 8.752$; $p = 0.0062$; Figure 3-18 b) mice consumed more fluid than *Tph2CON* mice in each sex group independent of aversive early-life experience. Again, Two-way ANOVA indicated no significant MS x treatment interactions in quantity of sucrose solution consumed by both male (Figure 3-18 c) and female (Figure 3-18 d) mice.

In the FST, both MS and Non-MS male mice put up comparative performance. No significant MS x treatment interactions in terms of latency (at first) to floating ($F_{(1,31)} = 0.3670$; $p = 0.5491$; Figure 3-19 a) and the duration of floating ($F_{(1,31)} = 1.702$; $p = 0.2017$; Figure 3-19 c). In female mice, MS and Non MS exhibited similar latency ($F_{(1,30)} = 2.043$; $p = 0.1632$; Figure 3-19 b) and duration of floating ($F_{(1,30)} = 0.3000$; $p = 0.5879$; Figure 3-19 d). These outcomes indicate that reduction in adult brain 5-HT level may not potentially predispose and individual to lack of pleasure that characterizes anhedonia and learned helplessness that characterizes depression.

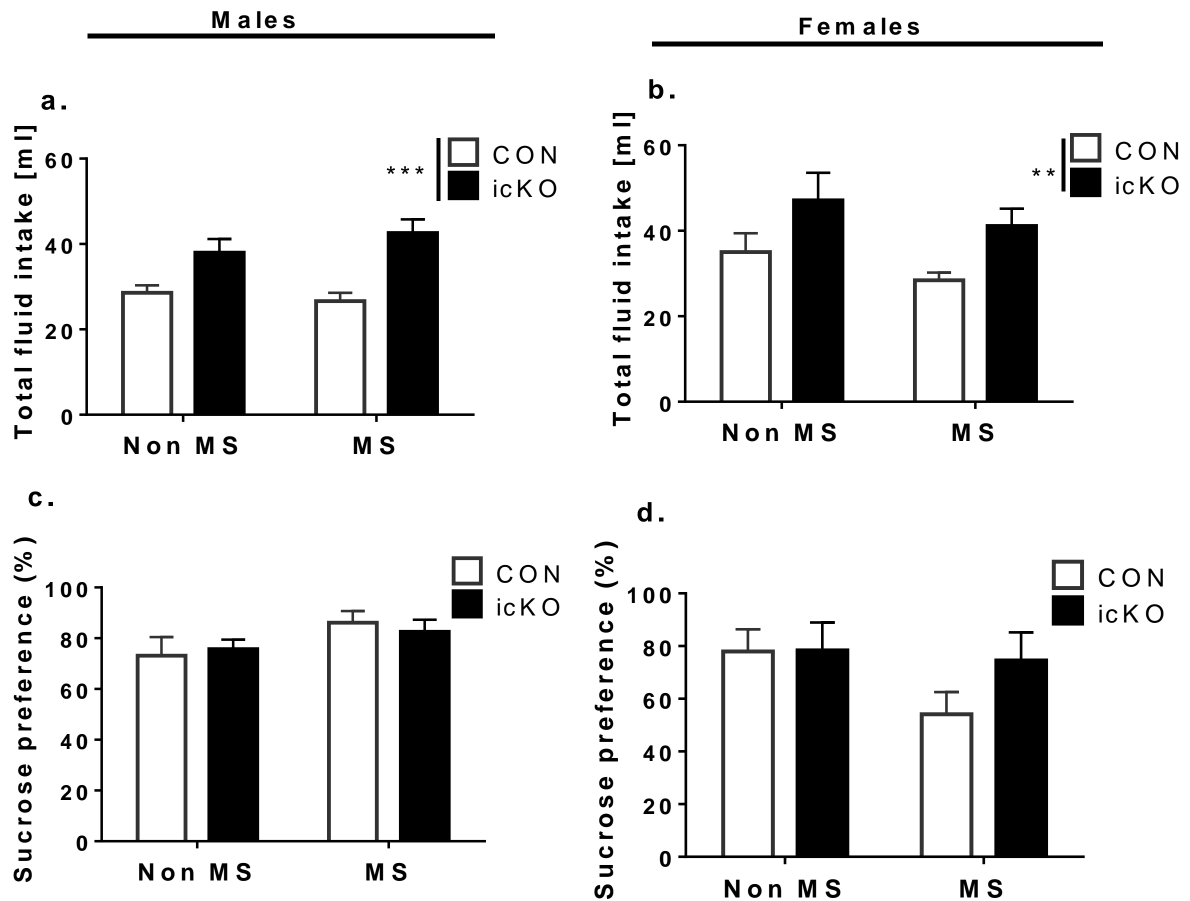

Figure 3-18: Sucrose preference in icKO mice. Measures of anhedonia in male (right column) and female (left column) mice. TAM-treated (a) male and (b) females consumed more fluid than Veh-treated mice. No obvious indication of anhedonia in (c) male and (d) female mice in the sucrose preference test. Data represented as mean ± SEM Number of mice per group; Male mice, **Non MS**: *Tph2icKO* = 9, *Tph2CON* = 9; **MS**: *Tph2icKO* = 10, *Tph2CON* = 8; Female mice, **Non MS**: *Tph2icKO* = 8, *Tph2CON* = 6; **MS**: *Tph2icKO* = 10, *Tph2CON* = 10. Two-way ANOVA, (*) indicate significant differences (**p<0.01, ***p<0.001).

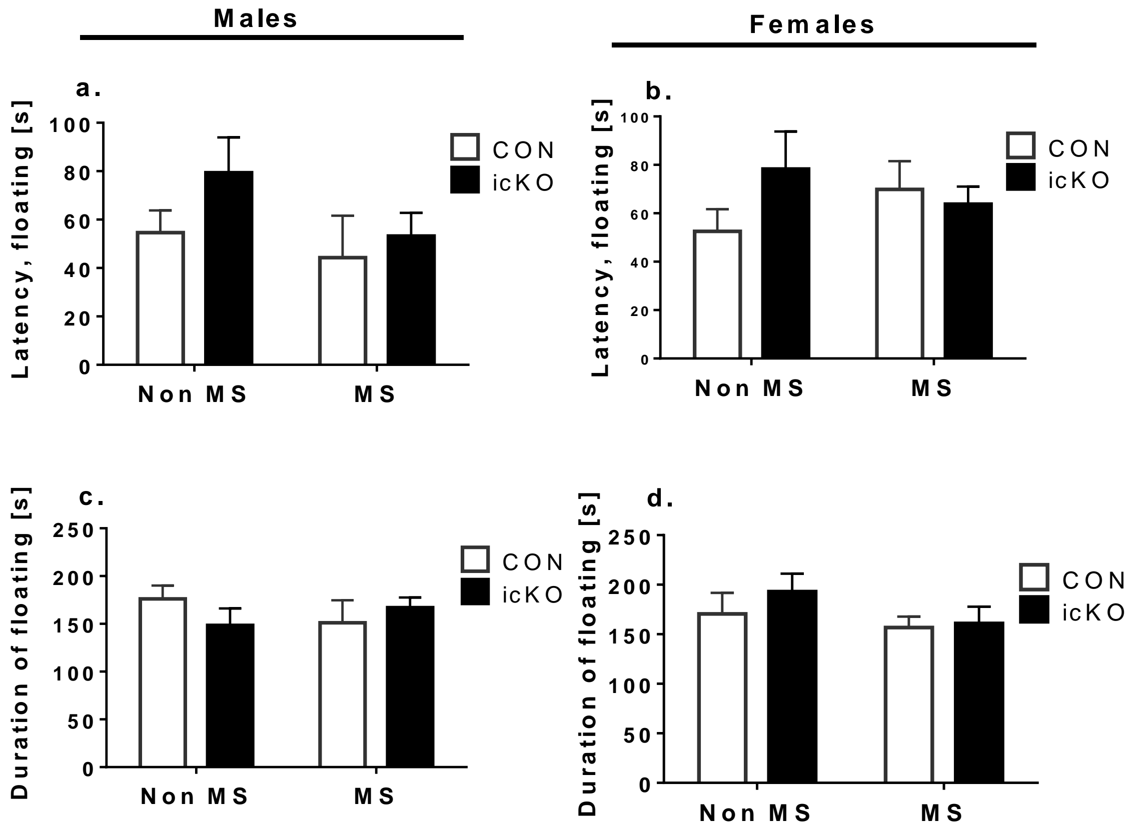

Figure 3-19: Performance of icKO mice in the FST. Measures of learned helplessness in male (right column) and female (left column) mice. No differences in (a&b) latency to floating and (b) duration of floating. Two-way ANOVA analysis. Data represented as mean ± SEM. Number of mice per group; Male mice, **Non MS**: *Tph2icKO* = 9, *Tph2CON* = 9; **MS**: *Tph2icKO* = 10, *Tph2CON* = 8; Female mice, **Non MS**: *Tph2icKO* = 8, *Tph2CON* = 6; **MS**: *Tph2icKO* = 10, *Tph2CON* = 10.

3.4 Gene expression study

To investigate the molecular mechanisms underlying the hyperlocomotor and altered anxiety-related behavior in novel environment, the expression of some selected genes in the raphe, hippocampus and amygdala was examined. The goal was to determine whether gene expression is influenced by MS (E), treatment/adult life 5-HT depletion (G) or both GxE interact to influence gene expression.

Tph2: In male mice, the relative levels of Tph2 mRNA in the raphe indicate no significant MS x treatment interactions effect ($F_{(1,31)} = 0.1104$; $p = 0.7419$; Figure 3-20 a). There was a significant main effect of treatment ($F_{(1,31)} = 30.05$; $p < 0.0001$) which resulted in significant reduction in relative levels of *Tph2* in *Tph2icKO* mice (MS and

Non MS mice) to a baseline minimal expression similar to the low expression level observed in hippocampus and amygdala. In the hippocampus, neither MS ($F_{(1,31)}$ = 1.45; p = 0.2377) nor Tam treatment ($F_{(1,31)}$ = 0.01329; p = 0.909) effects altered *Tph2* expression. Again, no main effect of MS x treatment interactions ($F_{(1,31)}$ = 0.08337; p = 0.7747) was observed (Figure 3-20 c). MS slightly altered the relative expression of *Tph2* in the amygdala (Figure 3-20 e). There was a trend in the effect of MS ($F_{(1,30)}$ = 3.542; p = 0.0696) on *Tph2* indicating potential effects of early-life adversity on 5-HT neurotransmission in amygdala. There was no significant MS x treatment interactions effect ($F_{(1,30)}$ = 1.179; p = 0.2863) nor main effect of treatment ($F_{(1,30)}$ = 2.68; p = 0.112).
Observation in female mice was similar to that in male mice. Tam treatment significantly reduced the levels of Tph2 mRNA in the raphe of MS and Non MS mice ($F_{(1,29)}$ = 48.39; $p < 0.0001$; Figure 3-20 b). In the hippocampus, neither main effect of MS x treatment interactions ($F_{(1,29)}$ = 2.734; p = 0.109) was observed (Figure 3-20 d) nor main effect of MS ($F_{(1,29)}$ = 0.00018; p = 0.989) and Tam induction ($F_{(1,29)}$ = 0.4937; p = 0.4879) effects on *Tph2* expression. The relative expression of *Tph2* in the amygdala was significantly reduced by early-life MS (Figure 3-20 f). There was a significant main effect of MS ($F_{(1,27)}$ = 5.726; p = 0.0239), but no effect of MS x treatment interactions ($F_{(1,27)}$ = 0.3178; p = 0.5439) or treatment ($F_{(1,27)}$ = 0.1356; p = 0.7156) effects was observed.

Htr1a: The relative expression of 5-HT receptor 1a gene (*Htr1a*) was assessed in the brain of mice. In both male (Figure 3-21 c&d) and female (Figure 3-21 d&f) mice, there was no difference in the relative expression of the gene in the hippocampus and amygdala. In the raphe, no significant MS x treatment interactions effect ($F_{(1,31)}$ = 2.269; p = 0.1421), but a significant main effect of treatment ($F_{(1,31)}$ = 4.345; p = 0.0456) and a trend in effect of MS ($F_{(1,31)}$ = 3.597; p = 0.0672) was observed in male mice (Figure 3-21 a).

Tam treatment resulted in reduced expression of *Htr1a* in raphe of female mice (Figure 3-21b). There was neither significant MS x treatment interactions effect ($F_{(1,29)}$ = 1.845; p = 0.1848) nor main effect of MS ($F_{(1,29)}$ = 1.512; p = 0.2288), but a main effect of treatment ($F_{(1,29)}$ = 8.075; p = 0.0081). This indicates reduction in adult brain 5-HT levels can alter expression of *Htr1a* in both males and females.

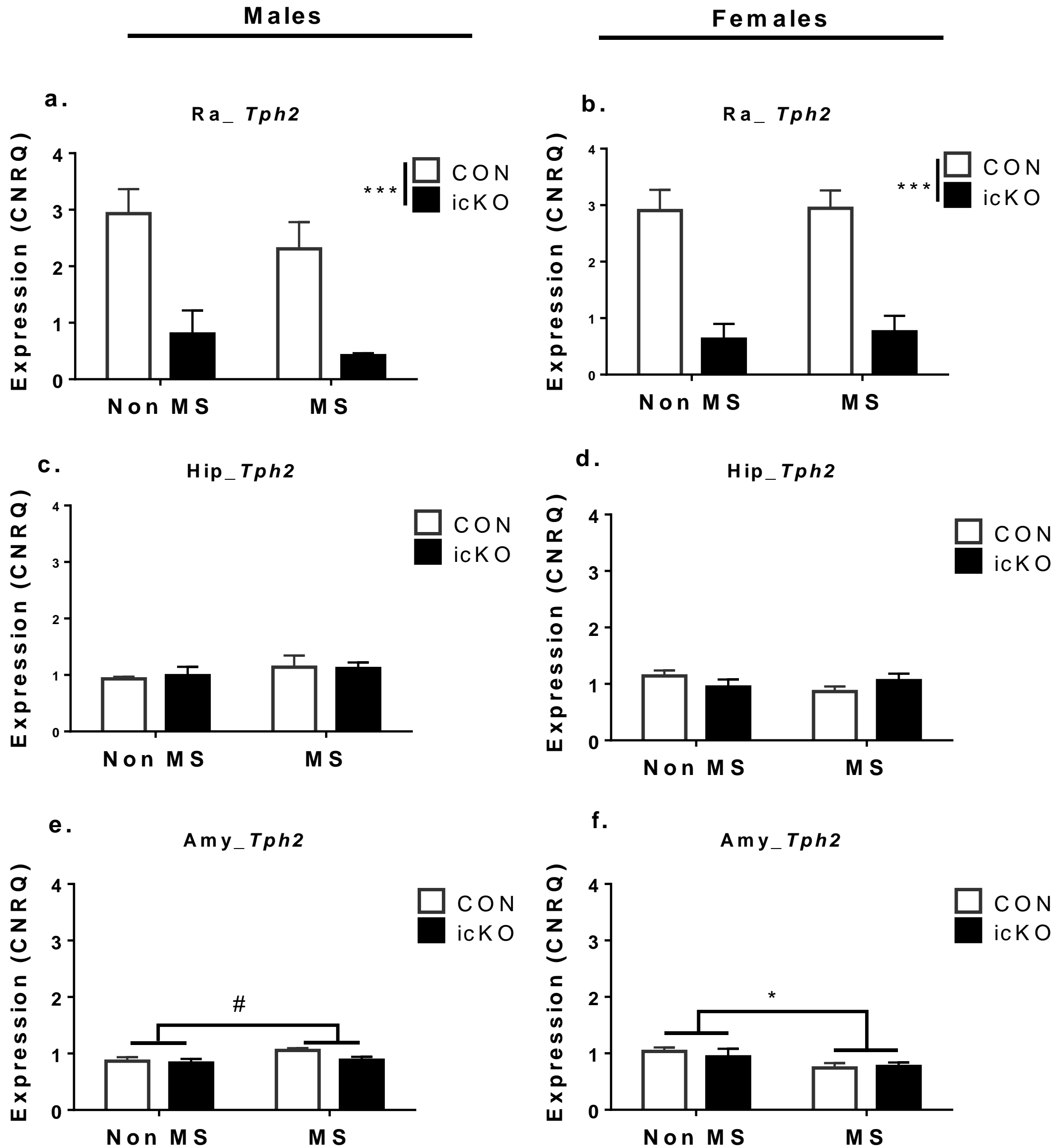

Figure 3- 20: Relative expression of *Tph2* in mouse brain. qRT-PCR analysis of *Tph2* expression in (a&b) raphe; (c&d) hippocampus and (e&f) amygdala of male (left column) and female (right column) mice. Number of mice per group; Male mice (Ra & Hip), **Non MS**: *Tph2icKO* = 9, *Tph2CON* = 9; **MS**: *Tph2icKO* = 10, *Tph2CON* = 8; Female mice (Ra & Hip), **Non MS:** *Tph2icKO* = 7, *Tph2CON* = 6; **MS**: *Tph2icKO* = 10, *Tph2CON* = 10. Male mice (Amy), **Non MS**: *Tph2icKO*=9, *Tph2CON*= 8; MS: *Tph2icKO* = 9, *Tph2CON* = 8; Female mice (Amy), **Non MS**: *Tph2icKO* = 6, *Tph2CON* = 6; M**S**: *Tph2icKO* = 9, *Tph2CON* = 9. Ra = raphe, Hip = hippocampus, Amy = amygdala. Data represented as mean ± SEM. Two-way ANOVA, (*) indicate significant differences ($^{\#}$p: 0.05≤p<0.1, *p<0.05, ***p<0.001).

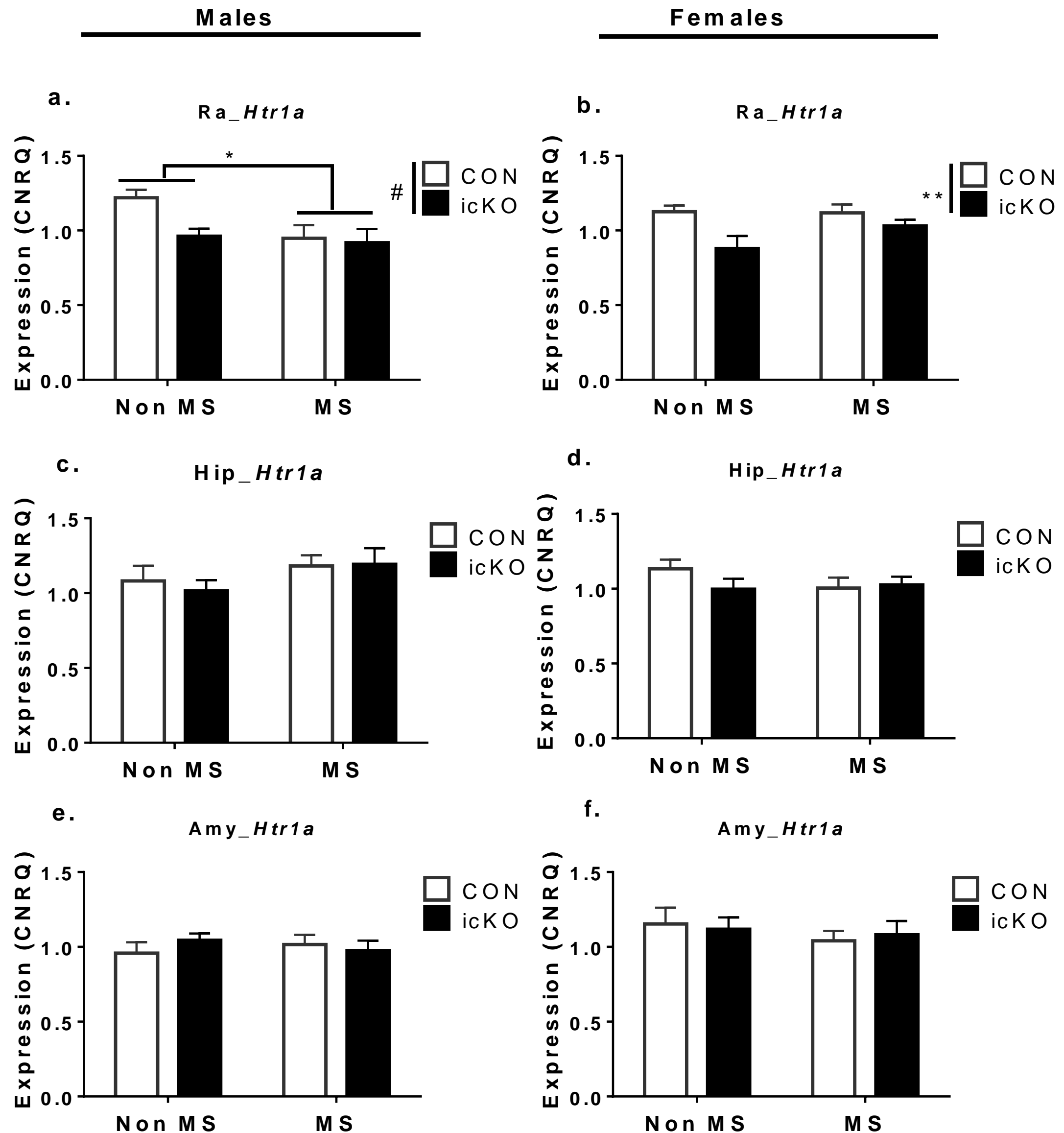

Figure 3-21: Relative expression of *Htr1a* in mouse brain. qRT-PCR analysis of *Htr1a* expression in (a&b) raphe; (c&d) hippocampus and (e&f) amygdala of male (left column) and female (right column) mice. Number of mice per group; Male mice (Ra & Hip), **Non MS**: *Tph2icKO* = 9, *Tph2CON* = 9; **MS**: *Tph2icKO* = 10, *Tph2CON* = 8; Female mice (Ra & Hip), **Non MS:** *Tph2icKO* = 7, *Tph2CON* = 6; **MS**: *Tph2icKO* = 10, *Tph2CON* = 10. Male mice (Amy), **Non MS**: *Tph2icKO* = 9, *Tph2CON* = 8; **MS**: *Tph2icKO* = 9, *Tph2CON* = 8; Female mice (Amy), **Non MS**: *Tph2icKO* = 6, *Tph2CON* = 6; M**S**: *Tph2icKO* = 9, *Tph2CON* = 9. Ra = raphe, Hip = hippocampus, Amy = amygdala. Data represented as mean ± SEM. Two-way ANOVA, (*) indicate significant differences ($^{\#}$p: 0.05≤p<0.1, *p<0.05, **p<0.01*).*

Htr2a: Relative mRNA expression of 5-HT receptor 2a genes (*Htr2a*) in male mice revealed no significant differences in the hippocampus (Figure 3-22 c) and amygdala (Figure 3-22 d). In the raphe, MS and Tam treatment, independent of each other, altered *Htr2a* expression (Figure 3-22 a). There was no significant MS x treatment interactions effect ($F_{(1,31)} = 0.3917$; $p = 0.536$), however, a significant main effect of MS ($F_{(1,31)} = 7.599$; $p = 0.0097$) and trend in effect of treatment ($F_{(1,31)} = 3.328$; $p = 0.0778$) was recorded.

A completely different pattern of expression of 5-HT receptor 2a was seen in female mice. In the raphe (Figure 3-22 b) and amygdala (Figure 3-22 f), the level of expression of the gene was comparatively similar between MS and Non MS mice irrespective of treatment. In the hippocampus, a significant main MS x treatment interactions effect ($F_{(1,29)} = 4.456$; $p = 0.0435$) was observed but post hoc analysis showed a weak effect between MS mice (Figure 3-22 d).

Maoa: Assessment of expression levels of *Maoa* in the raphe of male mice revealed a general effect of Tam treatment (Figure 3-23 a). No significant main MS x treatment interactions effect ($F_{(1,31)} = 1.852$; $p = 0.1833$), but a significant main effect of treatment ($F_{(1,31)}=13.39$; $p=0.0009$) was apparent. *Tph2icKO* mice in both MS and Non MS groups express lower levels of the gene. In the hippocampus, no significant main MS x treatment interactions effect ($F_{(1,31)} = 1.852$; $p = 0.1833$), but a significant main effect of MS ($F_{(1,31)} = 4.399$; $p = 0.0442$) was observed (Figure 3-23 c).
In female mice, the level of expression of *Maoa* in the hippocampus (Figure 3-23 d) and amygdala (Figure 3-23 f) did not differ between groups. In the raphe, a significant main MS x treatment interactions effect ($F_{(1,29)} = 8.79$; $p = 0.006$), and a significant main effect of MS ($F_{(1,29)} = 4.408$; $p = 0.0446$) was observed (Figure 3-23 a). Post hoc analysis revealed a significant difference between *Tph2icKO* mice in MS and Non MS groups ($p = 0.0051$).

Avpr1a: Arginine vasopressin receptor 1a receptor is reported to influence anxiety and aggression in humans and rodents. We investigated the expression of mRNA of *Avpr1a* in mice after they have been tested for anxiety-related behavior. In male mice, there was a significant main MS x treatment interaction ($F_{(1,31)} = 5.44$; $p = 0.0263$), and a significant main effect of treatment ($F_{(1,31)} = 4.807$; $p = 0.036$) was observed (Figure3-24 a). Post hoc analysis revealed *Avpr1a* expression in MS

exposed *Tph2CON* was significantly higher than MS exposed *Tph2icKO* mice ($p = 0.017$). In the hippocampus (Figure 3-24 c) and amygdala (Figure 3-24 e), no significant MS x treatment interactions effect ($F_{(1,31)} = 1.788$; $p = 0.197$) and ($F_{(1,29)} = 0.02332$; $p = 0.88$) respectively. All other statistics did not differ between MS and Non MS mice.

In female mice, no significant MS x treatment interactions effect ($F_{(1,29)} = 0.07936$; $p = 0.7802$; Figure 3-24 b) in the raphe. Again, the level of *Avpr1a* expression in the hippocampus showed no significant MS x treatment interactions effect ($F_{(1,29)} = 0.065$; $p = 0.8003$; Figure 3-24 d). In the amygdala a tendency towards significant MS x treatment interactions effect ($F_{(1,26)} = 0.3.286$; $p = 0.081$; Figure 3-24e) was observed.

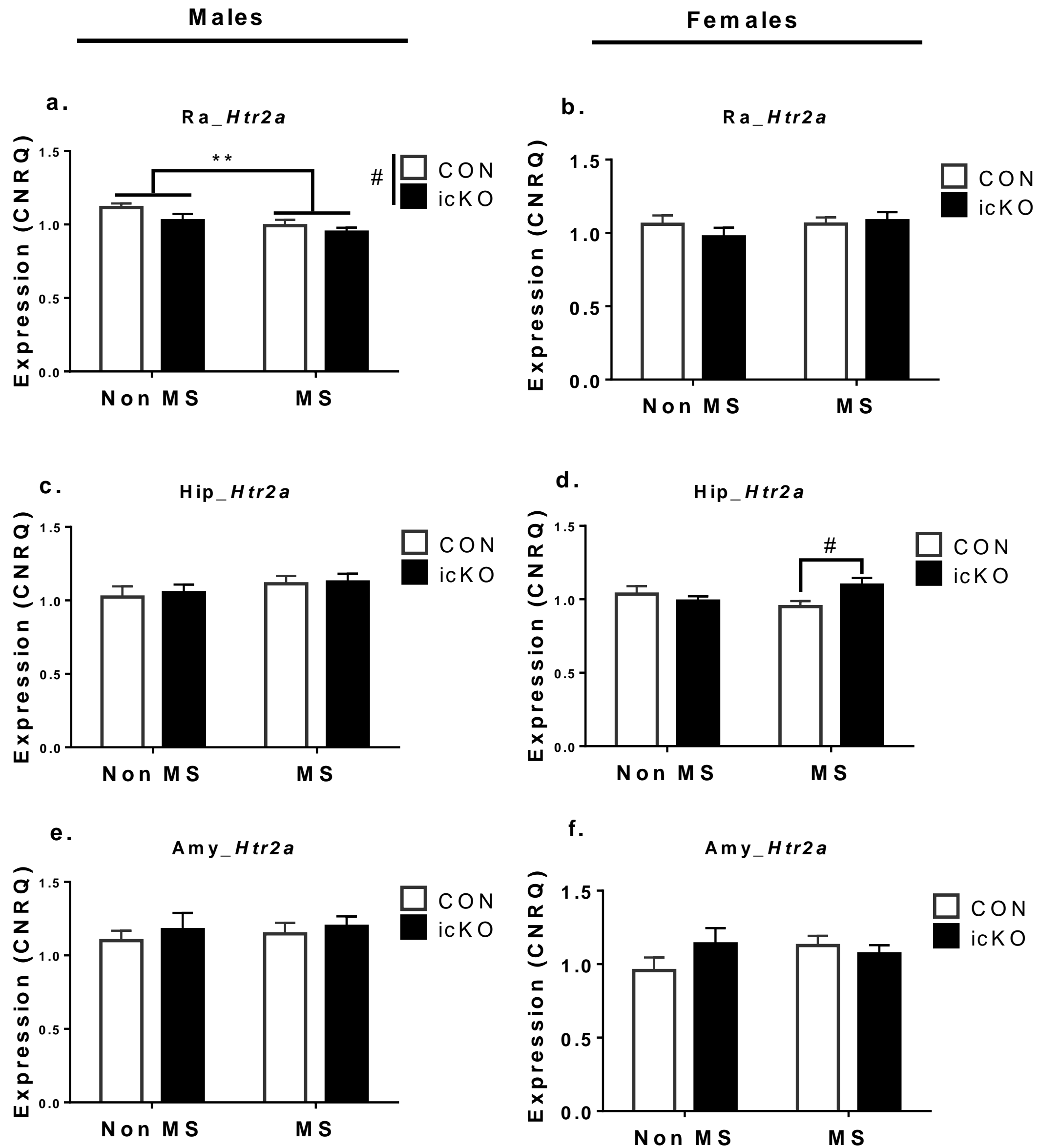

Figure 3-22: Relative expression of *Htr2a* in mouse brain. qRT-PCR analysis of *Htr2a* expression in (a&b) raphe; (c&d) hippocampus and (e&f) amygdala of male (left column) and female (right column) mice. Number of mice per group; Male mice (Ra & Hip), **Non MS**: *Tph2icKO* = 9, *Tph2CON* = 9; **MS**: *Tph2icKO* = 10, *Tph2CON* = 8; Female mice (Ra & Hip), **Non MS:** *Tph2icKO* = 7, *Tph2CON* = 6; **MS**: *Tph2icKO* = 10, *Tph2CON* = 10. Male mice (Amy), **Non MS**: *Tph2icKO* = 9, *Tph2CON* = 8; **MS**: *Tph2icKO* = 9, *Tph2CON* = 8; Female mice (Amy), **Non MS**: *Tph2icKO* = 6, *Tph2CON* = 6; M**S**: *Tph2icKO* = 9, *Tph2CON* = 9. Ra = raphe, Hip = hippocampus, Amy = amygdala. Data represented as mean ± SEM. Two-way ANOVA, (*) indicate significant differences ($^{\#}p$: $0.05 \le p < 0.1$, $^{*}p < 0.05$, $^{**}p < 0.01$).

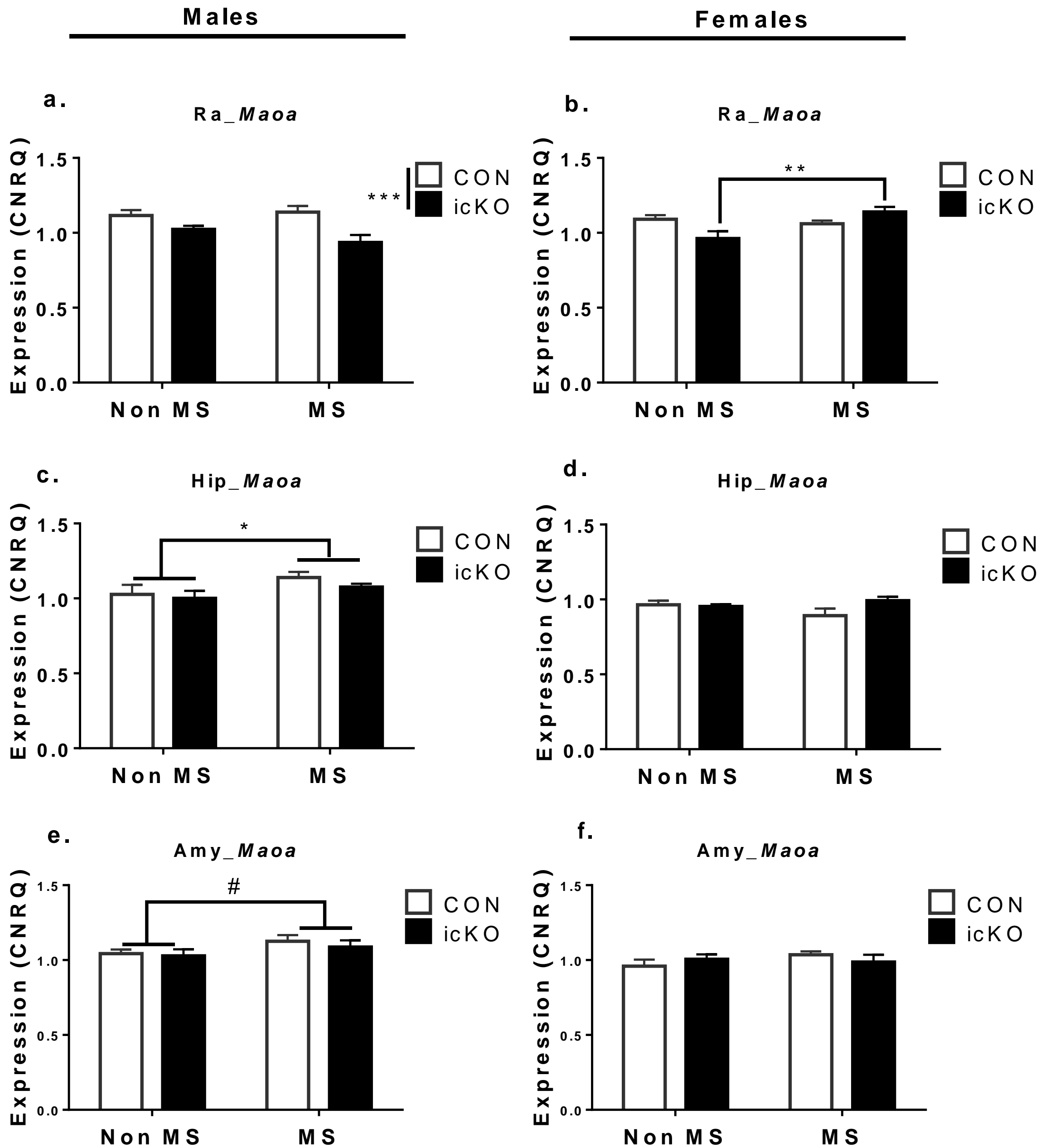

Figure 3-23: Relative expression of *Maoa* in mouse brain. qRT-PCR analysis of *Maoa* expression in (a&b) raphe; (c&d) hippocampus and (e&f) amygdala of male (left column) and female (right column) mice. Number of mice per group; Male mice (Ra & Hip), **Non MS**: *Tph2icKO* = 9, *Tph2CON* = 9; **MS**: *Tph2icKO* = 10, *Tph2CON* = 8; Female mice (Ra & Hip), **Non MS:** *Tph2icKO* = 7, *Tph2CON* = 6; **MS**: *Tph2icKO* = 10, *Tph2CON* = 10. Male mice (Amy), **Non MS**: *Tph2icKO* = 9, *Tph2CON* = 8; **MS**: *Tph2icKO* = 9, *Tph2CON* = 8; Female mice (Amy), **Non MS**: *Tph2icKO* = 6, *Tph2CON* = 6; M**S**: *Tph2icKO* = 9, *Tph2CON* = 9. Ra = raphe, Hip = Hippocampus, Amy = Amygdala. Data represented as mean ± SEM. Two-way ANOVA, (*) indicate significant differences ($^{\#}$p: 0.05≤p<0.1, *p<0.05, **p<0.01).

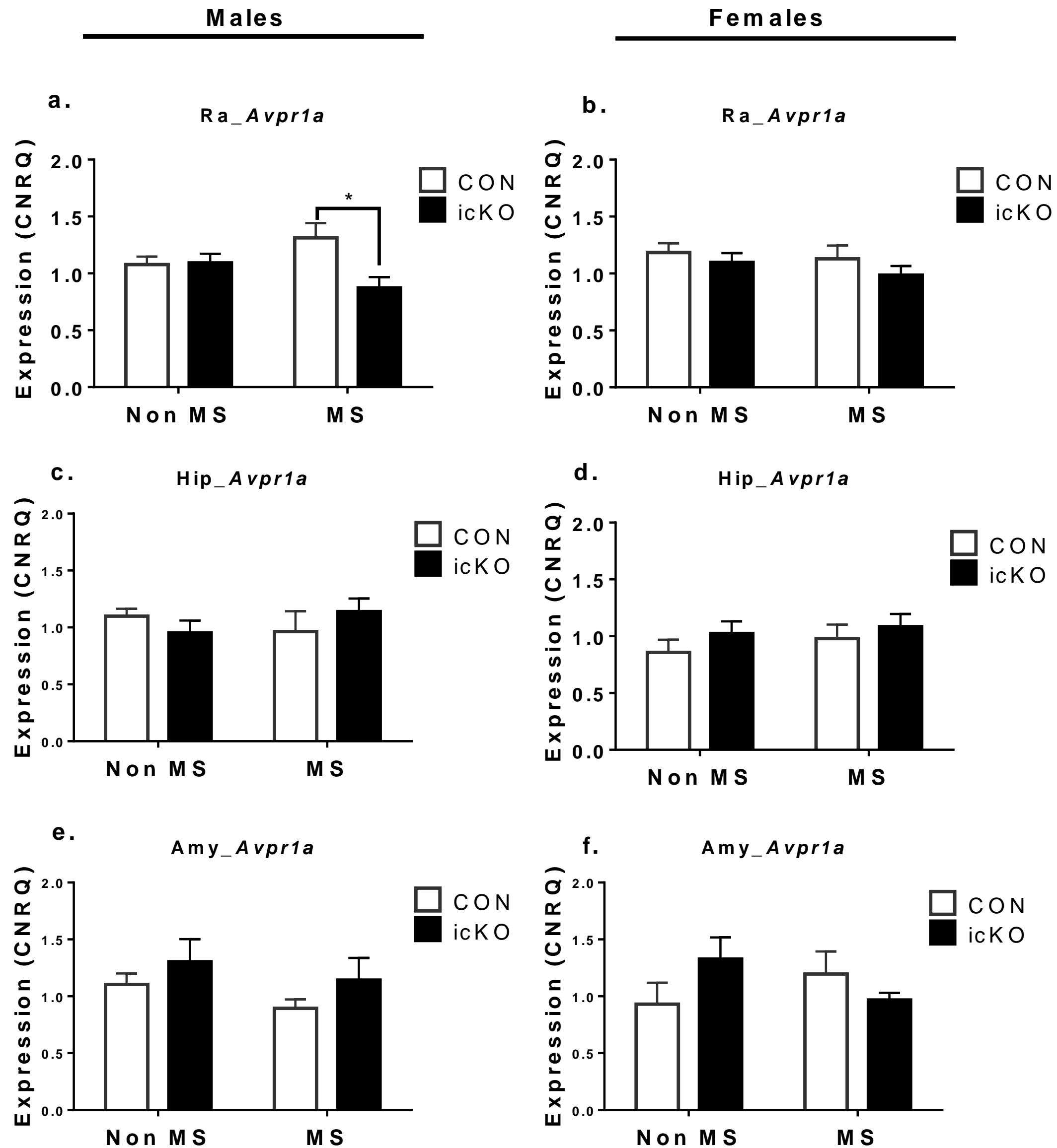

Figure 3-24: Relative expression of *Avpr1a* in mouse brain. qRT-PCR analysis of *Avpr1a* expression in (a&b) raphe; (c&d) hippocampus and (e&f) amygdala of male (left column) and female (right column) mice. Number of mice per group; Male mice (Ra & Hip), **Non MS**: *Tph2icKO* = 9, *Tph2CON* = 9; **MS**: *Tph2icKO* = 10, *Tph2CON* = 8; Female mice (Ra & Hip), **Non MS:** *Tph2icKO* = 7, *Tph2CON* = 6; **MS**: *Tph2icKO* = 10, *Tph2CON* = 10. Male mice (Amy), **Non MS**: *Tph2icKO* = 9, *Tph2CON* = 8; **MS**: *Tph2icKO* = 8, *Tph2CON* = 8; Female mice (Amy), **Non MS**: *Tph2icKO* = 7, *Tph2CON* = 5; M**S**: *Tph2icKO* = 8, *Tph2CON* = 9. Ra = raphe, Hip = hippocampus, Amy = amygdala. Data represented as mean ± SEM. Two-way ANOVA, (*) indicate significant differences ($^{\#}$p:0.05≤p<0.1, *p<0.05, **p<0.01)

4 Discussion

4.1 Impact of induced *Tph2* inactivation on monoamine systems

The first part of this study capitalized on the Tam-inducible Cre-mediated inactivation of *Tph2* to significantly reduce 5-HT synthesis in adult mouse brain. An induction protocol which gives efficient outcome (Leone et al. 2003; Mori et al. 2006) was adopted and this resulted in up to 96% reduction in number of brain 5-HT ir cells in anterior raphe nuclei with only few patches of 5-HT ir cells remaining especially in the dorsolateral wing of the DR. One possible explanation could be ineffective nuclear translocation of CreERT2 in some cells leading to incomplete recombination of *Tph2* (Weber et al. 2011). Again, brain 5-HT neurons are of different genetic lineage, heterogeneous and have subtype-specific functions (Bang et al. 2012; Okaty et al. 2015) which makes it difficult manipulating them all at the same time.

Direct measurement of monoamines concentrations was determined by using HPLC on samples from four different brain regions. In the 2nd week, 5-HIAA levels were significantly low but 5-HT levels remained unchanged in *Tph2icKO* mice. Significant differences in the level of 5-HT occurred from 4 weeks onward after Tam treatment. This appears to be an immediate adaptive neurochemical activity towards retention of 5-HT in the brain regions in response to reduction in the 5-HT synthesis. Significant reduction of 5-HT and 5-HIAA within 4 weeks of Tam treatment has been reported, (Song et al. 2016) indicating that Tam inducible approach requires a relatively longer time before significant differences could be detected by HPLC. This present finding contradicts an earlier report (Whitney et al. 2016) which achieved dramatic reduction in adult brain 5-HT and 5-HIAA concentration within 2 weeks after treatment. Since the authors used young mice and measured 5-HT concentration in whole brain, it would be difficult to compare this data with their work. Notwithstanding this, the induction protocol used could be the possible explanation for differences in the observations above. The authors used adeno-associated virus (AAV) which delivers Cre directly to neurons specifically in the raphe of adult mouse brain (Ahmed et al. 2004) as opposed to systemic modification of intraperitoneal Tam injection adopted in this study. The work of Song and coworkers (Song et al. 2016) revealed significant reduction in brain 5-HT only after high dose of Tam was injected to Pet1-CreERT mice. Again, the above mentioned authors are the only known reporters who

measured central monoamine levels by HPLC after induced *Tph2* deletion in mice and they reported opposite outcome on levels of NE, therefore comparative studies with reference to their works should be done with caution.

In *Tph2*$^{-/-}$ mice, which lack brain 5-HT synthesis even at embryonic stages, some detectable levels of brain 5-HT from HPLC has been reported (Mosienko et al. 2012; Alenina et al. 2009) and this has been attributed to factors such as; accidental contamination from 5-HT in blood platelets due to inefficient PBS perfusion of the brain; detection of pseudo-monoamines with the same retention time as 5-HT; possibility that 5-HTP derived from Tph1 enzymatic activity in the blood could cross the blood brain barrier and get converted into 5-HT by AADC (Alenina et al. 2009; Gutknecht et al. 2012) or alternative splicing of Tph2 RNA can result in synthesis of non-functional or a compound structurally related to 5-HT which can be detected by HPLC (Pelosi et al. 2015). The possibility of these factors contributing to our observed outcomes cannot be ruled out.

In the brain, 5-HIAA (main metabolite of 5-HT) levels in the *Tph2icKO* mice remained significantly low throughout the study, indicating reduction in 5-HT metabolism. Compared with *Tph2icKO* mice, levels of 5-HIAA in *Tph2CON* mice were high in the 2nd week but dropped drastically low in the 4th week and increased dramatically again in the 6th week. These inconsistencies are unexpected and contradict a report by (Song et al. 2016) who reported clear differences in the fourth week. 5-HT turnover rate (5-HIAA/5-HT), an index of neuronal activity (Kim et al. 2005), indicates rate of 5-HT synthesized and released after induction of *Tph2* inactivation. 5-HT turnover rate in *Tph2icKO* mice was significantly lower than that of the *Tph2CON* in all forebrain regions, which indicates inactive release of 5-HT and very low levels of 5-HT in the synapses. It is likely that decrease in 5-HT release and turnover are inherent neural mechanisms to retain some already synthesized 5-HT in axons.

The concentration of DA was comparatively similar between *Tph2icKO* mice and *Tph2CON* mice in all brain regions except for the amygdala, but concentration of NE in*Tph2icKO* mice were significantly higher than *Tph2CON* mice. These observations differ from that which has been reported in *Tph2*$^{-/-}$ mice (Gutknecht et al. 2012). Germline deletion of *Tph2*$^{-/-}$ resulted in concurrent reduction in brain concentrations of DA and NE in the mice. It is not clear why such a difference occurred in this study, but a reciprocal interaction between 5-HT and DA systems has been reported. 5-HT

inhibit DA activity via 5-HT_2 receptors (Seo, Patrick, and Kennealy 2008; Kapur and Remington 1996) and pharmacological treatment which caused increased DA levels in prefrontal cortex caused severe decrease in both 5-HT and NE concentrations of the cortex (Nayyar et al. 2009) which confirms the reciprocal inhibition hypothesis. Therefore, it is expected that high levels of 5-HT (2nd week) in *Tph2icKO* mice would result in decreased concentration of DA in amygdala. The observed similarities in DA concentration between *Tph2CON* and *Tph2icKO* mice could indicate that reduction in 5-HT synthesis may not affect DA neurotransmission or may partially alter DA in amygdala.

It has been postulated that under baseline conditions, an intact 5-HT system is required for normal NE system functioning. Pharmacological models which increase 5-HT concentration concurrently increase NE concentration (Conway et al. 1990; Guiard et al. 2008; Xue et al. 2016). In this study reduced 5-HT concentration due to Tam treatment caused a significant reduction in NE concentration especially in the second week. In contrast decreased levels of NE were reported by (Whitney et al. 2016) in the 6th week, while Song et al.2016 reported no changes in NE and DA concentrations after 6 weeks. This apparent contradiction might have arisen form stressful injection method. Acute stressors alter levels of NE, DA and 5-HT in brain of rodents (Miura et al. 1993; Konstandi et al. 2000). The injection protocol adopted in this study is a form of acute stressor to the mice and that could account for the increased concentration of NE in the brain coupled with expected reduction in 5-HT concentration.

Such stress might have caused significant increases in synthesis of 5-HT in *Tph2CON* mice in the second week (Clement et al. 1993; Lanfumey et al. 2008; Inoue, Tsuchiya, and Koyama 1994; Pei, Zetterstrom, and Fillenz 1990) which in turn caused reduction in NE levels. Endogenous 5-HT neurotransmission in *Tph2icKO* mice was significantly reduced, but not abolished entirely and this could serve as a model for assessing the interactions between monoamines under basal and (acute) stressful conditions.

4.2 Disruption in brain 5-HT metabolism and altered behavior in mice

The second part of this study examined the behavioral consequences of adult brain 5-HT depletion on anxiety, depression and fear in male mice. Later, effects of MS

stress, 5-HT depletion and their interaction on behavior were assessed in both male and female mice.

4.2.1 Anxiety-related and locomotor hyperactive behavior in icKO mice

Anxiety-related behavior was tested in mice which have severe disruption in adult brain 5-HT neurotransmission using EPM, LDB and OFT paradigms. Evaluation of behavior revealed no differences in anxiety indices in *Tph2icKO* compared with *Tph2CON* mice. However, a hyperactive phenotype (*Tph2*$^{fl/-}$) which covered longer distance than all other genotypes in the OFT was observed. Again *Tph2*$^{fl/-}$ and heterozygote *Tph2*$^{+/-}$ also performed more vertical activities as indicator of increased exploratory but also panic-like responses. Indeed, *Tph2*$^{-/-}$ mice with a lifelong absence of 5-HT synthesis showed similar hyperactivity and panic-like responses in potentially threatening aversive inescapable environment (Waider et al. 2017), which confirms the crucial involvement of 5-HT in responses to acute inescapable threat (Baldwin and Rudge 1995; Deakin 1998a; Deakin 1998b; Baratta et al. 2009).

A limited number of studies exist which looked specifically at manipulation of adult brain 5-HT system and its effect on anxiety and hyperactive locomotor behavior. In a recent study Whitney and colleagues (Whitney et al. 2016) reported lack of observable anxiety in AAV-cre *Tph2-CKO* compared with *Tph2-CON*. Both groups of mice did not significantly differ from each other in anxiety performance indices. More interestingly, the authors reported that *Tph2-CKO* exhibited increased locomotor hyperactivity characterized by increased total distance travelled and number of entries to the center of novel open field arena. This hyperactive behavior was also evident in home cage activities which resulted in disruption in normal nocturnal siesta, as well as periods of rapid eye movement (REM) and non-REM sleep. On the contrary, Song and her coworkers (Song et al. 2016) reported anxiolysis in LDB but not in EPM in icKO mice. The observed discrepancy could be attributed to the high dose (250mg/kg) of Tam administered to the mice. In humans, Tam is known to induce cognitive impairment and high dose of Tam in mice imposes stress on cells of the nervous systems (Denk et al. 2015). The findings from this study supports the observation of (Whitney et al. 2016), indicating that short period of disruption in adult brain 5-HT metabolism may not be sufficient to impact anxio-phenotype in mice, however it can prone an individual to restlessness, lack of concentration and hyperactivity.

Earlier pharmacological studies involved use of neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT) and para-chlorophenylalanine (PCPA) to induce brain 5-HT depletion in rats. PCPA and 5,7-DHT treatment enhanced locomotor activity in rats (Dringenberg et al. 1995; Steigrad et al. 1978) while 5,7-DHT has anxiolytic effects (Sziray et al. 2010). The neurotoxin, 5,7-DHT, destroys neurons so the observed anxiolytic-phenotype has been seriously questioned (Choi, Jonak, and Fernstrom 2004).

The outcome from this study differs completely from anxiolytic, and aggressive phenotypes seen in *Tph2*$^{-/-}$ mice (Angoa-Perez et al. 2012; Mosienko et al. 2012; Mosienko et al. 2015; Gutknecht et al. 2015, Weidner 2018 submitted), and that of mice with conditional perturbation of central 5-HT synthesis (CKO) (Dai, Han, et al. 2008). This suggests that brain 5-HT during development period is needed to establish neural circuits which control normal state anxiety. Once these circuits are fully established in adult brain, acute disturbance may not be sufficient to dysregulate their functions. Similarly, doxycycline (DOX) inducible conditional manipulation of 5-HT_{1A} autoreceptor in the raphe (Richardson-Jones et al. 2010; Deneris 2011), and 5-HT_{1A} heteroreceptor (Richardson-Jones et al. 2010) of mice has no effect on expression anxiety. Mice that expressed high (1A-High) and low (1A-Low) level of the receptor performed comparatively similar in baseline anxiety-related behavioral indices indicating that manipulation of *Tph2* and 5-HT_{1A} autoreceptor is not sufficient to impact conflict anxiety behavior. Adult *Pet-1*$^{fl/-}$*;ePet::CreER*^{T2} mice exhibit exaggerated anxiety compared with vehicle treated controls (Liu et al. 2010). It has been postulated that *Pet-1* influence emotional behavior via downstream transcriptional targets such as reduction in *5-Htt* and *Vglut3* (Liu et al. 2010), and therefore reduced function in adulthood can render mice vulnerable to anxiety. However, further studies are needed to decipher *Pet-1* dependent transcription modulation of ascending 5-HT neurons and its influence on behavior. The outcome from this study dissociates functions of 5-HT in baseline anxiety-related behavior in adulthood from an early developmental stage.

4.2.2 Depression-like phenotype but lack of anhedonia in icKO mice

Evidence from patients suffering from major depression as well as analysis of postmortem brain tissue of depressed individuals have established a strong link between dysregulation in 5-HT metabolism and the disease. Some of the available

evidence include reduced concentrations of 5-HT and 5-HIAA in postmortem brain tissue, reduced concentrations of 5-HIAA in cerebrospinal fluid (CSF) of patients who are not on antidepressants (inhibitors of 5-HT uptake). Again, chronic treatment with antidepressants augments 5-HT neurotransmission and improve mood (Owens and Nemeroff 1994; Fraser 2003; 'The highs and lows of the serotonin theory of depression' 2015). Acute tryptophan depletion (ATD), i.e. ingesting tryptophan depleted diet, has been established to study effect of reduced 5-HT synthesis on mood. In humans, ATD causes relapse in patients who respond positively to antidepressants, however, ATD does not worsen the situation in depressed patients who are not on medication (Bell, Abrams, and Nutt 2001; Hughes et al. 2003; Mace et al. 2010, 2011). Again, ATD challenge in healthy participants caused little or no change in mood, but in those with family history of depression, lowering of mood has been reported although this remains within normal mood range (Young 2013b, 2013a). A direct association between ATD and reduced extracellular 5-HT concentration has not been established, and ATD challenge in mice failed to produce the desired anxiolytic and depressogenic phenotypes (van Donkelaar et al. 2010) although other studies reported otherwise (Biskup et al. 2012; Ardis et al. 2009). Tryptophan is a precursor for kynurerine which could also play a role in mood disturbance independent of 5-HT (for reviews (Oxenkrug 2013; Dantzer et al. 2011) and therefore caution is necessary when interpreting results from ATD challenge (van Donkelaar et al. 2011).

In this study, inducible depletion of adult brain 5-HT caused no difference in latency to float, but significant difference in duration of floating. The *Tph2icKO* mice showed a trend in duration of floating compared with*Tph2CON* (*Tph2*$^{+/-}$) mice, suggesting a tendency to behavior despair. Lack of behavior despair in FST has been reported in high dose Tam treated hTM-DTA^{ipet1} mice (Song et al. 2016). These slight differences in the outcome could be attributed to method used in scoring behavior. Manual method was used in the study, however it is not clear whether the authors scored behavior manually or used behavior assessing software. An observation similar to the finding in this study was seen in mice with DOX inducible inactivation of 5-HT$_{1A}$ auto- and hetero-receptors (Richardson-Jones et al. 2010) which suggests a possible compensatory mechanism in the case of dysregulation in adult brain 5-HT neurotransmission. Other studies involving *Tph2*$^{-/-}$ mice also reported lack of depression in the FST (Angoa-Perez et al. 2014; Mosienko et al. 2014); marginally

reduced anxiety- and depression-like behavior (Gutknecht et al. 2015) and lack of responsiveness to SSRIs (Angoa-Perez et al. 2014) which seriously questions the exact role of 5-HT in depression in mice.

Sucrose preference (pleasure seeking) has been used to also assess altered mood in mice. *Tph2*$^{fl/fl}$, *Tph2*$^{fl/-}$ and *Tph2*$^{+/-}$ mice consumed more sucrose solution than *Tph2*$^{+/+}$ mice, which suggests lack of anhedonia in these mice. However, it is difficult to attribute the observed behavior to lack of depression in *Tph2icKO* mice. These mice exhibited locomotor hyperactivity and therefore the above observation could best explain the energy need of these mice. This is confirmed by the fact that *Tph2icKO* (*Tph2*$^{fl/-}$) mice drank more water and ate more food than all other groups. Similar to other study, sucrose preference was same in WT and *Tph2*$^{-/-}$ mice, and *Tph2*$^{-/-}$ consumed a slightly greater quantity of food (Angoa-Perez et al. 2014). This supports the assertion that minimal alteration in 5-HT metabolism underlies pathophysiology of eating disorders (anorexia and bulimia nervosa) through various hormonal and receptor systems (Hernandez et al. 2016; Steiger 2004).

4.2.3 Altered fear-like responses in icKO mice

It has been postulated that altered 5-HT neurotransmission especially in the amygdala distorts defense mechanisms in response to environmental threats. In humans and rats, acute increase in central 5-HT concentration (through pharmacological moieties) before cued fear conditioning paradigm intensifies conditioned fear acquisition and expression (Bocchio et al. 2016) but chronic exposure blunts fear expression (Burghardt et al. 2004). Again injection of 5-HT-depleting agent (5,7-DHT) into amygdala of rats caused reduction in acquisition of fear (decreased freezing time) during conditioning as well as fear retrieval (Johnson et al. 2015). In our inducible model, a cue/tone was used as conditioned stimulus and 2 s electric foot shock was used as unconditioned stimulus. Pre-shock fear responses to the novel environment were similar in all mice. Post-shock freezing increased with time but no differences were observed between *Tph2icKO* and control mice, which indicate fear acquisition was same in all mice. In the context dependent fear retrieval, *Tph2icKO* mice froze for a significantly longer period than *Tph2CON* mice and the fear was sustained throughout the period without signs of diminishing. CKO mice differed in fear responses only after foot shock was presented more than 3

times and the mice showed enhanced context dependent fear memory (Dai, Han, et al. 2008) which is in line with outcome from this study. Similar observation was seen in hTM-DTA^{ipet1} mice and enhanced context dependent fear memory persisted even 30 days after fear conditioning (Song et al. 2016). This suggests that acute ablation of 5-HT in adult brain facilitates hippocampus dependent long term fear memory consolidation; therefore this inducible approach could serve as a model for studying the neurobiological basis for PTSD. Genetic ablation of brain 5-HT synthesis in mice resulted in increased cued fear conditioning and retention (Gutknecht et al. 2015), which is contrary to the observation in *Tph2icKO* mice. The increased cued fear conditioning in *Tph2*$^{-/-}$ mice stems from lifelong depletion in 5-HT metabolism as opposed to short term effects of Tam treatment.

When humans and rodents are exposed to SSRIs, there is an initial exacerbation of anxiety and fear symptoms (Ravinder et al. 2013), which indicates that anxiety and fear are primed on similar neural circuits (Steimer 2002; Apps and Strata 2015; Tovote, Fadok, and Luthi 2015). Since no obvious anxiety-like phenotype was observed in this study, it can be hypothesize that there are alternate fear pathways which are modulated separately by 5-HT. Enhanced activation of various amygdala nuclei (Tye and Deisseroth 2012; Haubensak et al. 2010; Ciocchi et al. 2010) and bed nucleus of stria terminalis (BNST) (Ravinder et al. 2013) in rodents has been reported during fear conditioning and fear expression. Again, some 5-HT receptors selectively modulate either cued or contextual fear (Homberg 2012). These reports suggest that future studies should look beyond signal inputs to amygdala nuclei to include other circuits which can gate fear acquisition and expression. In this study, conclusions on fear expression are drawn with caution since the number of mice involved is small due to uncontrollable technical challenges. Therefore, further studies involving large number of mice are required to unearth the neural substrates and networks incorporated in fear acquisition and expression in *Tph2icKO* mice.

4.3 Interaction of MS stress and aberrant adult brain 5-HT on behavior

There are reports about a child’s early social context being a sensitive factor for adverse effects of later trauma, which are usually controlled by genetic susceptibility factors (Gross and Hen 2004). MS has been commonly used to study GxE interaction on anxiety and depression in mice and varied outcomes have been reported due to variations in methodology (Fabricius, Wortwein, and Pakkenberg 2008; George et al.

2010). In this study, mice of both sexes were either separated from their mother for 180 s daily or left undisturbed and they were later treated with either Tam or Veh. MS exposed mice (both sexes) had reduced locomotor activity in EPM and LDB. Male *Tph2icKO* mice showed hyperactivity, but an opposite effect was seen in female *Tph2icKO* mice. Again female *Tph2icKO* mice seemed more anxious indicating sex specific effect of brain 5-HT depletion. Thus MS exposure has anxiolytic effect but reduced 5-HT neurotransmission may abolish this effect in MS-exposed female mice. Reports in ovariectomized MS exposed female mice indicated increased baseline anxiety in the OFT (Tsuda and Ogawa 2012) which is in line with this study. Tam is a selective estrogen receptor modulator used in breast cancer treatment and prevention (Gjerde et al. 2010) and it may act as partial agonist/antagonist of estrogen receptor in Tam exposed female mice. However, its effect might have worn-out by the time mice were tested for anxiety and depression. The hydrophilic nature of Tam facilitate its rapid elimination from the body (Jordan 2007).
Male and female rodents seem to have different stress coping strategies and behave differently in many behavioral experiments (Archer 1975, 1977; Genn et al. 2003; Johnston and File 1991). A recent study reported sex-specific MS effect on anxiety and social behavior. The authors reported that MS exposure did not alter anxiety, but reduced exploratory behavior in male mice. In female mice MS exposure had anxiogenic effect, and increased social behavior (Bondar, Lepeshko, and Reshetnikov 2018). MS exposed *Tph2* knock-in (TPH2KI) mice, which have 60-80% reduction in brain 5-HT, produced behavior similar to that of male T*ph2icKO* mice towards the time spent in center of OFT (Sachs et al. 2013). On the contrary, increased baseline anxiety found in TPH2KI mice (Beaulieu et al. 2008; Sachs et al. 2013) was absent in *Tph2icKO* mice, which indicates that MS exposure in male mice differentially controls anxiety-related behavior in the two genetically manipulated mice. Loss of function via gene knock-in might have caused distortion in the formation of anxiety networks during development.

MS, 5-HT depletion and their interactions had no significant effect in SPT, however *Tph2icKO* mice (both sexes) tend to consume more fluid than *Tph2CON* mice. Again, both male and female mice exhibited lack of behavior despair or depression in the FST. One assumption is that after each daily MS, maternal care on reunion with pups increases and this could annul MS effects or the MS protocol used was not robust

enough to impact depression-like behavior in the mouse strains (Millstein and Holmes 2007). Here pups were placed in new cages together with their bedding materials so the major stress factor was the absence of mother and therefore proper maternal care of pups after each separation period might have buffered the stress effects. Results from other studies support outcome from our study. MS exposure did not affected hedonic behavior in rats (Shalev and Kafkafi 2002), neither did it impact anxiety and depression-related behavior in C57BL/6 mice but BALB/c mice were emotionally affect (Savignac, Dinan, and Cryan 2011), suggesting strain specific effects. In another study, MS exposure enhanced anxiety and depressive-like behaviors only in WT mice, while TPH2 KI mice exhibited no such behaviors (Wong et al. 2015). On the contrary, other studies have strongly associated MS exposure to abnormal behavior and stress induced alteration in neurotransmitter levels (Daniels et al. 2004; Veenema, Bredewold, and Neumann 2007). Mice which were separated from their mother for just one day (24 h) in their tender age showed behavior despair in FST during adulthood (Macri and Laviola 2004). Separation from mother ($MS_{2–15}$) for 6 h daily caused increased sera corticosterone; altered immune system parameters and depression and anxiety-like behaviors in mice (Roque et al. 2014).

Noteworthy is that most rodents studies that found strong association between MS and behavioral deficits were largely done in rats (Holmes et al. 2005; de Kloet et al. 2005; Veenema, Bredewold, and Neumann 2007). As was observed in this study, C57BL/6J mice appear to be resilient to neonatal MS stress and therefore several studies reported lack of association between MS stress and behavior (Anisman et al. 1998; Millstein and Holmes 2007; Vetulani 2013; Millstein et al. 2006; Savignac, Dinan, and Cryan 2011; Shawn Tan 2017; Parfitt DB 2007). That notwithstanding some of these discrepancies hinge on methodological differences in the MS paradigm and handling of control mice, which has been described in details (Vetulani 2013; Hall 1998). The duration of separation of pups from mother per day are varied (Vetulani 2013); the number of days for separation of pups from mother either begins on postnatal day 3 to 15 ($MS_{3–15}$) or even from postnatal day 7 to 20 days ($MS_{7–20}$) (Roque et al. 2014) and time of permanent separation from mother (Shawn Tan 2017; Carlyle et al. 2012; George et al. 2010) vary between groups. Control mice are also either left undisturbed other than regular cage change (Own, Iqbal, and Patel 2013) or they are handled briefly at variable length of time (Arborelius and Eklund 2007; Bondar, Lepeshko, and Reshetnikov 2018) and all these variables affect

behavior differently. Brief handling (usually maximum 15 minutes) of pups positively influence behavior in rodents.

The HPA axis modulates various physiological responses to ELS and its dysfunction is highly implicated in anxiety and depression related disorders. Gonadal hormones differentially alter HPA-axis activity such that estrogen increase basal HPA-axis activity while testosterone decreases it (Goel et al. 2014; Schroeder et al. 2018) and this may explain why female rodents are more prone to depression (Liu et al. 2018). One caveat of this study is the failure to measure corticosterone levels in blood or fecal samples as a measure of HPA axis activity. However, administration of corticosterone (CORT) to male C56BL/6J mice impacted no anxiety and depression behavior (Sturm et al. 2015), neither did protracted exposure of female C56BL/6J to CORT affect behavior nor hippocampal neurogenesis (Mekiri et al. 2017). Again, no *5-htt*×ELS interaction effects for plasma CORT levels was found in rodents (van der Doelen et al. 2014) and various studies found no *5-htt*×ELS interaction effects on negative behavior in mice (Kloke et al. 2013; Houwing et al. 2017). The above findings support our data, suggesting that if there was any alteration in CORT levels due to MS, injection protocol or Tam in the mice used in this study, it was not sufficient to impact behavior despair and that other stress mechanisms played a more important function in behavior observed, rather than stress-hormone levels per se. For example, female mice are gregarious and therefore their single housing could have triggered innate depression which did not change during the period of testing.

The findings from this study support the match-mismatch theory of psychiatric disease and the predictive adaptive response hypothesis, which suggest that exposure to early-life stress may induce selective advantages which can improve fitness or confer adaptive value in later stage of development within certain individuals (Rana et al. 2015; Gluckman, Hanson, and Spencer 2005; Nettle, Frankenhuis, and Rickard 2013, 2014).

4.4 Gene expression in raphe, hippocampus and amygdala

The expression of *Htr1a, Htr2a, Maoa and Avpr1a* revealed no differences in MS-naive mice. An earlier study (Kriegebaum, Song, et al. 2010) also reported no change in expression of *Htr1a and Htr2a* in Tam treated mice, which suggests that adult brain depletion is not sufficient to alter the expression of genes relevant to 5-HT system. It

was observed that relative expression of *Tph2* in raphe nuclei of both male and female was not altered by MS exposure, however, *Tph2icKO* mice showed significant reduction in expression of *Tph2* in both MS exposed and *Tph2CON* mice. This is a confirmation of efficiency of the Tam inducible technique. Lack of effect of MS on *Tph2* expression in WT mice has been reported (Wong et al. 2015). However, one study found association between MS exposure and reduced raphe *Tph2* expression in C56BL/6J mice. The authors studied expression patterns of *Tph2* and *5htt* in the following regions; dorsal raphe dorsalis (DRd), ventralis (DRv), lateral wings (DRlw), median raphe (MR), and paramedian raphe (pMR) and found significant reduction in *Tph2* as well as *5htt* in only DRd and DRv regions while other regions remained unaffected (Own, Iqbal, and Patel 2013). Considering the small size of these raphe (DRd and DRv) regions (Alonso et al. 2013), the overall expression of *Tph2* in raphe of these mice would yield no difference between groups. In female mice, MS significantly altered to *Tph2* expression in the amygdala, while only a tendency was seen in amygdala of male mice. These observations could be artefactual because expression of the genes was estimated with reference to ubiquitously expressed housekeeping genes in the cell. The levels of *Tph2* in amygdala and hippocampus were similar to that seen in the raphe of in Tam treated mice. It could mean that these observations are baseline limits set with reference to the housekeeping gene. It can also be argued that alternate splicing produced some stable Tph2 transcripts which were transported along axons to target sites. Here elimination of exon 5 of *Tph2* was predicted to create a shift in the reading frame which results in synthesis of a truncated non-functional Tph2 protein (Gutknecht et al. 2008).

In male mice, MS caused reduction in expression of *Htr1a* in raphe and a tendency towards increased *Htr1a* expression in *Tph2CON* mice, but in female mice, *Tph2icKO* had reduced *Htr1a* expression which could result in low quantities of $5HT_{1A}$ autoreceptor levels in the mice. Genetic ablation (Parks et al. 1998; Ramboz et al. 1998; Toth 2003) and pharmacological inhibition or enhanced expression of 5-HT_{1A} receptors has shed light on its role in anxiety and exploratory behavior (Gross et al. 2002a; Lo Iacono and Gross 2008; Parks et al. 1998). Selective antagonism of 5-HT_{1A} autoreceptors increases anxiety, suggesting enhanced 5-HT function via 5-HT heteroreceptors activation (Albert 2012; Richardson-Jones et al. 2011). Here altered levels of *Htr1a* in raphe were associated with sex-specific hyperlocomotion

and altered anxiety, which indicates male and female mice respond differently to stress and acute brain 5-HT depletion. Expression of *Htr1a* in hippocampus and amygdala was not altered which could indicate that 5-HT_{1A} heteroreceptor function was not compromised and that explains the lack of depression in the mice (Gross et al. 2002b).

The expression of *Htr2a* in raphe of male mice followed the same pattern as that of *Htr1a* in raphe, with no changes in hippocampus and amygdala. MSx5-HT depletion interactions resulted in increased *Htr2a* expression in hippocampus of female mice. This means MS stress reduces *Htr2a* expression but this effect can be potentially abolished by reduced 5-HT neurotransmission in female mice. On the contrary, MS induced increase in *Htr2a, Crhr1 and Crhr2* expression, but reduced *Nr3c1* and *Htr1a* expression in the hippocampus of mice has been reported (de Almeida Magalhaes et al. 2018). The difference stems from post-MS handling of the mice. The authors sacrificed the mice immediately after MS while in this study gene expression was done 16 weeks post MS. *Htr2a* codes for 5-HT_{2A} receptors and little expression of this receptor in the brain is associated with increased anxiety, fear and cognition, while reduced 5-HT_{2A} receptors in hippocampus and platelets have been reported in patients suffering from major depression (Mintun et al. 2004; Raote, Bhattacharya, and Panicker 2007). It was found that female mice which displayed increased *Htr2a* expression had increased inhibition in conflict anxiety paradigms and this contravenes an earlier report (Weisstaub et al. 2006). Since 5-HT_{2A} receptors are widely distributed in the brain and are constitutively active, the increased hippocampal *Htr2a* expression was probably a compensatory measure against depression (Weisstaub et al. 2006; Zhang and Stackman 2015).

In male mice, *Maoa* expression in raphe was higher in *Tph2CON* than *Tph2icKO* mice. On the other hand, MS exposure significantly increased *Maoa* expression in hippocampus, with only a tendency in amygdala. In female mice, MSx5-HT interactions altered *Maoa* in raphe only. Brain 5-HT depletion reduced *Maoa* expression, an effect abolished in MS exposed *Tph2icKO* female mice, suggests that environmental stressors influence *Maoa* expression in regulation of behavior. Maoa KO mice are much anxious, depressive in FST and show intense contextual fear (Seif and De Maeyer 1999), but also hyperactive and very aggressive to intruders (Godar et al. 2016; Gingrich and Hen 2001). In this study, increased *Maoa*

expression in raphe of female mice was associated with increased anxiety-like behavior. Similarly, stress exposure during adolescence in rats caused increased *Maoa* expression in prefrontal cortex and this was associated with increased anxiety (Marquez et al. 2013). Mice raised in safe environment followed by later-life adverse experience had reduced anxiety and reduced *Maoa* expression in hippocampus (Bodden et al. 2017). This indicates that environmental stressors influence behavior via regulation of *Maoa* functions. Aggression and injuries were observed among male mice (personal communication) especially during and after the injection period. Since MS influenced *Maoa* expression in hippocampus and amygdala, the observed aggression could be attributed to MS and acute stress due to injection.

One other gene that has been associated with aggression and panic is *Avpr1a* (Zhang-James et al. 2018; Scordalakes and Rissman 2004). Early beneficial and late adverse life history in mice significantly altered *Avpr1a* expression in the hippocampus and this had anxiolytic effect on the mice (Bodden et al. 2017). MS caused increased alcohol consumption in mice and this was associated with increased *Avpr* in hippocampus (de Almeida Magalhaes et al. 2018). *Avpr1a* knockout as well as pharmacological blockade of *Avpr1a* function in rodents reduced aggression and had anxiolytic and anti-depression effects (Bielsky et al. 2005; Charles et al. 2014). Here alteration in *Avpr1a* was seen only in the raphe of MS exposed male mice but not in hippocampus which suggests that environmental factors differentially alter brain *Avpr1a* expression. MS increased *Avpr1a* expression but Tam treatment decreased this effect and the mice were less anxious in the OFT. This suggests that *Avpr1a* could serve as therapeutic target for treating anxiety especially in case of altered 5-HT metabolism.

4.5 Conclusion and perspectives

This study sought to generate inducible conditional *Tph2* knockout mice which have severe deficits in brain 5-HT neurotransmission, and to investigate the interplay between this effect and MS stress in regulation of behavior. Using the cre-loxP system, *Tph2icKO* had a significant reduction in the level brain 5-HT and 5-HIAA in adult mice. However, this effect did not alter baseline anxiety and depression-related behavior, which indicates that the disruption in 5-HT synthesis was sufficient to completely abolish brain 5-HT neurotransmission. Interestingly, *Tph2icKO* mice

showed locomotor hyperactivity but also increased context-dependent fear-like responses which indicate that this inducible model may be a good tool for studying the neural basis of conditions such as ADHD, PTSD and cognitive impairment.

MS stress produced hypolocomotor phenotype in both male and female mice irrespective Tam or vehicle treatment. However, a sex-specific anxiety effect emerged such that female *Tph2icKO* mice showed anxio-phenotype while male mice remained unaffected. This anxiogenic effect in female mice was aggravated by interplay between MS exposure and adult brain 5-HT depletion while male mice proved less anxious.

Lastly, there was interplay between MS and brain 5-HT depletion in regulating the expression of *Htr2a, Maoa* and *Avpr1a* in the brain in a sex-specific manner. This outcome shows that these genes may be prominent in GxE control of anxiety- and depression related behavior in mice. It must be emphasized that in this study, low levels of Tam was used to achieved desired results, however, the immediate and/or protracted effect of Tam on the neuroendocrine system of female mice was not assessed. Future study is required to examine effect of Tam on the HPA axis function and alterations in gene expression in female mice. This will explain factors that may confound research outcomes.

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6.3 Abbreviations

5-HIAA - 5-hydroxyinole acetic acid
5-HT - 5-Hydroxytryptamine (serotonin)
5-HTP - 5-hydroxytryptophan
5-HTTLPR - 5-HTT linked polymorphic region
5-HTT - Serotonin transporter
5-HIAL - 5-hydroxyindole-3-acetaldehyde
AAAD - Aromatic L-amino acid decarboxylase
AADC - Aromatic aminoacid decarboxylase
AANAT - Serotonin N-acetyltransferase
AcCoA - Acetyl coenzyme A
ADHD - Attention-deficit/hyperactivity disorder
ALDH - Aldehyde dehydrogenase
Amy - Amygdala
ANOVA - Analysis of variance
BDNF - Brain-derived neurotrophic factor
BH4 - Tetrahydrobiopterin
cDNA - Complementary deoxyribonucleic acid
CeA - Central nucleus of the amygdala
c-Fos - Cellular FBJ murine osteosarcoma
CKO - Conditional knockout
cre - Cyclization recombinase
CNS - Central nervous system
CLN - Caudal linear nucleus
CAMKII - Calcium/calmodulin-dependent protein kinase II
CS - Conditioned stimulus
CMV - Cytomegalovirus promoter
Ctrl - Control
DA - Dopamine
DAPI - 4',6-Diamidino-2-Phenylindole
DOX - Doxycycline
DTPA - Diethylenetriaminepentaacetic acid
DR - Dorsal raphe

Abbreviations

DRN - Dorsal raphe nucleus
EDTA - Ethylendiamintetraacetat
ELS - Early life stress
EPM - Elevated plus maze
EPSP - Excitatory post synaptic potential
FAD - Flavin adenine dinucleotide
FC - Fear conditioning
floxed - loxP-flanked
FST - Forced swim test
GABA - Gamma-aminobutyric acid
GPCR - G -protein coupled receptor
GAPDH - Glyceraldehyde-3-phosphate dehydrogenase
H_3PO4 - Orthophosphoric acid
HIOMT - Hydroxy-indole-O-methyl transferase
Hip - Hippocampus
HPA - Hypothalamo-pituitary-adrenal axis
HPLC - High-performance liquid chromatography
IHC - Immunohistochemistry
icKO - Inducible conditional knockout
IPSP - Inhibitory post synaptic potential
KO - Knockout
Lmx1b - LIM homeodomain transcription factor 1 beta
loxP - Locus of cross-over of bacteriophage P1
LSD - Lysergic acid diethylamine
MAO - Monoamine oxidase
MRN - Median raphe nucleus
mRNA - Messenger ribonucleic acid
MS - Maternal separation
PKA - Protein kinase A
NaH_2PO_4- Sodium dihydrogen Phosphate
NE - Norepinephrine
OFT - Open field test
PAH - Phenylalanine hydroxylase
PCPA - Parachlorphenylalanine

Abbreviations

PBS - Phosphate buffered saline

Pet 1 - Plasmacytoma expressed transcript 1

PFA - Paraformaldehyde

Phe - Phenylalanine

RT-qPCR - Quantitative real-time polymerase chain reaction

RT - Room temperature

RR -Rostral raphe

SCD - Superior cerebellar decussation

SDS- Sodium dodecyl sulfate

SERT - Serotonin transporter

SLC6A4 - Serotonin transporter gene

SNP - Single nucleotide polymorphism

SSRI - Selective serotonin reuptake inhibitor

TAM - Tamoxifen

TBS - Tris buffered saline

TH - Tyrosine hydroxylase

TPH - Tryptophan hydroxylase

Trp - Tryptophan

TST - Tail suspension test

US - Unconditioned stimulus

VMAT - Vesicular monoamine transporter

WT - Wildtype

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Curriculum vitae

6.6 Affidavit

Affidavit

I hereby confirm that my thesis entitled “Behavioral and physiologic consequences of inducible inactivation of the *Tryptophan hydroxylase* 2 gene in interaction with early-life adversity” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or meterials applied are listed specified in the thesis.

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

Würzburg, 04.09.2018

Place, Date Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „Verhaltens und physiologische Konsequenzen einer induzierbaren Inaktivierung des *Tryptophan hydroxylase* 2-Gens Interaktion mit frühkindlichen Stresses“ eigenständig, d.h. insbensondere selbständig und ohne Hilfe eines kommerziellen Promotinsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg, 04.09.2018

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