The effect of fluoxetine treatment on persistent contextual fear conditioning in post-traumatic stress disorder modulated through the serotonergic system

Foreword

This bachelor's thesis is written as part of the pre-master Biomedical Sciences at the University of Groningen. Robbert Havekes sparked my interest in the neuroscience during the course Neurobiology, which made me want to dive deeper into the underlying fields of neurological disorders.

Abstract

Post-traumatic stress disorder (PTSD) is a disabling psychiatric condition which is characterized by persistence of contextual conditioned fear after the exposure to a physical, physiological or sexual traumatic event due to poor fear memory extinction. The selective serotonin reuptake inhibitor (SSRI) fluoxetine is widely used in the treatment of PTSD, but the therapeutic mechanism remains poorly understood. Brain structures involved in contextual fear conditioning, including the hippocampus, amygdala, bed nucleus of the stria terminalis (BNST) and the prefrontal cortex (PFC), contain a high number of serotonin (5-HT) receptors. Furthermore, serotonergic dysfunction has been associated with PTSD, indicating that 5-HT plays an important role in contextual fear conditioning. Investigating the neurocircuitry of contextual fear conditioning and the role of 5-HT demonstrated that contextual fear conditioning processes are mainly modulated via 5-HT1, 5-HT-2 and 5-HT3 receptors. Multiple experimental animal models showed that the 5-HT1A heteroreceptor mediates a reduced fear acquisition and expression, the 5-HT2A and 5-HT3A receptors regulate fear memory extinction and the 5-HT2C receptor is involved in mediating anxiety-like behavior. Not surprising, humans carrying a genetic variation in essential genes for the development of serotonergic neurons and 5-HT signaling such as Pet1, Thp2, Vmat2 and 5-HTT displayed to be predisposed to a serotonergic dysfunction leading to PTSD-like behavior. Acute fluoxetine treatment immediately increases extracellular 5-HT levels, but worsens patients' symptoms via the internalization and activation of 5-HT1A autoreceptors in the raphe nucleus. In contrast, chronic fluoxetine treatment leads to the deactivation of 5-HT1A autoreceptors resulting in normal 5-HT signaling. In addition, fluoxetine blocks 5-HT2C receptors leading to a reduction in anxiety-like behavior. In rodents, chronic fluoxetine treatment displayed to have beneficial effects on contextual fear conditioning. However in human PTSD patients, chronic fluoxetine treatment resulted in contradicting findings indicating that there probably exist additional underlying dysfunctions which require more research.

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1. Introduction

Anxiety and fear-related disorders among which post-traumatic stress disorder (PTSD) are disabling psychiatric conditions that lead to an impaired workplace performance and high costs to the society and individuals (Kogan, Stein, & Maj, 2016). Worldwide, approximately 5-10% of the population suffers from PTSD wherein women are affected two to three times more often than men (Yehuda, Hoge, & McFarlane, 2015) (Olff, 2017). One of the main factors in the development of PTSD is the persistence of contextual fear after the exposure to a physical, physiological or sexual traumatic event (Shin & Liberzon, 2009) (Maeng & Milad, 2017).

Fear is a primitive emotion which has been conserved throughout evolution, but is different and distinguishable between species and individuals (Adolphs, 2013). The expression of fear is depending on several processes such as fear conditioning, consolidation, retrieval, discrimination, extinction and generalization which have extensively been studied using contextual fear conditioning experiments in rodents (Britton, Evans, & Hernandez, 2014). Contextual fear conditioning represents many similar situations in humans in which an initially neutral context, the conditioned stimulus (CS), becomes threatening through the association with another negative stimulus, the unconditioned stimulus (US), and generates fear (Tronson, Corcoran, & Jovasevi, 2012). In rodents, the standard fear response is freezing behavior, also referred to as the conditioned response (CR), and is characterized by an immobility of the animal except for breathing. Upon re-exposure to the CS without the US, the degree of conditioned fear retrieval and expression is often weakened with time due to fear memory extinction resulting in less freezing behavior (Homberg, 2012). However, patients suffering from PTSD demonstrate a persistence of contextual conditioned fear due to poor fear memory extinction and/or increased fear acquisition and consolidation (Maeng & Milad, 2017).

One of the most widely used drug in the treatment of PTSD and other fear-related disorders is the antidepressant fluoxetine, which acts as a selective serotonin reuptake inhibitor (SSRI) (Ravindran & Stein, 2009). Serotonin (5-hydroxytryptamine; 5-HT), is a monoamine neurotransmitter of the serotonergic system of the central nervous system (CNS) and has important modulatory effects in many essential brain functions such as autonomic and cognitive control, sensory processing, motor activity, sexual behavior and emotional regulation of *e.g.,* mood, fear, anxiety, stress and aggression (Olivier, 2015). Brain structures involved in contextual fear conditioning, including the hippocampus, amygdala, bed nucleus of the stria terminalis (BNST) and prefrontal cortex (PFC), contain a high number of 5-HT receptors (Berumen, Rodríguez, & Miledi, 2012) (Puig & Gulledge, 2011) (Asan, Steinke, & Lesch, 2013). Furthermore, serotonergic dysfunction has been associated with PTSD, indicating that 5-HT plays an important role during physiological contextual fear conditioning processes (Ressler & Nemeroff, 2000). However, the cause of a serotonergic dysfunction as well as the precise therapeutic mechanism of fluoxetine treatment in PTSD patients remains poorly understood (Homberg, 2012).

This raises the question, how does fluoxetine treatment affects the neurocircuitry of contextual fear conditioning? To answer this question, it is important to understand the neurocircuitry of contextual fear conditioning. Secondly, it is essential to know how the serotonergic system modulates contextual fear conditioning and the causes of a serotonergic dysfunction. Lastly, the effect of fluoxetine treatment on contextual fear conditioning is investigated to increase the understanding of the therapeutic mechanism on persistent contextual fear conditioning and to eventually, optimize the treatment of PTSD. It is hypothesized that fluoxetine restores a physiological functioning of contextual fear conditioning processes by counteracting the serotonergic dysfunction.

2. The neurocircuitry of contextual fear conditioning

Already a long time ago, the hippocampus has been linked to spatial learning and memory in both humans and rodents (O'Keefe & Nadel, 1978). The hippocampus, which is composed of four cornu ammonis regions (CA1-CA4), the dentate gyrus (DG) and the subiculum can be divided into a dorsal (septal) and ventral (temporal) region in rodents which correspond to the posterior and anterior hippocampus in humans, respectively (Seok & Cheong, 2020) (Lee, Lew, & Wickenheisser, 2019). Although it was first assumed that the dorsal hippocampus was essential for spatial processing and the ventral hippocampus for modulating anxiety-related behaviors, Lee et al., demonstrated that the dorsal and ventral hippocampus function as a single integrated structure which is involved in spatial processing and acquisition and therefore, essential for contextual fear conditioning processes (Lee, Lew, & Wickenheisser, 2019).

First, the neurocircuitry of contextual fear conditioning starts with the input from cortical areas to the hippocampus via the perforant pathway. In specific, the entorhinal cortex layer II signals to the

DG and CA3 and the entorhinal cortex layer III to CA1 (Kajiwara, Wouterlood, & Sah, 2008). From the hippocampus, contextual representations of the CS are projected to the amygdala (Kim & Cho, 2020). Since damage to the ventral hippocampus blocks the transfer of contextual representations from the dorsal hippocampus to the amygdala, only the ventral hippocampus can project contextual representations directly to the amygdala (Fanselow & Dong, 2010).

Second, the US which is often a foot shock, is processed in the somatosensory cortex and projected to the lateral nucleus of the amygdala (LA) where the convergence of the CS-US acquisition is mediated (Romanski, Clugnet, & Bordi, 1993). Thereafter, projections from the basolateral nucleus of the amygdala (BLA) and LA are relayed to the central nucleus of the amygdala (CE), which functions as the main output. Furthermore, the BNST also receives strong excitatory projections from the BLA which signals to the CE (Sullivan, Apergis, & Bush, 2004).

Third, fear expression is mediated through the CE via the disinhibition of fear output neurons of the medial division (CEM) which project to several fear systems in the hypothalamus and brain stem, including the periaqueductal grey (PAG). Then, PAG regulates freezing behavior, the conditioned response (CR) (Ciocchi, Herry, & Grenier, 2010). In addition, increased activity of glutamatergic neurons in the BLA also increase freezing behavior (Herry, Ciocchi, & Senn, 2008).

Lastly, the CE projects directly to the PFC which can be subdivided into the infralimbic prefrontal cortex (IL) and prelimbic prefrontal cortex (PL) region in rodents and have opposite functions. The PL stimulates the BLA and CE resulting in the facilitation of fear expression whereas the IL is important for fear memory extinction. Fear memory extinction is regulated via the inhibition of CEM fear output neurons, a process that is dependent on the IL and will lead to a decrease in the CR. Inactivation of the BLA has also shown to block the acquisition of fear memory extinction, indicating the important contribution of the BLA in this process (Figure 1) (Parkes & Westbrook, 2010).

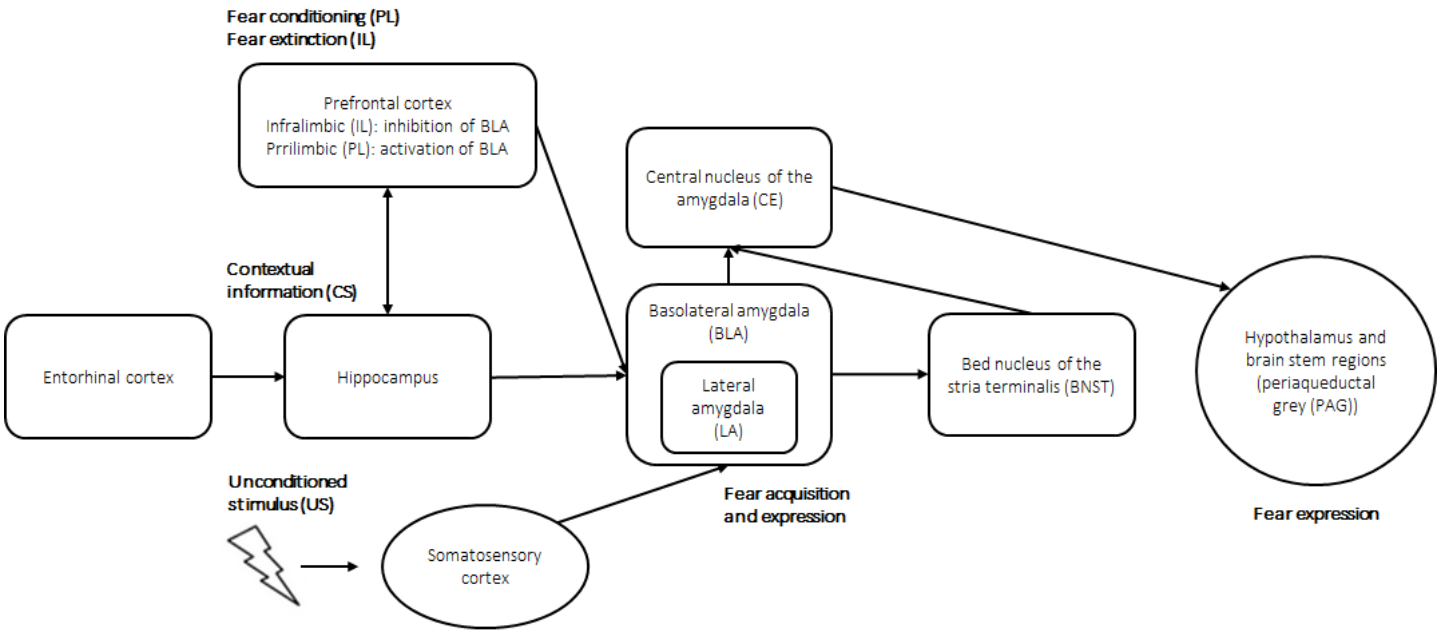

FIGURE 1: THE NEUROCIRCUITRY OF CONTEXTUAL FEAR CONDITIONING.
FIGURE COMPOSED USING SEVERAL SOURCES (KIM & CHO, 2020) (ROMANSKI, CLUGNET, & BORDI, 1993) (SULLIVAN, APERGIS, & BUSH, 2004) (CIOCCHI, HERRY, & GRENIER, 2010) (PARKES & WESTBROOK, 2010)

The acquisition and consolidation of fear memory extinction is therefore characterized by a hippocampus-dependent IL inhibition of the CE resulting in decreased freezing behavior. Exactly this process is impaired in PTSD patients leading to persistent contextual fear conditioning (Milad, Rauch, & Pitman, 2006). The hippocampus, amygdala, BNST and PFC are all innervated by the serotonergic system and contain a high number of 5-HT receptors, indicating that 5-HT plays an important role during physiological contextual fear conditioning (Berumen, Rodríguez, & Miledi, 2012) (Puig & Gulledge, 2011) (Asan, Steinke, & Lesch, 2013) (Britton, Evans, & Hernandez, 2014).

3. Serotonergic modulation of contextual fear conditioning

Serotonergic neurons which synthesize 5-HT are located along the midline of the brain stem particularly in the raphe nucleus, with their axons innervating almost every region of the CNS. As a result, the serotonergic system affects a great variety of behaviors including contextual fear learning and memory (Frazer & Hensler, 1994).

In the development of serotonergic neurons, the transcription factors LIM homeobox transcription factor 1-bèta (Lmx1b) and pheochromocytoma 12 ETS factor-1 (Pet1) are essential. Limxb1 is expressed earlier than Pet1 and has an overall broader role in the development. In contrast, Pet1 is specifically expressed in serotonergic neurons for the activation of important genes involved in serotonergic metabolism and neurotransmission, including tryptophan hydroxylase 2 (Thp2), the vesicular monoamine transporter 2 (Vmat2) and the 5-HT transporter (5-HTT) (Hendricks, Fyodorov, & Wegman, 2003).

When serotonergic neurons are completely developed, 5-HT is synthesized from the essential amino acid tryptophan via the enzyme Thp2 (Natarajan, de Boer, & Koolhaas, 2009). After its synthesis, 5-HT is packaged into vesicles and transported near the presynaptic membrane by Vmat2. Upon the arrival of an action potential to the presynaptic serotonergic neuron, vesicles fuse with the cell membrane and release 5-HT in the synaptic cleft. Then, 5-HT can exert multiple effects by binding to one or more of the 7 types of 5-HT receptors (5-HT1-5-HT7), among which 17 receptor subtypes, on postsynaptic neurons in mammalians. Except for the 5-HT3 receptors which are ligand-gated ion channel receptors, all 5-HT receptors are G-protein coupled receptors (Kriegebaum, Gutknecht, & Schmitt, 2010). Eventually, 5-HT signaling termination is regulated via the binding of 5-HTT to extracellular 5-HT resulting in the recycling or breakdown of 5-HT by monoamine oxidase (MAO) (Adell, Celada, & Abellàn, 2002).

Brain structures involved in contextual fear conditioning processes, including the hippocampus, amygdala, BNST and PFC, contain a large number of several 5-HT receptors making them a principal target of 5-HT (Hensler, 2006). However, to comprehend the serotonergic modulation of contextual fear conditioning, it is important to know the individual effect of 5-HT on 5-HT receptor (sub)types since each type has unique characteristics and a distinct distribution pattern in the CNS. This is an overall complex process because 5-HT receptor (sub)types can be present on both excitatory glutamatergic as inhibitory GABAergic interneurons and multiple 5-HT receptor (sub)types can be located on one cell (McDonald & Mascagni, 2007). In the hippocampus, amygdala, BNST and PFC especially 5-HT1, 5-HT2 and 5-HT3 receptor (sub)types are abundantly expressed (Huang, Tan, & Huang, 2007). Therefore, the following paragraphs focus on the effects of these specific 5-HT receptor (sub)types on the several processes involved in contextual fear conditioning.

3.1 The effects of 5-HT1 receptor (sub)types on contextual fear conditioning

In humans, the 5-HT-1 receptor can be divided into 5 receptor subtypes (5-HT1A-5-HT1F). The 5-HT1A receptor exists both as an autoreceptor on presynaptic serotonergic neurons in the raphe nucleus as on postsynaptic neurons in limbic and cortical regions. In the raphe nucleus, 5-HT1A autoreceptors regulate the inhibition of the firing of serotonergic neurons upon 5-HT release, which is autoregulated by the 5-HT1B and 5-HT1D receptor in rodents and humans, respectively (Kriegebaum, Gutknecht, & Schmitt, 2010). On the other hand, 5-HT1A heteroreceptors in limbic and cortical regions regulate local responses to 5-HT like the 5-HT1B and 5-HT1D receptor (McDonald & Mascagni, 2007).

The local response of the 5-HT1A heteroreceptor on contextual fear conditioning has been studied in a 5-HT1A knock-out mouse model by Klemenhagen et al. In this study, 5-HT1A knock-out mice demonstrated increased fear responses (Klemenhagen, Gordon, & David, 2005). In addition, injections of the selective 5-HT1A receptor agonist flesinoxan into the hippocampus and amygdala after contextual fear conditioning resulted into reduced freezing behavior during re-exposure to the CS in male Sprague-Dawley rats. However, no differences in fear responses were observed when flesinoxan was infused into the PFC (Li, Inoue, & Abekawa, 2006). Furthermore, administration of the 5-HT1A agonist cannabidiol in the BNST after contextual fear conditioning also caused reduced freezing behavior during re-exposure to the CS in male Wistar rats (Gomes, Reis, & Alves, 2010). These findings indicate that the 5-HT1A heteroreceptor exerts its effect via the hippocampus, amygdala and BNST and mediates a reduced fear acquisition and thereby fear response after re-exposure to the CS.

3.2 The effects of 5-HT2 receptor (sub)types on contextual fear conditioning

The 5-HT2A, 5-HT2B and 5-HT2C receptor are subtypes of the 5-HT2 receptor and mainly found in the BLA where they are located both on excitatory glutamatergic as on inhibitory GABAergic interneurons. In the BLA, the primarily response to 5-HT is an inhibitory response due to the depolarization of parvalbumin-positive GABAergic interneurons which also induce the inhibition of excitatory glutamatergic neurons (Stutzmann & LeDoux, 1999) (McDonald & Mascagni, 2007).

The specific role of the 5-HT2A receptor on contextual fear memory consolidation and extinction has been investigated in male C57BL/6J mice. For this, the 5-HT2A receptor agonist TCB-2 was administered intraperitoneally before re-exposure to the CS and increased the acquisition of fear memory extinction. Furthermore, injections of the 5-HT2A receptor antagonist MDL 11939 before re-exposure to the CS decreased the acquisition of fear memory extinction. Besides, administration of the TCB-2 agonist after contextual fear conditioning enhanced contextual fear memory, indicating that the 5-HT2A receptor facilitates contextual fear memory consolidation. However, no differences in fear memory retrieval were observed (Zhang, Asgeirsdottir, & Cohen, 2013). These findings indicate that serotonergic activation of the 5-HT2A receptor has particularly an important role on fear memory consolidation and extinction learning. It is unclear which brain structures were involved and mediated fear memory acquisition and extinction because the 5-HT2A receptor agonist was administered intraperitoneally. Nevertheless, fear memory extinction is regulated by a hippocampus-dependent IL inhibition of the CE resulting in decreased fear expression (Milad, Rauch, & Pitman, 2006). Therefore, 5-HT2A receptors regulating fear memory extinction are most likely present in the hippocampus, amygdala and PFC.

More recently, the role of hippocampal 5-HT2A receptors in specific was investigated by Jing et al., using contextual fear conditioning in male Sprague-Dawley rats. The specific 5-HT2A receptor agonist TCB-2 or the antagonist MDL 11939 was administered in the hippocampus before contextual fear conditioning. Administration of the 5-HT2A agonist TCB-2 enhanced whereas the antagonist MDL 11939 blocked contextual fear learning and memory (Jiang, Wang, & Yin, 2020).

On the contrary, a 5-HT2C knock-out mouse model demonstrated reduced anxiety-like behavior characterized by a decreased c-Fos expression in the BNST and CE upon a threatful stimuli (Heisler, Zhou, & Bajwa, 2007). In addition, administration of the 5-HT2C receptor antagonist asenapine before re-exposure to the CS reduced freezing behavior in male Sprague-Dawley and Wistar rats, indicating that the 5-HT2C receptor mediates fear expression (Ohyama, Kondo, & Yamauchi, 2016).

3.3 The effects of 5-HT3 receptor (sub)types on contextual fear conditioning

The 5-HT3 receptor is comprised of two subtypes, the 5-HT3A and 5-HT3B receptor. Particularly the 5-HT3A receptor is expressed on postsynaptic neurons located in the hippocampus, amygdala and PFC, indicating its essential role in contextual fear conditioning processes (Tecott, Marica, & Julius, 1993). In the BLA and CE, 5-HT3 receptors are almost

exclusively expressed on inhibitory GABAergic neurons (Mascagni & McDonald, 2007).

To investigate the role of 5-HT3A on contextual fear conditioning, Kondo et al., used a 5-HT3A knock-out mouse model. The 5-HT3A receptor appeared not to be involved in fear memory acquisition and retrieval, but displayed to be crucial for contextual fear memory extinction (Kondo, Nakamura, & Ishida, 2014). Since PTSD patients are characterized by impaired contextual fear memory extinction, Wu et al., studied if stimulation of the 5-HT3 receptor in the dorsal hippocampus stimulates contextual fear memory extinction in male Sprague-Dawley rats. Infusion of the selective 5-HT3 agonist SR57227 into the dorsal hippocampus after a single prolonged stress treatment was followed by contextual fear conditioning after three weeks. Treated-rats demonstrated enhanced contextual fear memory extinction, indicating that the 5-HT3 receptor is particularly involved in fear memory extinction (Wu, Yang, & Cui, 2017).

4. Genetic predisposition to serotonergic dysfunction and persistent contextual fear conditioning

As described above, the serotonergic system is essential for physiological processing of contextual fear conditioning. Remarkably, some individuals seem to be more vulnerable for the development of PTSD after a traumatic event than others, indicating that there exists a genetic predisposition (Steimer, 2002). Therefore, genetic predispositions to serotonergic dysfunction have widely been studied for their risk in the development of PTSD (Waider, Araragi, & Gutknecht, 2011).

4.1 Genetic variations in the development of serotonergic neurons

The Pet1 transcription factor is essential in the development of serotonergic neurons by activating 5-HT regulating genes (Hendricks, Fyodorov, & Wegman, 2003). In humans, a common Pet1 single nucleotide polymorphism (rs860573) has been associated with threat-related amygdala activity and processing and psychopathology. To investigate this genetic predisposition of Pet1 to serotonergic dysfunction and persistent contextual fear conditioning, Pet1 knock-out mice were submitted to contextual fear conditioning. Pet1 knock-out mice were characterized by a significant loss of serotonergic neurons and demonstrated increased fear acquisition and memory. In addition, Pet1 knock-out mice displayed a higher number of fear recovery after fear memory extinction, indicating that Pet1 gene variations can decrease the number of serotonergic neurons in the CNS leading to persistent contextual fear conditioning (Wellman, Camp, & Jones, 2013).

4.2 Genetic variations in 5-HT synthesis

Serotonergic neurons synthesize 5-HT from tryptophan via the enzyme Thp2. Therefore, Thp2 gene defects and variants influence 5-HT synthesis and the serotonergic modulation of contextual fear conditioning (Hendricks, Fyodorov, & Wegman, 2003).

In humans, a rare mutation in the Thp2 gene (R441H) is identified in a few patients suffering from a major depressive disorder. The effect of this mutation on 5-HT synthesis was investigated in a knock-in mouse model which expressed a hypomorph allele (R439H) of the Thp2 gene resembling the mutation in humans. Homozygous R439H Thp2 knock-in mice demonstrated a decrease of ~ 80% in 5-HT synthesis in the striatum, PFC and hippocampus whereas heterozygote R439H Thp2 knock-in mice showed only a 40% reduction. In addition, homozygous knock-in mice were associated with an overall lower 5-HT level in the striatum, PFC and hippocampus, but heterozygous knock-in mice demonstrated only a reduction in the PFC, indicating that the serotonergic modulation differs among several brain structures (Beaulieu, Zhang, & Rodriguiz, 2008). Since PTSD is often accompanied by a major depression, these findings suggest that this Thp2 mutation can also have a pathophysiological role in PTSD and more psychiatric disorders (Segi-Nishida, 2017).

Furthermore, inbred mouse lines such as the BALB/c and DBA/2 strains have found to be homozygous for the specific 1473G Thp2 gene variant. These mice demonstrate a 40% decrease in 5-HT levels in the cortex and striatum compared to C57BL/6J and 129S1/SvJ mouse lines which are homozygous for the 1473C Thp gene variant. Therefore, these

findings confirm the idea that there exists a relation between naturally occurring Thp2 gene variants and serotonergic dysfunction leading to reduced 5-HT synthesis (Zhang, Beaulieu, & Sotnikova, 2004)

However, the effect on contextual fear conditioning has only been investigated via a Thp2 knock-out model by Waider et al. in male mice with a mixed Sv129/C57BL/6N genetic background. 5-HT deficient mice displayed increased contextual fear conditioning and memory, indicating that a Thp2 induced 5-HT deficiency causes a dysfunction of the 5-HT receptor mediated inhibition of the neurocircuitry which controls contextual fear conditioning (Waider, Popp, & Mlinar, 2019).

4.3 Genetic variations in 5-HT storage

Before the arrival of an action potential to serotonergic neurons and the release of 5-HT, 5-HT is stored into vesicles and transported close to presynaptic membranes by Vmat2 (Hendricks, Fyodorov, & Wegman, 2003). A previous study already associated certain genetic variations in the SCL1842 gene, the gene encoding for Vmat2, as a risk factor for the development of PTSD (Solovieff, Roberts, & Ratanatharathorn, 2014). More recently, a decrease in Vmat2 protein expression was also observed in postmortem brains of PTSD patients compared to postmortem brains of non-PTSD patients and thereby associating a Vmat2 dysfunction with PTSD (Bharadwaj , Jaffe, & Chen, 2016).

Therefore, the effect of Vmat2 dysfunction on the serotonergic modulation of contextual fear conditioning was investigated in transgenic mice that expressed either 5% (VMAT2-LO) or 200% (VMAT-HI mice) of wild type Vmat2 protein levels. VMAT2-LO mice demonstrated reduced Vmat2 protein levels in the hippocampus and amygdala, impaired vesicular storage capacity in the striatum and PFC and excessive contextual fear expression on the behavioral level compared to control mice. In contrast, VMAT2-HI mice were characterized by increased Vmat2 protein levels in the brain and higher vesicular storage capacity compared to control mice. However, on the behavioral level, VMAT2-HI mice demonstrated similar responses as wild type mice. These findings indicate that a reduced presynaptic function of Vmat2 is associated with increased contextual fear conditioning and PTSD-like behavior (Branco, Burkett, & Black, 2020).

Nevertheless, Vmat2 is not only expressed in serotonergic neurons but in all monoaminergic neurons, which was confirmed in a homozygous Vmat2 knock-out mouse model wherein mice demonstrated a significant lack of all monoamines and were not even viable (Alvarez, Vitalis, & Fon, 2002). On the other hand, heterozygous Vmat2 knock-out mice showed a 34% reduction in 5-HT, 42% in dopamine and 23% in norepinephrine, but were viable (Fon, Pothos, & Sun, 1997). It must therefore be taken into account that the development of PTSD-like behavior in VMAT2-LO mice as described before, is in all probability not only due to a serotonergic dysfunction.

4.4 Genetic variations in 5-HTT function

Serotonin signaling termination is regulated via 5-HTT. Therefore, 5-HTT has an essential role in serotonergic system homeostasis and 5-HTT gene variations can directly influence extracellular 5-HT concentrations, 5-HT signaling and contextual fear conditioning processes (Shan, Guo, & van den Heuvel, 2018).

One extensively studied 5-HTT gene variation is a functional polymorphism in the promoter region of the 5-HTT transporter gene (5-HTTPR). This polymorphism is characterized by a short (s) allele of the 5-HTT promoter region and is associated with a decreased transcription of the 5-HTT gene in contrast to the long (l) allele, resulting in a decreased 5-HTT function (Margoob & Mushtaq, 2011). Shan et al., investigated whether 5-HTT knock-out mice, resembling the 5-HTT s allele, demonstrated impaired fear memory extinction by measuring the c-Fos expression. 5-HTT knock-out mice demonstrated a reduction in fear memory extinction after contextual fear conditioning which was characterized by a decreased c-Fos activity in the PFC and increased c-Fos activity in the CE (Shan, Guo, & van den Heuvel, 2018).

In contrast, humans carrying a certain 5-HTT gene variant which results in a higher 5-HTT expression demonstrated lower amygdala reactivity in response to threatful stimuli. The neurocircuitry underlying this effect was investigated in a 5-HTT

overexpression mouse model (5-HTTOE) resembling the human gene variation. Increased 5-HTT expression showed to impair 5-HT2A mediated recruitment of parvalbumin-positive interneurons in the amygdala during fear resulting in inhibition of the BLA and the fear response (Bocchio, Fucsina, & Oikonomidis, 2015).

Taking these findings together, the s allele of the 5-HTTPR is a potential risk factor for PTSD which is probably due to a decreased PFC inhibition of the parvalbumin-positive interneurons in the BLA leading to impaired fear memory extinction (Shan, Guo, & van den Heuvel, 2018).

5. The effect of fluoxetine treatment on persistent contextual fear conditioning

Genetic predispositions to serotonergic dysfunction have shown to be associated with PTSD-like behavior (Branco, Burkett, & Black, 2020) (Shan, Guo, & van den Heuvel, 2018). Therefore, one of the most widely used drug in the treatment of PTSD is the SSRI fluoxetine. Fluoxetine blocks the 5-HTT and thereby the reuptake of 5-HT into presynaptic serotonergic neurons causing an increase in extracellular 5-HT levels that repeatedly stimulate postsynaptic 5-HT receptors (Ohira, Hagihara, & Miwa, 2019). In addition, fluoxetine is a competitive 5-HT2C receptor antagonist leading to reduced fear expression mediated by the amygdala (Ni & Miledi, 1997) (Ohyama, Kondo, & Yamauchi, 2016).

Although acute fluoxetine treatment results in an immediate increase of extracellular 5-HT levels, an initial worsening of the patients' symptoms are observed instead of the relief of persistent fear (Hervàs & Artigas, 1998). This initial worsening is caused by the activation of 5-HT1A autoreceptors in the raphe nucleus leading to inhibited firing of serotonergic neurons and in response, inhibited 5-HT signaling (Czachura & Rasmussen, 2000).

Under normal conditions, these 5-HT1A autoreceptors are particularly located on extrasynaptic parts of the somatodendritic plasma membrane of serotonergic neurons in the raphe nucleus (Figure 2A). However, one hour after the administration of a single dose of the 5-HT1A receptor agonist 8-OH-DPAT or fluoxetine, a partial internalization of 5-HT1A autoreceptors in rat serotonergic neurons was observed by Descarries et al (Figure 2B-C). Administration of 8-OH-DPAT and fluoxetine together did not lead to a higher number of 5-HT1A internalized 5-HT1A autoreceptors in the raphe nucleus (Figure 2D). Furthermore, prior administration of the 5-HT1A receptor antagonist WAY 100635 before 8-OH-DPAT or fluoxetine treatment blocked the internalization of 5-HT1A autoreceptors in the raphe nucleus, indicating that the internalization of 5-HT1A autoreceptors is the effect of 5-HT1A autoreceptor activation (Figure 2E-F) (Descarries & Riad, 2012). This same finding has been observed using [^{18}F]MPPF PET imaging of 5-HT1A autoreceptors in the raphe nucleus in healthy human volunteers who received a single administration of fluoxetine (Sibon, Benkelfat, & Gravel, 2008).

However, a therapeutic effect can be observed after chronic fluoxetine treatment in PTSD patients which is likely the result of the desensitization of 5-HT1A autoreceptors in the raphe nucleus leading to normal 5-HT signaling (Santos, Martinez, & Brandão, 2006). Therefore, Descarries et al., investigated in an additional experiment whether chronic fluoxetine treatment prevents the internalization and thereby activation of 5-HT1A autoreceptors in the raphe nucleus. After 3 weeks of fluoxetine treatment, 5-HT1A autoreceptors were observed mainly on the somatodendritic plasma membrane of rat serotonergic neurons (Figure 2I) equivalent to the control (Figure 2G). As before, acute fluoxetine treatment resulted in the internalization of 5-HT1A autoreceptors in the raphe nucleus (Figure 2H) (Descarries & Riad, 2012). These results suggest that the repeated internalization of 5-HT1A autoreceptors in the raphe nucleus during chronic fluoxetine treatment eventually leads to the desensitization of 5-HT1A autoreceptors and physiological 5-HT signaling.

Therefore, chronic fluoxetine treatment has widely been studied on contextual fear conditioning in rodents to explore the therapeutic effect on persistent contextual fear conditioning in PTSD patients through modulation of the serotonergic system.

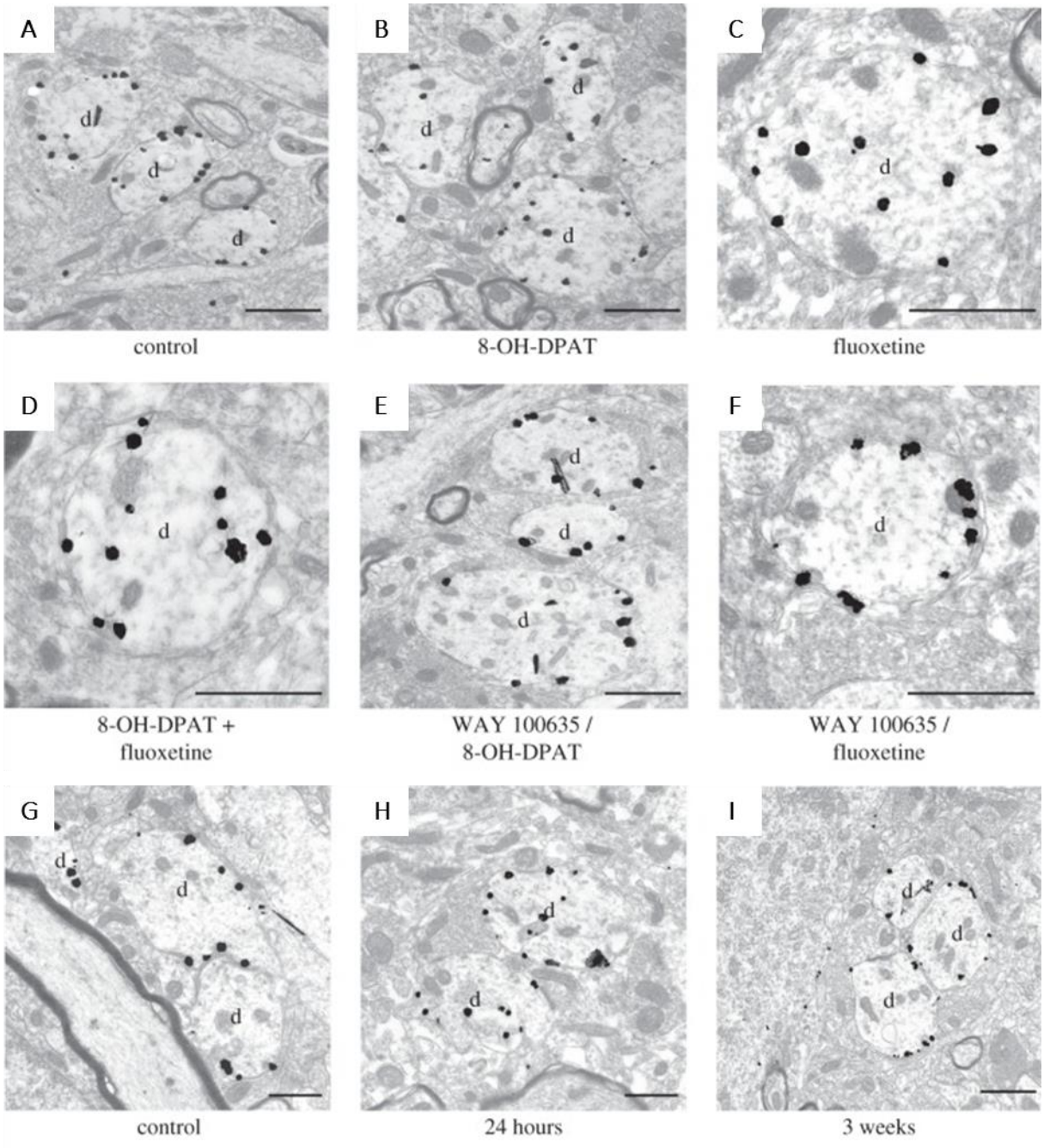

FIGURE 2: INTERNALIZATION AND ACTIVATION OF 5-HT1A AUTORECEPTORS IN RAT DORSAL RAPHE NUCLEUS DENDRITES (D) AFTER ACUTE 8-OH-DPAT AND/OR FLUOXETINE TREATMENT, BUT DEACTIVATION AFTER CHRONIC FLUOXETINE TREATMENT. SUBCELLULAR DISTRIBUTION OF 5-HT1A AUTORECEPTORS IN THE RAPHE NUCLEUS 1 HOUR AFTER THE ADMINISTRATION OF THE 5-HT1A RECEPTOR AGONIST 8-OH-DPAT (B) OR FLUOXETINE (C) VISUALIZED USING IMMUNO-ELECTRON MICROSCOPY. IN A CONTROL RAT, 5-HT1A AUTORECEPTORS ARE LOCALIZED AT THE PLASMA MEMBRANE (A). AFTER THE ADMINISTRATION OF 8-OH-DPAT (B), FLUOXETINE OR BOTH DRUGS TOGETHER, INTERNALIZATION OF 5-HT1A AUTORECEPTORS IS OBSERVED (B-D). HOWEVER, ADMINISTRATION OF THE 5-HT1A RECEPTOR ANTAGONIST WAY 100635 10 MINUTES PRIOR TO 8-OH-DPAT (E) OR FLUOXETINE (F) TREATMENTS BLOCKS THIS INTERNALIZATION. AN ADDITIONAL EXPERIMENT DEMONSTRATED THE SAME SUBCELLULAR LOCATION OF 5-HT1A AUTORECEPTORS AT THE PLASMA MEMBRANE OF RAT DORSAL RAPHE NUCLEUS DENDRITES IN A CONTROL (G) AND 5-HT1A AUTORECEPTOR INTERNALIZATION 24 HOURS AFTER FLUOXETINE TREATMENT (H). HOWEVER, DEACTIVATION OF THE 5-HT1A AUTORECEPTORS WAS OBSERVED AFTER 3 WEEKS OF FLUOXETINE TREATMENT (DESCARRIES & RIAD, 2012).

Sanders et al., investigated the effect of chronic fluoxetine treatment prior to contextual and tone-cued fear conditioning in C57BL/6J male mice. Fluoxetine-treated mice demonstrated a significant decrease in freezing behavior during re-exposure to the CS. On the other hand, no differences in freezing behavior were observed during tone-cued fear conditioning, indicating that chronic fluoxetine treatment seems to specifically reduce contextual fear conditioning by blunting of hippocampal-mediated fear memory processes (Sanders & Mayford, 2016).

However, fluoxetine is not administered before the traumatic event in PTSD patients. It is therefore important to investigate whether chronic fluoxetine administration after the traumatic event would facilitate fear memory extinction upon re-exposure to the CS. This was in fact studied by Yu et al., who treated C57BL/6J male mice for 14 days with fluoxetine after contextual fear conditioning . During the situational reminder phase which resembled the re-experience of the traumatic event, fluoxetine-treated mice demonstrated a significant decrease in freezing time compared to control mice (Figure 3). These findings suggest that two weeks of fluoxetine treatment resulted in a decrease of contextual fear acquisition and consolidation (Yu, Ou, & Shyu, 2020).

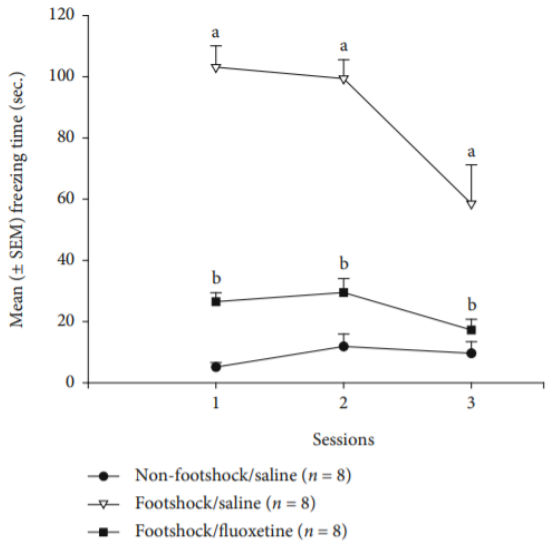

FIGURE 3: THE MEAN (± STANDARD ERROR OF THE MEAN (SEM)) FREEZING TIME IN SECONDS DURING THREE SITUATIONAL REMINDER PHASE SESSIONS OF THE NON-FOOT SHOCK/SALINE (N=8), FOOT SHOCK/SALINE (N=8) AND FOOT SHOCK/FLUOXETINE (N=8) (YU, OU, & SHYU, 2020).

In addition, the severity of contextual fear learning, memory and expression after a traumatic event can differ between individuals due to a genetic predisposition (Steimer, 2002). Therefore, Santos et al., administered fluoxetine subchronic after moderate and high contextual fear conditioning which was depending on the amplitude of the foot shock in Wistar rats. The effect on freezing behavior was measured upon re-exposure to the CS. After moderate contextual fear conditioning, fluoxetine-treated rats demonstrated no significant differences in freezing time compared to control rats. However, after high contextual fear conditioning fluoxetine-treated rats showed a significantly lower time of freezing behavior. These findings suggest that fluoxetine restored serotonergic functions in brain structures that are involved during high contextual fear conditioning and lead to a decrease in contextual fear acquisition and an increase in fear memory extinction (Santos, Martinez, & Brandão, 2006).

In humans, the effectiveness of chronic fluoxetine treatment has predominantly been investigated in adult PTSD patients, but resulted in contrary outcomes. In a 12-week double-blind, randomized, placebo-controlled study, patients were predominantly male and had mainly been exposed to a combat-related traumatic event. The findings of this trial demonstrated that fluoxetine-treated patients displayed improvements on the behavioral level compared to placebo-treated PTSD patients (Martenyi, Brown, & Zhang, 2002). However, in another 12-week double-blind, placebo-controlled trial of chronic fluoxetine treatment in adult PTSD patients, no differences were observed between fluoxetine-treated and placebo-treated PTSD patients on the behavioral level. In contrast to the above mentioned trial, this trial consisted mainly of women instead of men (Martenyi, Brown, & Caldwell, 2007).

6. Discussion and conclusion

This study focused on the effect of fluoxetine treatment on persistent contextual fear conditioning in PTSD through modulation of the serotonergic system. It was hypothesized that fluoxetine counteracts a serotonergic dysfunction and thereby

restores a physiological functioning of contextual fear conditioning processes.

The neurocircuitry of contextual fear conditioning has mainly been investigated in rodents which demonstrated that it is a complex process regulated by several brain structures, including the hippocampus, amygdala, BNST and PFC (Adolphs, 2013). Although human fear expressions are not identical to those of rodents and other animals when they are exposed to the same threat, there has been proposed that fear is a biologically primitive emotion characterizing for all humans and other animals, indicating that the feeling of emotional states can be the same (Ekman, 1992). Therefore, the use of rodents for discovering the neurocircuitry of contextual fear conditioning is applicable on humans.

In the case of PTSD, the hippocampal-dependent IL inhibition of the amygdala is impaired leading to poor fear memory extinction and in response, persistent contextual fear conditioning (Milad, Rauch, & Pitman, 2006). The serotonergic system has shown to modulate contextual fear conditioning processes especially via the 5-HT1, 5-HT2 and 5-HT3 receptor in the hippocampus, amygdala, BNST and PFC. The 5-HT1A heteroreceptor in the hippocampus, amygdala and BNST showed to mediate a reduced fear acquisition and in response, decreased fear expression (Li, Inoue, & Abekawa, 2006) (Gomes, Reis, & Alves, 2010). In the raphe nucleus, the 5-HT1A autoreceptor is present on serotonergic neurons as well and modulates the inhibition of the firing of serotonergic neurons and thereby 5-HT signaling (Kriegebaum, Gutknecht, & Schmitt, 2010). Moreover, the 5-HT2A and 5-HT3A receptors are particularly important for fear memory extinction whereas the 5-HT2C receptor is mainly involved in mediating anxiety-like behavior (Jiang, Wang, & Yin, 2020) (Ohyama, Kondo, & Yamauchi, 2016) (Wu, Yang, & Cui, 2017).

Following the important modulating role of the serotonergic system on contextual fear conditioning processes and the given that some individuals are more vulnerable for the development of PTSD after a traumatic event than others, it was hypothesized that there exists a genetic predisposition for a serotonergic dysfunction leading to increased contextual fear conditioning (Steimer, 2002). This was confirmed by multiple researchers who found genetic variations in the Pet1, Thp2, Vmat2 and 5-HTT gene which were all associated with a serotonergic dysfunction and PTSD-like behavior (Wellman, Camp, & Jones, 2013) (Beaulieu, Zhang, & Rodriguiz, 2008) (Branco, Burkett, & Black, 2020) (Shan, Guo, & van den Heuvel, 2018).

Therefore, one of the most widely used drug in the treatment of PTSD is the SSRI fluoxetine (Ohira, Hagihara, & Miwa, 2019). It was hypothesized that fluoxetine exerts its therapeutic mechanism by elevating extracellular 5-HT levels which eventually results in normal 5-HT signaling and contextual fear conditioning processes. Acute fluoxetine treatment was found to induce the internalization and activation of 5-HT1A autoreceptors in the raphe nucleus leading to an initial worsening of patient's symptoms through the inhibition of 5-HT signaling. However, chronic fluoxetine treatment demonstrated to block the internalization of 5-HT1A autoreceptors which resulted in deactivation of 5-HT1A autoreceptors (Descarries & Riad, 2012). In addition, chronic fluoxetine treatment resulted in less freezing behavior upon re-exposure to the CS after contextual fear conditioning in rodents (Yu, Ou, & Shyu, 2020) (Santos, Martinez, & Brandão, 2006) (Sanders & Mayford, 2016). Taking together, these findings suggest that the delayed therapeutic effect of chronic fluoxetine treatment is due to the desensitization of 5-HT1A autoreceptors with time, the increase in extracellular 5-HT levels resulting in normal 5-HT signaling and the blockade of 5-HT2C receptors.

Although 5-HT receptors instantly play a key role in the modulation of contextual fear conditioning and improvements after chronic fluoxetine treatment, it is more likely that the therapeutic effects on the longer-term are the result of downstream pathways mediated by the serotonergic system. This is indeed the case as demonstrated by other research which showed that chronic fluoxetine treatment stimulated neurogenesis in the hippocampus and cortex of male mice with a mixed C57BL/6J and 129S6/SvEvTac genetic background (Sachs & Caron, 2015). In addition, chronic fluoxetine treatment also demonstrated to induce synaptic plasticity and

hippocampal dendritic spine density (Ohira, Hagihara, & Miwa, 2019) (Pedraza, Sierra, & Giachero, 2019). Since these molecular and structural changes specifically take place in the hippocampus after chronic fluoxetine treatment, this can possibly explain why chronic fluoxetine treatment did not seem to have any beneficial effects on amygdala-dependent tone-cued fear conditioning (Sanders & Mayford, 2016).

In addition to 5-HT, fluoxetine treatment has also shown to increase extracellular norepinephrine and dopamine levels in the PFC (Bymaster, Zhang, & Carter, 2002). Since norepinephrine is able to facilitate fear learning, it is important to keep in mind that any beneficial effect on persistent contextual fear conditioning and extinction in PTSD patients is not only due to the serotonergic system (Giustino & Maren, 2018).

Furthermore, a lot of studies have investigated the effect of chronic fluoxetine treatment on fear memory extinction upon re-exposure to the CS after contextual fear conditioning as mentioned before. However, the effect of chronic fluoxetine treatment on fear overgeneralization, the process in which PTSD patients are unable to restrict their fear expression to harmful situations related to their trauma leading to fear expression in neutral environments non-related to their trauma, has been investigated to a much lower extent. Recently, Pedraza et al., did investigate this to test a new pharmacological approach of PTSD. In this study, chronic fluoxetine treatment after contextual fear conditioning led to contextual discrimination upon exposure to a new context whereas control rats displayed fear generalization due to the disability to discriminate between the contexts (Pedraza, Sierra, & Giachero, 2019). These findings indicate that chronic fluoxetine most likely will also have a beneficial effect on fear overgeneralization, but more research is necessary to confirm this.

More surprisingly are the contradicting results that have been found regarding the efficacy of chronic fluoxetine treatment in human trials including adult PTSD patients (Martenyi, Brown, & Caldwell, 2007) (Martenyi, Brown, & Zhang, 2002). These results indicate that there exist in all probability more underlying dysfunctions instead of a serotonergic dysfunction only. Multiple studies indeed have shown a large number of genetic variations which are associated with PTSD-like behavior (Cornelis, Nugent, & Amstadter, 2010). Another striking difference is that the effect of chronic fluoxetine on contextual fear conditioning in rodents has mostly been investigated in male mice or rats. However, women are affected two to three times more often in the human population with PTSD (Olff, 2017). It is important to take these differences into consideration since men demonstrated to have a 52% higher brain 5-HT synthesis than women which can contribute to the better outcomes in rodents (Nishizawa, Benkelfat, & Young, 1997).

In conclusion, contextual fear conditioning processes are modulated via the serotonergic system, particularly via the 5-HT1, 5-HT2 and 5-HT3 receptor. Not surprising, humans carrying a genetic variation in essential genes for the development of serotonergic neurons and 5-HT signaling such as Pet1, Thp2, Vmat2 and 5-HTT displayed to be predisposed to a serotonergic dysfunction leading to PTSD-like behavior. Acute fluoxetine treatment immediately increases 5-HT levels in several brain regions, but worsens patients' symptoms via the internalization and activation of 5-HT1A autoreceptors in the raphe nucleus. In contrast, chronic fluoxetine treatment leads to the deactivation of 5-HT1A autoreceptors and an increase in extracellular 5-HT levels resulting in normal 5-HT signaling. In addition, fluoxetine blocks 5-HT2C receptors leading to a reduction in anxiety-like behavior. In rodents, chronic fluoxetine treatment displayed beneficial effects on contextual fear conditioning. However in human PTSD patients, chronic fluoxetine treatment resulted in contradicting findings indicating that there probably exist additional underlying dysfunctions which require more research.

Afterword

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