Neural circuits underlying learning and memory – the role of dorsal raphe nucleus and serotonin in fear conditioning

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Preface

This thesis was performed in the Neuroscience programme at the Kavli Institute of Systems Neuroscience at the Norwegian University of Science and Technology (NTNU) in Trondheim, Norway, and was written under the supervision of Prof. Dr. Emre Yaksi and co-supervision of PhD candidate Fabrizio Palumbo.

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Abstract

Learning and memory processes are essential for survival for any species, and the ability to learn and predict which situations or environmental contexts are profitable or hazardous can be beneficial. Both neurotransmitter 5-HT and its main producing brain structure, the dorsal raphe nucleus (DRN). has been implicated to play a role in such learning processes. The DRN, a structure part of the raphe nuclei, is further known to widely innervates cortical areas and structures of the brain stem and given its wide circuitry to areas serving different functions in the vertebrate CNS, it is deemed to exert a role involving a range of cognitive processes, thereunder learning. However, it is not fully understood how 5-HT DRN neurons are involved in these processes, and to what extent the neurotransmitter 5-HT plays a role. To test the hypothesis of the 5-HT neurons of the DRN being involved in learning, a conditioned place avoidance (CPA) behavioral assay was used for testing operant fear conditioning, to examine the role of 5-HT DRN neurons in learning.

By experimental work in a host lab, 3-4 week-old transgenic zebrafish larvae were used and non-invasive chemogenetical manipulations were performed to specifically ablate 5-HT DRN neurons in these animals. In response to the chemogenetic ablation there were no signs of motor disease, survival, growth problems for the animals. In a CPA protocol involving the use of a mild aversive stimulus (electrical shock), 5-HT DRN ablated zebrafish showed a significant decrease in learning performance compared to control animals, and the impairment in learning was evident for multiple parameters of animal performance. Furthermore, by pharmacological manipulations by use of drugs targeting 5-HT, we aimed to explore whether the deficit in learning performance was related to the neurotransmitter 5-HT alone. 5-HT DRN ablated zebrafish treated with both buspirone and fluoxetine in separate experiments showed no improvement in learning performance and did not show any substantial difference in the CPA protocol. Lastly, immunohistochemical staining targeting phosphorylated ERK revealed activity patterns in the dorsal telencephalon (forebrain) of 5-HT DRN neurons different from control animals, showing elevated patterns of activity in the Dm and Dl areas of the zebrafish dorsal telencephalon. In conclusion, the results of this thesis provide evidence that 5-HT neurons in the DRN are involved in learning that involves a fear component.

Sammendrag

ærings- og hukommelsesprosesser er avgjørende for å overleve for enhver art, og evnen til å lære og forutsi hvilke situasjoner eller miljømessige sammenhenger som er lønnsomme eller farlige kan være fordelaktig. Både nevrotransmitter 5-HT og den viktigste produserende hjernestrukturen, dorsal raphe nucleus (DRN). har blitt involvert til å spille en rolle i slike læringsprosesser. DRN, en strukturdel av raphe-kjernene, er videre kjent for å innehaver kortikale områder og strukturer i hjernestammen, og gitt sitt brede kretsløp til områder som betjener forskjellige funksjoner i virveldyrets CNS, anses det å utøve en rolle som involverer et område av kognitive prosesser, derunder læring. Imidlertid er det ikke helt forstått hvordan 5-HT DRN-nevroner er involvert i disse prosessene, og i hvilken grad nevrotransmitteren 5-HT spiller en rolle. For å teste hypotesen om 5-HT-nevroner fra DRN som var involvert i læring, ble en betinget atferdsanalyse (CPA) atferdsanalyse brukt for å teste operatør fryktkondisjonering, for å undersøke rollen til 5-HT DRN nevroner i læring.

Ved eksperimentelt arbeid i et vertslaboratorium ble 3-4 uker gamle transgene sebrafisklarver brukt og ikke-invasive kjemogenetiske manipulasjoner ble utført for spesifikt å ablere 5-HT DRN nevroner i disse dyrene. Som respons på den kjemogenetiske ablasjonen var det ingen tegn til motorsykdom, overlevelse og vekstproblemer for dyrene. I en CPA-protokoll som involverte bruk av en mild aversiv stimulans (elektrisk sjokk), viste 5-HT DRN-abstrakt sebrafisk en signifikant reduksjon i læringsytelse sammenlignet med kontrolldyr, og svekkelsen i læring var tydelig for flere parametere for dyreprestasjoner. Ved farmakologiske manipulasjoner ved bruk av medisiner rettet mot 5-HT siktet vi videre å undersøke om underskuddet i læringsytelse var relatert til nevrotransmitteren 5-HT alene. 5-HT DRN abstrakt sebrafisk behandlet med både buspiron og fluoksetin i separate eksperimenter viste ingen bedring i læringsytelsen og viste ingen vesentlig forskjell i CPA-protokollen. Til slutt avslørte immunhistokjemisk farging rettet mot fosforylerte ERK aktivitetsmønstre i dorsaltelencephalon (forhjernen) av 5-HT DRN-nevroner forskjellig fra kontrolldyr, og viser forhøyede aktivitetsmønstre i Dm- og Dl-områdene i sebrafisk ryggtelencephalon. Avslutningsvis gir resultatene fra denne oppgaven bevis på at 5-HT-nevroner i DRN er involvert i læring som involverer en fryktkomponent.

List of abbreviations

5-HT serotonin
AFW artificial fish water
cAMP cyclic adenosine monophosphate
CNS central nervous system
CPA conditioned place avoidance
CS conditioned stimulus
DAPI 4',6-diamidino-2-phenylindole
DHB dorsal habenula
DHBl dorsolateral habenula
DMSO dimethyl sulfoxide
DP dorsal pallium
DPF days post fertilization
ERK extracellular signal-regulated kinase
GFP green fluorescent protein
GCaMP GFP, calcium-binding protein calmodulin (CaM), CaM-interacting peptide
GPb globus pallidus
Hb habenula
Hpf hours past fertilization

LA Lateral amygdala
LC locus coerelus
LMP low melting point
LTP long-term potentiation
LTD long-term depression
MAPK Ras/mitogen-activation protein kinase
NTR nitroreductase
O/N over night
RO H2O Reverse Osmosis water
TPH2 tryptophan hydroxylase 2
TH thalamus
US unconditioned stimulus
Wt wild type
WPF weeks post fertilization

1. Introduction

1.1 An introduction to learning

1.1.1 Associative and non-associative learning

The ability of an individual to predict threats and rewards, and adjust behavior according to changing environmental contexts, is crucial for ensuring survival. Learning such relationships based on past experiences provides the possibility to predict which situations and environmental contexts that are harmful or profitable and allows the individual to shape its behavior accordingly. As this type of learning is essential for survival, it has both developed and been conserved throughout evolution across different animal species (Waddell, 2019; Maren 2001). Learning has been a focus of scientific studies for a long period of time, and multiple types of learning ranging have been observed and studied. Examples range from such as fear learning in rodents, that establish a freezing behavior by observing other rodents receiving foot shocks (Jeon et al., 2010), to simple forms of sensory response learning in invertebrates (Brembs, 2003). Through studies of learning, a distinction of two types has been established: associative and non-associative learning. In non-associative learning, the animals does not establish a connection between the stimulus and its behavior. An example of a type of non-associative learning is habituation, where a decline is seen over time in a behavioral response after repeated exposure to a non-hazardous stimulus (Glanzman, 2009). In a classical study from 1970, habituation was first described in the species *Aplysia californica,* or the marine snail, where it was shown that the repeated delivery of a tactile stimulation to the skin of the organism caused a decline in a defensive withdrawal reflex of the gill of the animal (Pinsker et al., 1970). Habituation is widely regarded as one of the simplest forms of learning and has since its first description been shown in a variety of both vertebrate and invertebrate species, of both short-term and long-term (Christoffersen, 1997, Glanzman, 2009).

In contrast to habituation, associative learning entails processes where the animal does successfully establish a connection between two stimuli that occur closely in time and is able to learn and predict one stimuli from the other. (Fanselow 2016, Maren 2001). Associations being formed cannot be observed in itself however and observing changes in behavior becomes the only way to tell if an association has been formed. Therefore, paradigms studying associative learning have largely revolved and been

designed around tasks where clear changes in behavior can be observed in response to occurring stimuli (Fanselow, 2016).

1.1.2 Classical and operant conditioning

One commonly used and well-established paradigm designed for studying associative learning is conditioning, of which there are two main types (Pavlov, 1927; Skinner, 1984; Catania, 1984; Maren, 2001):

- Classical conditioning, or 'Pavlovian conditioning', describes a type of learning paradigm where the animal learns to associate two experienced stimuli that occur closely in time, and predict the one from the other (Pavlov, 1927; Fanselow, 2016).
- Operant conditioning, or 'instrumental conditioning', is a type of conditioning where the animals is instructed to perform a task to obtain a reward, or an instrumental outcome, named so because the behavioral is instrumental for obtaining the outcome (Fanselow, 2016, Skinner 1984, Catania 1984).

In both classical and operant conditioning, an association is formed between a biologically relevant and an irrelevant stimulus, as they are occurring closely in time. Classical and operant conditioning differ however in the contexts and pre-conditions of how the associations are to be formed. In classical conditioning, the animal learns to establish a connection between a conditional stimulus (CS), which is mostly a neutral stimulus, with a biologically relevant unconditional stimulus (US) (Pavlov, 1927, Maren, 2001). In such a paradigm, the chosen neutral stimulus does not lead to a change in behavioral response of the animal, whereas the unconditional stimulus does. Through the conditioning however, the neutral and unconditioned stimulus occur together (or closely in time), which leads to the behavioral response also appearing when the neutral stimulus is presented alone. The neutral stimulus is now considered a conditioned stimulus (CS) (Fanselow, 2016; Maren, 2001). In contrast, operant conditioning, it is the behavior of the animal that is decisive for the stimulus being experienced, meaning that the animal learns to associate that it is its own action that leads to a certain outcome (Skinner, 1984; Catania, 1984; Maren, 2001).

To illustrate the difference between classical and operant conditioning, consider Figure 1.1 depicting an example of each paradigm:

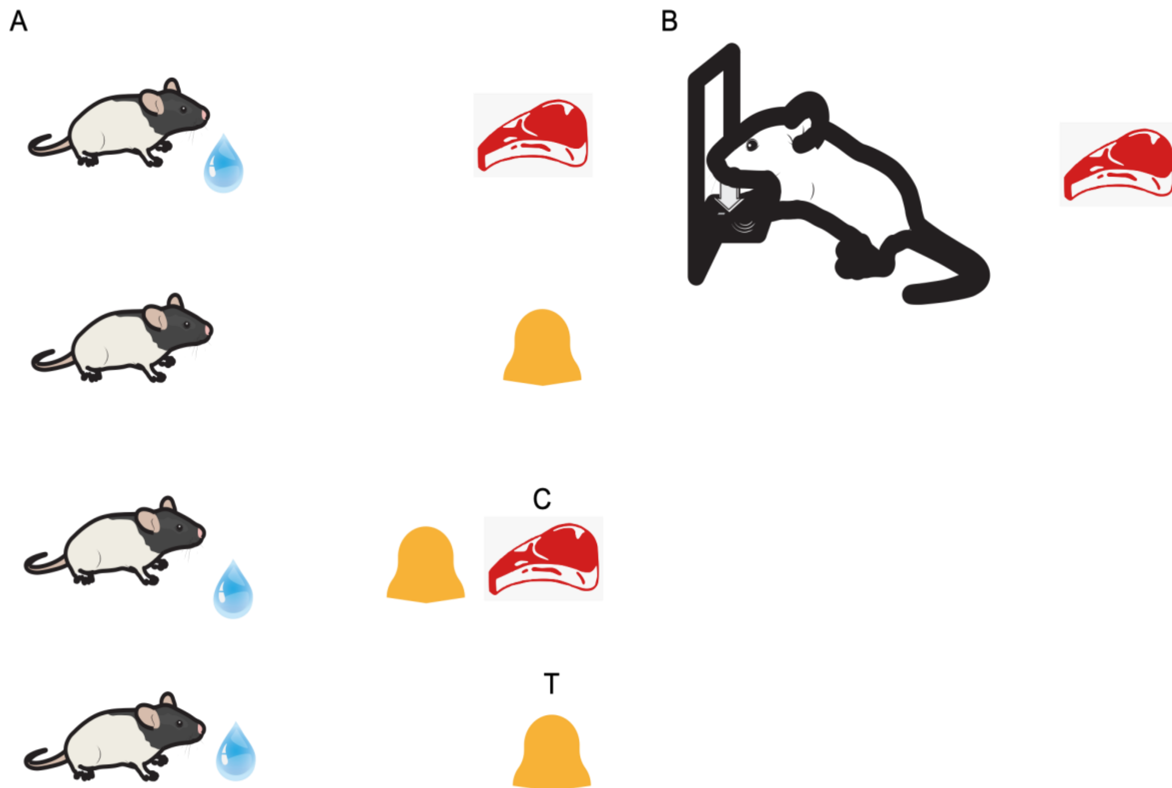

Figure 1.1 – Classical and operant conditioning: *A) Classical conditioning paradigm, based on Pavlov (1927). Food, serving as the conditional stimulus, elicits saliva production in the animal, but the tone of a bell does not. After conditioning trials where the tone and the food is presented closely in time, the animal learns to associate the two stimuli, and subsequent trials with the tone alone is enough to elicit the same response. B) Operant conditioning paradigm showing a mouse that has learned to press a trigger in order to receive food, associating its behavior with desired outcome. Based on Malkki et al., (2010). Illustrations adapted from scidraw.io.*

Depending on what type of stimulus is chosen as the unconditioned stimulus, the likelihood of a behavior response being elicited can be either increased or decreased, by use of reinforcers or punishers, respectively (Ferster, 1973). Reinforcers can be positively or pleasantly linked stimulus, such as food, and punishers can be stimulus such as electric shocks, that are unpleasant or even hazardous. This is determined positive and negative reinforcement of behavior. However, it can be difficult to set a clear distinction between positive and negative reinforcers. Often, positive reinforcers also involve some degree of punishment. For example, for operant conditioning there

are two main types of conditioning used for studies: fear conditioning and appetitive conditioning. In general, these two differ in that the former is regarded as involving a punishment, whereas appetitive conditioning is regarded as involving a positive reward. For appetitive conditioning to have an effect however, it requires the animal to be food deprived, which in itself involves a sort of negative reinforcement (Fanselow, 2016; Ferster 1973; Perone, 2003).

1.2 Fear conditioning and its underlying neural circuits

1.2.1 The amygdala

The brain mechanisms underlying fear learning have been extensively studied by use of Pavlovian fear conditioning (LeDoux, 2014; Maren, 2001). Studies point to the amygdala, a structure part of the limbic system, as an important structure for fear conditioning, with work in both rodents, mammals and humans confirming this (Maren, 2001). Work of studies nearly five decades ago already established that learning and memory which involves fear is specifically reliant on the amygdala structure (Blanchard & Blanchard, 1972; Maren, 2001). This has been shown in classical studies by simply documenting that in both animals or even humans that has damage or lesions to the amygdala are incapable of learning Pavlovian fear conditioning (Amorapanth, LeDoux & Nader, 2000; Bechara et al., 1995; Büchel & Dolan, 2000,). The amygdala however is a complex structure, that consists of several different subnuclei, which has various sensory and motor inputs from different brain areas (LeDoux, 2007). Although there are discussions about how the amygdala should be divided, roughly, the amygdaloid nuclei can be sorted into two different subsystems: the basolateral complex (BLA) and the central nucleus (CE) (Maren, 2001; LeDoux, 2007). The BLA consists of the subnuclei lateral, basolateral and basomedial nuclei. The BLA through its different subnuclei receives various sensory inputs from cortical areas, such as the somatosensory, auditory, gustatory and perirhinal cortices. It also receives inputs from structures such as the hippocampus, as well as medial geniculate nucleus of the thalamus (Maren, 2001). Given the wide range of sensory inputs the BLA receives, it is plausible that it is the structure where the association of conditioned and unconditioned stimulus is established. The BLA has further intra-amygdaloid connections to the CE (Figure 1.2). CE, in turn, further has connection to the hypothalamus and several brainstem nuclei, which mediate the fear responses seen in fear conditioning (Figure 1.2; Maren, 2001). These structures include the

periaqueductal grey (PAG), known to mediate freezing behavior (Watson et al., 2016), the bed nucleus of the stria terminalis (BNST), which contributes to the general stress response seen in fear conditioning (Goode & Maren, 2017), as well as the hypothalamus, which is essential for regulating heart rate and blood pressure (LeDoux, 2000), also seen as a response in fear conditioning. The BNST again is known to be interconnected with the dorsal raphe nucleus (DRN) and receives serotonergic input from this structure, as well as the lateral habenula (Maren, 2001). In terms of fear conditioning, the amygdala is then widely regarded as a hub receiving multiple sensory and motor inputs, which again sends projections to multiple output structures, contributes to the fear response seen in fear conditioning. Although much is known about this circuitry already and its role in fear conditioning, the investigations of the several interconnected structures could reveal more about this form of learning.

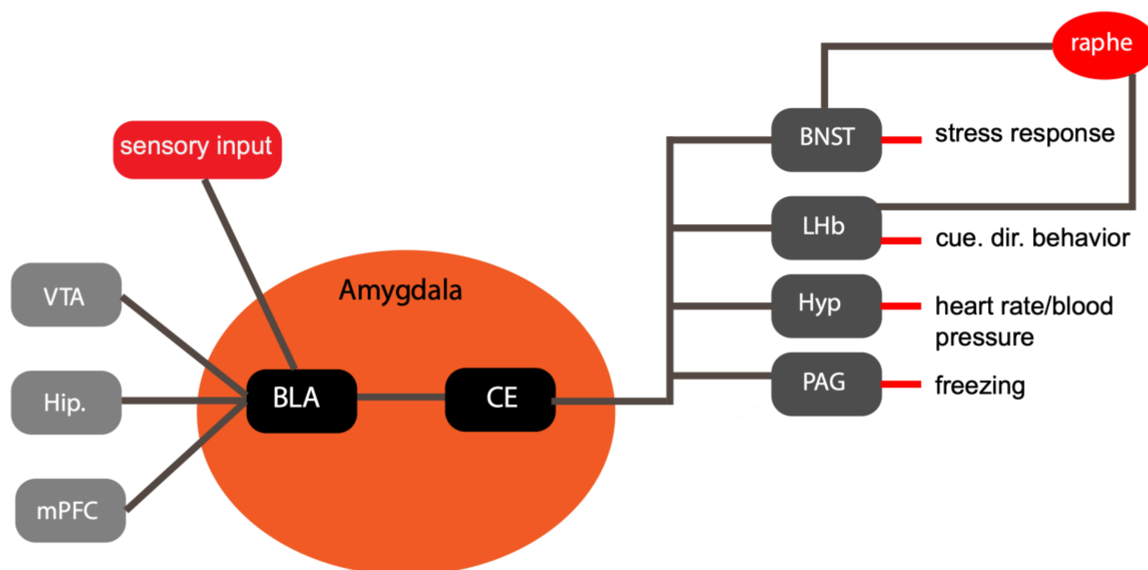

Figure 1.2 – Amygdaloid circuit figure: *Scheme showing amygdaloid intraconnections of amygdala subnuclei, with projecting input regions in light grey and output structures in dark grey. Abbreviations: VTA: Ventral tegmental area. Hip: Hippocampus. mPFC: Medial prefrontal cortex. BLA: Basolateral complex. CE: Central nucleus. PAG: Periaqueductal grey. Hyp: Hypothalamus. LHb: Lateral habenula. BNST: Bed nucleus of the stria terminalis. Raphe: raphe nuclei populations. Based on Maren, 2001.*

1.3 The serotonergic system in brain function and behavior

1.3.1 Serotonin (5-HT)

Serotonin (5-hydroxytryptamine, 5-HT) is a monoaminergic neurotransmitter found throughout the body and the CNS, that constitutes an important part of the evolutionary conserved serotonergic system (Lillesaar, 2011). In humans and mammalian species, 5-HT exerts a modulatory role in a wide range of physiological functions ranging from contributions to changes in mood, happiness, to aspects of cognition, such as learning and memory (Pawluwski, Li & Lonstein, 2019; Bauer et al., 2014). Furthermore, 5-HT has been implicated in several neuropsychiatric disorders, including depression, anxiety and eating disorders (Veenstra-VanderWeele et al., 2000). Several of the most common drugs used for treating psychiatric conditions target 5-HT, such as the selective-serotonin reuptake inhibitors (SSRIs), such as fluoxetine, or anxiolytic drugs such as buspirone. Although these drugs are known and commonly prescribed since they exert good effect in alleviating symptoms, their exact pharmacological effects are still not entirely certain (Bauer et al., 2014). Investigations into these pharmacodynamics could reveal possibly new therapeutic uses or reveal insights into how 5-HT modulates activity in the brain.
In the body, 5-HT is synthesized firstly by the enzyme tryptophan hydroxylase (TPH), of which there have been found two gene variants. The variant TPH1 mainly synthesizes the 5-HT of the peripheral nervous system (Abireault, Sibon & Cote, 2013), whereas the variant TPH2 is responsible for the synthesis of 5-HT in the CNS (Walther et al., 2003). A wide range of serotonin receptor subtypes have also been discovered and extensively studied, with varying functions being implicated to the different subtypes (Jacobs & Azmitia, 1992; Palacios, 2015). Due to the variety of subtypes, the function of 5-HT and the serotonergic system in general can be considered quite complex. In total, there have been found 17 different subreceptors for 5-HT, and these are further categorized into 7 families of receptors (Bauer et al., 2014). For example, the 5-HT_{1A} receptor has been implicated to be involved in learning (see Ögren et al., 2008 for a review). Interestingly, the anxiolytic drug buspirone is known to be a full-agonist for this subtype 5-HT receptor (Loane & Politis, 2012).

1.3.2 Serotonin in fear conditioning

As 5-HT as a neurotransmitter is interlinked with various physiological and cognitive functions and has various projections to important cortical and brainstem structures, it is perhaps not surprising that it has been found to also have an involvement in fear conditioning specifically (Bauer, 2014). Given the vast array of subtypes found for this neurotransmitter, the overall role of 5-HT in terms of fear conditioning can be quite extensive to cover. Firstly, it is known that there is an increase in 5-HT levels in the amygdala in response to encounters of unconditioned stimuli. Secondly, multiple subreceptors such as the 5-HT_{2A} and 5-HT_{2C} have been implicated in processes of memory, and expression of anxiety-like behavior respectively (Bauer et al., 2014),, suggesting an important role for 5-HT in the processes underlying fear conditioning.

1.3.3 Serotonin in pain perception.

As fear conditioning often involve the use of aversive stimulus, the role of 5-HT in pain perception should be considered. 5-HT is known to be involved in pain perception, and lesions of the DRN in rats have shown to contribute to an increased sensitivity to pain when exposed to an electrical shock (Telner, Lepore & Guillemot, 1979). However, newer findings suggest that 5-HT likely is involved in both inhibtion and promotion in pain perception, and that the exact involvement of 5-HT is currently considered as uncertain (Marks et al., 2009).

1.3.4 Serotonin as target for pharmacological manipulations

Although there are differences between the serotonergic system of zebrafish and mammals, studies have shown that pharmacological manipulations targeting 5-HT yield similar effects across species, suggesting further that the functional role of the system is evolutionary conserved (Maximino et al., 2013). Recently, work has beend done examining the possibility of 5-HT effects being modulatory depending on whether the synaptic release is phasic or tonic (Lima-Maximino et al., 2020). In this theory, 5-HT phasic and tonic release is deemed to acts as neurobiological switch toward cautious exploration when aversive stimulus is no longer present (Lima-Maximino et al. 2020). Although still a novel examination, this theory can explain how 5-HT in the CNS can exert such differentiated effects as seen in various studies.

1.3.4.1 Buspirone

Buspirone hydrochloride is a high-affinity agonist of the 5-HT_{1A} receptor, and a commonly prescribed anxiolytic drug used for treatment of anxiety disorders (Loane & Politis, 2012). It works as a full-agonist on presynaptic 5-HT_{1A} receptors, and partial agonist for postsynaptic 5-HT_{1A} receptors. 5-HT_{1A} receptors are G-protein coupled receptors, and buspirone acts by binding to these, which in turn decreases cAMP concentrations intracellularly (Liu et al., 1999). In addition, it has been suggested that buspirone also works antagonistic on dopamine D2 autoreceptors, as well as having weak affinity for 5-HT_2 receptors (Loane & Politis, 2012). Despite having shown to alleviate symptoms of anxiety, depression, social phobia, the exact pharmacological underlying mechanisms are still not fully elucidated for buspirone, which could be important steps in certifying the various uses of it. The 5-HT_{1A} receptor is known to be involved in learning. Buspirone has shown an anxiolytic effect in zebrafish (Maximino et al., 2013) in a novel tank test (NTT), by decreasing bottom-dwelling, which is an indicator of anxiety-like or defensive behavior in zebrafish. Although in clinical applications, buspirone must be taken for several weeks, zebrafish have different metabolism, and acute treatment can exert effects differently than in humans.

1.3.3.2 Fluoxetine

Fluoxetine hydrochloride (Tocris, product no. 0927) is a commonly prescribed antidepressant, known under the name Prozac, which functions as an SSRI (Selective serotonin reuptake-inhibitor). In clinical use it has been used for treatment of symptoms of major depressive disorder (MDD), obsessive compulsive disorder (OCD) and panic disorders, among others (Wong et al., 1995). Its pharmacological function relates to 5-HT, by delaying the reuptake after vesicular release in synaptic clefts. This means the 5-HT persists longer in the cleft, allowing more of the neurotransmitter to bind to postsynaptic receptors, such as the 5-HT_{1A} and exerting the postsynaptic effect of 5-HT. Fluoxetine has been used in behavioral experiments involving zebrafish, where it has shown to have an anxiolytic effect. In a light-dark test, a conspecific alarm substance led to an increase in anxiety-like behavior in zebrafish. Pre-treatment of fluoxetine blocked this effect, as well as appearing anxiolytic in several physiological parameters and extracellular levels of 5-HT were found to be elevated (Maximino et al., 2014). As with buspirone, fluoxetine in clinical use is taken for weeks before showing to have effects of alleviating symptoms, but studies reveal that these drugs

indeed exert the same effects even for short periods of treatment (Maximino et al., 2013; 2014; 2020).

1.3.5 The dorsal raphe nucleus

The dorsal raphe nucleus (DRN) is a brainstem nucleus, located in the of the mammalian brain, and constitutes a major part of the raphe nuclei and the serotonergic system of the CNS (Jacobs & Azmitia, 1992). Although considered a nucleus on its own, it is further anatomically divided into medial, lateral and caudal components. Importantly, the DRN is known to contain the major serotonergic populations of neurons in the brain (Dahlstrom & Fuxe, 1964; Dorocic et al., 2014), and is thus an important part of the serotonergic system, along with the medial raphe (MR). Although the DRN is considered the major serotonin producing of the brain, its population of serotonergic neurons is in minority considering its other populations of neurons, as populations of dopaminergic, GABAergic and peptidergic neurons are also prevalent (Fu et al., 2010). It has been observed that the largest cells in terms of size and length of projections for all of the raphe nuclei are serotonergic (see Jacobs & Azmitia, 1992). In rats, it is estimated that from between 30-50 % of the total neuronal population in the DRN are serotonergic (Steinbusch et al., 1980).

1.3.5.1 The mammalian and teleost DRN circuits

In mammals, the projections of DRN are largely considered as widespread and extensively connected with other brain structures, although diffuse (Andrade & Haj-Dahmane, 2013; Jacobs & Azmitia, 1992). In zebrafish and mammals both, it is established that the DRN is responsible for a majority of the serotonergic projection to the forebrain, or dorsal telencephalon, but there also has a range of projections to structures such as the hindbrain and the hypothalamus (Lillesaar et al., 2009). In terms of afferents, the DRN is known to receive projections from areas such as the periaqueductal gray (PAG), central amygdala nucleus (CEA), ventral tegmental area (VTA), prefrontal cortex (PFC), hypothalamic areas and the lateral habenula (LH), as showed in studies of the mouse brain (Dorocic et al. 2014, see Figure 1.4). In general terms, the overall similarity in terms of connectivity and connected the two systems can be considered homologous (see Figure 1.3).

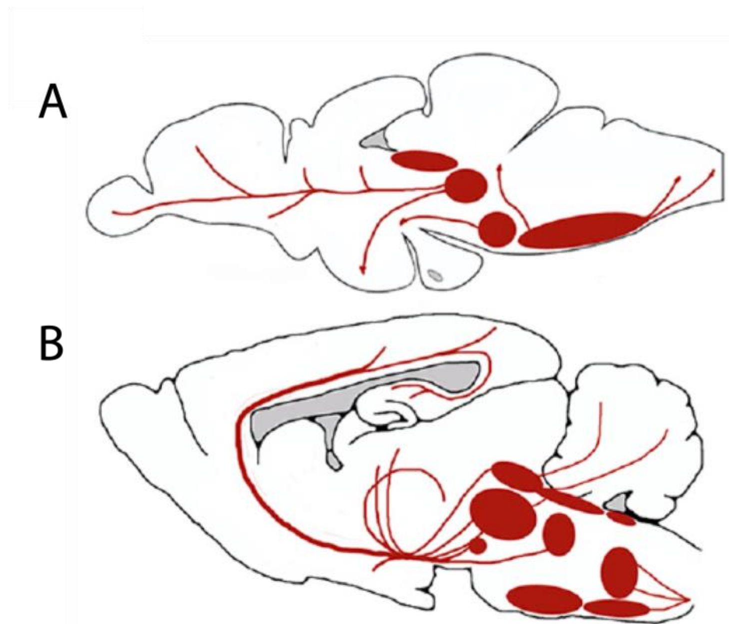

Figure 1.3 – Serotonergic projections: *Scheme showing a lateral view the serotonergic projections of the A) adult zebrafish brain and the B) adult rat brain. Marked structures in red signify raphe populations in both figures. Adapted and modified from Parker et al., 2015.*

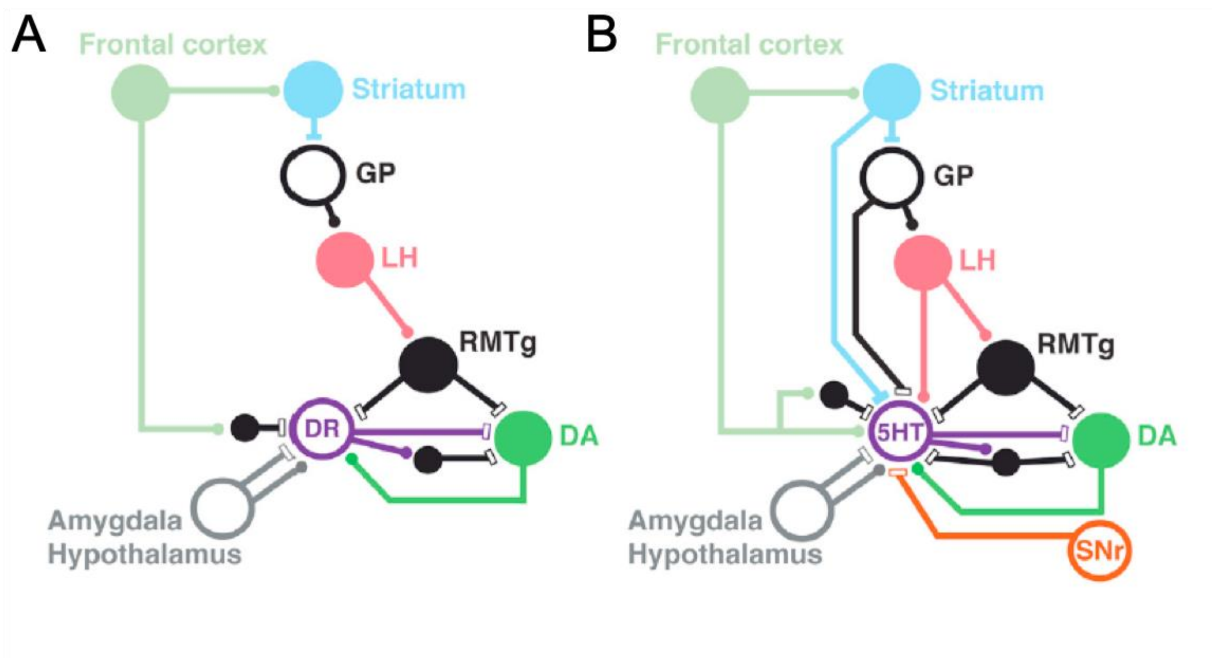

Figure 1.4 – Dorsal raphe receiving inputs: *Scheme showing a conventional model of efferent inputs that the DRN receives in mouse brain. A) Conventional model of DR inputs. B) Cell-type specific model of input to 5-HT system. Abbreviations: DR: dorsal raphe nucleus, DA:*

dopaminergic pathway LH: lateral habenula, GP: globus pallidus, RMTg: rostromedial tegmental nucleus, SNr: substantia nigra retic. Adapted and modified from Dorocic et al., 2014.

1.3.5.2 Dorsal raphe nucleus in brain function and behavior

Studies have implicated certain behaviors related to activity in the DRN. It is widely known to be involved in behavior related to sensory responsiveness, and in a study involving zebrafish larvae it was shown to be necessary for responding to visual cues when aroused (Yokogawa et al., 2012). Chemogenetic ablation in zebrafish larvae by use of metronidazole (MTZ) was shown to alter the tendency to increase visual sensitivity to relevant visual cues during arousal (Yokogawa et al., 2012). Various studies have implicated that the DRN also has an involvement in learning. (Kawashima et al., 2016) By whole-brain calcium imaging, it has been shown that specifically neurons of the DRN are activated during a task of locomotive learning in zebrafish larvae. Further investigations showed that chemogenetic ablation by use of MTZ in zebrafish larvae lead to a significant impairment in the locomotive learning, showing the DRN ablated animals struggling to respond with appropriate swimming bout-strength to match an experienced flow, compared to non-ablated animals (Kawashima et al. 2016). Furthermore, in a study using rodents, ablation of the DRN by tetrodotoxin injection lead to a significant spatial learning impairment in a Morris water maze (MWM) task (Ghaderi et al. 2012).

Overall, the DRN is seen as implicated in a variety of learning-related tasks. Neurons of the DRN have also widely been linked to aspects of reward and reward acquisition. Much of this research have studied the different effects 5-HT neurons of the DRN exerts with phasic and tonic release of the neurotransmitter. In a study using mice, 5-HT neurons of the DRN were shown to signal that a reward was delivered (or acquired). After the mice learn to wait for the delivery of a pleasant stimulus (sucrose), 5-HT neurons were shown to mostly fire in a tonic manner whilst the animals were waiting for the reward, and then phasically when the reward was acquired. (Li et al., 2016). Similar findings have been done in studies involving punishment as stimulus, where in the DRN of anesthetized rats, most active 5-HT neurons were shown to be phasically excited by noxious foot shocks, whereas most bursting (or tonically active) 5-HT neurons were shown to be inhibited (Schweimer & Ungless, 2010). These studies

reveal that the different cell populations present in the DRN might contribute to the complexity and wide range of roles this structure exerts, and that both. Similar studies done in reward processing specifically mice have implicated that both the serotonergic and glutamatergic neurons of the DRN contribute to the reward processing, as shown by optogenetic stimulations of Pet-1 neurons reinforced mice to explore a spatial region paired with a stimulus (Liu et al., 2014). Overall, the DRN is known as a vastly, and although the examinations into

1.3.5.3 Dorsal raphe nucleus in zebrafish

Zebrafish became an interesting model to study the function of DRN. In contrast to mammalian brain development, the zebrafish brain develops externally, allowing the study of and various experimental manipulations (Schmidt et al., 2013). Anatomically in the zebrafish brain, the DRN is located along the ventral midline of the brainstem, ventrally to the optic tectum and posterior of the forebrain (Yokogawa et al., 2012). Due to this relatively accessible anatomical location, the DRN can be quite easily located in the developing brain of zebrafish larvae, which allows for various imaging methods to be applied to study this structure. Although there exists extensive literature covering the efferent connections of the serotonergic nuclei in the mammalian brain (Dorocic et al., 2014; Jacobs & Azmitia, 1992), there are less available reviews covering these connections in the zebrafish brain. Lillesaar et al., (2009) have shown that the projections of the raphe nuclei populations are similar to that of the mammalian brain, asserting that the serotonergic system is evolutionary conserved, and comparable for these two model organisms (Yokogawa et al., 2012).

1.4 The molecular mechanisms of learning and memory

1.4.1 Synaptic transmission

By use of neurotransmitters, which are chemical substances, a presynaptic neuron can signal to a postsynaptic neuron. This process is called synaptic transmission (Purves, 2013). By release of neurotransmitter into the synaptic cleft between a presynaptic and a postsynaptic neuron, the probability that neurotransmitters bind to the postsynaptic receptors increases, allowing for a signalling between the two cells. By various molecular processes, the binding of neurotransmitter leads to a change in membrane potential in the postsynaptic neuron, ultimately leading to a transfer of signal between

the neurons (Purves, 2013). Calcium (Ca^{2+}) constitutes an important part in regulating this cellular signalling process (Simons, 1988). Following neuronal activation, Ca^{2+} levels are elevated intracellularly, which in turn modifies various cellular processes, and is essential for the excitability of the neuron and the release of neurotransmitter (Simons, 1988). This happens through various ways, but one way is through voltage gated channels, called LTCs. Ca^{2+}- calmodulin binds to LTCs and is an essential step in the activation of Ca^{2+} regulated signalling pathways. From this stage, various signalling pathways are activated by Ca^{2+}, from with calcium signals the fate of a neuron, one of those being the the MAPK/Erk pathway (Suo et al., 2019; Gao, 2009).

1.4.2 MAPK/ERK signalling pathway

The MAPK/ERK signalling pathway involves different kinases and cyclases. One of such is extracellular signal-regulated kinase (ERK) is which is involved in the phosphorylation of transcription factors (TFs) such as cyclic adenosine monophosphate (cAMP) and response element binding protein (CREB) (Hutton et al., 2017) These TFs are involved in the transcription of genes essential for neuronal survival, and the plasticity of the nervous system. In addition, they contribute to the expression of early genes, such as c-Fos and Arc, which are widely used in scientific applications as indicators of neuronal activity and markers of neurons undergoing synaptic plasticity (Thomas & Huganir, 2004). Neurons critical for fear, memory and sleep have been identified by the expression of IEGS such as c-Fos and Arc (Guzowski et al., 2005; Randlett et al., 2015). The problem with staining techniques focusing on these IEGS however is the poor temporal resolution and low sensitivity of methods using c-Fos or Arc, which makes them limited applicable in studies using zebrafish (Hudson, 2019). Using phosphorylated ERK can also be used as a method of localizing activated neurons and can have a better temporal resolution as the signal is present within 5-10 minutes after activation (Randlett et al., 2015).

1.4.2 Synaptic plasticity

A capacity of the mammalian brain is the ability of neural circuits to be modified following certain neural activity. This process is called synaptic plasticity and is deemed to be the molecular underpinning to the processes of learning and memory (Citri & Malenka, 2008; Johansen et al., 2011). Specifically, synaptic plasticity entails how the efficacy of synaptic transmission can be modified or strengthened in response to

activity (Citri & Malenka, 2008). The concept of synaptic plasticity originally traces back to the work of Donald Hebb, which famously quoted that “neurons that fire together, wire together”, and proposed that associative memories being formed was by a mechanisms involving a strengthening of connections between presynaptic and postsynaptic neurons, following two coincident events, such as the pairing of stimuli seen in a conditioning session (Hebb, 1948; Citri & Malenka, 2008). Synaptic plasticity can be distinguished in terms of longevity, into short-term plasticity and long-term plasticity. Short-term plasticity has been observed in numerous forms in species ranging from invertebrates, to mammals (Zucker & Regehr, 2002). Short-term plasticity is however thought to be the molecular underpinning of short-lasting memories, or transient changes in behavioral states (Citri & Malenka, 2008). The lasting changes in synapses and neurons needed for memories and learning to be enduring is deemed to be reliant on long-term plasticity. Although originally proposed by Hebb, there was lacking evidence of such a mechanism in the mammalian brain until the classical work done by by Tim Bliss and Terje Lømo in 1973 showed that by repeated activation of excitatory synapses in the rabbit hippocampus, a potentiation of the synapses occurred, which was shown to last for several days (Bliss & Lømo, 1973). Since then, the concept of this finding has been called LTP, for long-term potentiation, and massive investigations have been conducted at is believed to be the molecular mechanism for how memories are formed (Whitlock et al., 2006)

1.5 Zebrafish as a model organism

1.5.1 The zebrafish

Zebrafish, or *danio rerio,* is a freshwater fish which natively lives in the Himalayan region of South Asia. It is known to be a relatively rapidly developing vertebrate species and undergoes metamorphosis from larvae to juvenile stage at around 3-4 weeks of age post fertilization (Schmidt et al., 2013).

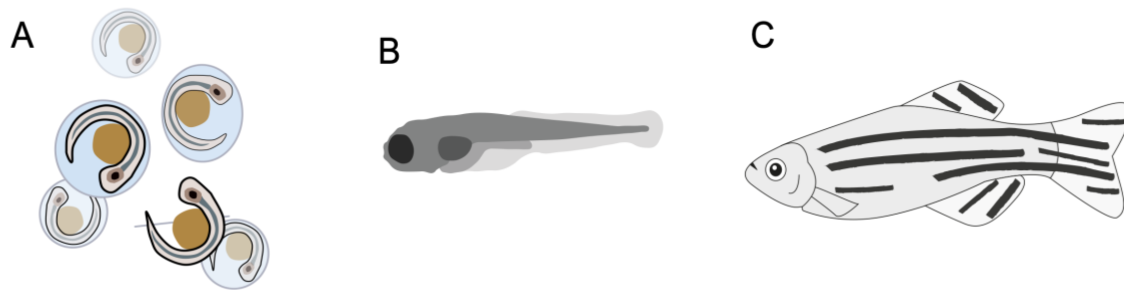

Figure 1.5 – Zebrafish development from embryonal to adult stage. *A) Illustration of zebrafish eggs. B) Illustration of a zebrafish larvae. C) Illustration of an adult zebrafish. Sizes are not scaled and comparable. Illustrations adopted from scidraw.io.*

1.5.2 Use of zebrafish studying learning and memory

1.5.2.1 Teleost brain regions involved in leaning

As an animal model, zebrafish has been used in a variety of scientific areas, but has recently gained a lot of research in different studies of learning, with a widespread use of larval and juvenile zebrafish (Lawrence, 2007; Roberts, Bill & Glanzman, 2013; Valente et al., 2012; Yashina et al., 2019). Although widely different in complexity, there are certain similarities between the mammalian brain and the teleost brain of zebrafish (Wullimann, Rupp & Reichert; 1996; Wullimann & Muller, 2004: Mueller, Dong, Berberoglu & Guo, 2011) A major difference between mammalian and zebrafish brain development is however in the formation of ventricles. In zebrafish the telencephalon, or forebrain, is formed by eversion, whereas the mammalian forebrain is formed by evagination. Zebrafish telencephalon contains areas and assemblies of neurons that have been found to be homologous to limbic mammalian structures. Due to this developmental difference, the homologous of limbic mammalian structures are located much closer to the skull and the top of the brain, making them more accessible for various experimental manipulations and.

Considering the homologues of the zebrafish brain, firstly, the medial part of the dorsal telencephalon (Dm) is considered a homologue to the mammalian amygdala (Lal et al., 2018). Studies performed in goldfish, where the Dm homologue medial pallium (MP) was lesioned, showed a learning deficit of ablated animals in a task of conditioned avoidance (Portavella, Torres & Salas et al., 2004), implicating the importance of this region as important for this type of learning. Secondly, the lateral

part of the dorsal telencephalon (Dl) is known to be a homologue of the mammalian hippocampus (Lal et al., 2018). The hippocampus is widely known to be important for spatial learning and memory (O'Keefe & Nadel, 1978; Burgess, Maguire & O'Keefe, 2002). In neuroscience, studies brain circuitry and structures often use mammalian species such as rodents for studies learning, due to its similarities with the human brain. However, an increasing amount of studies has been conducted in teleost fish and zebrafish in particular with studies describing that these species are capable of both active avoidance and Pavlovian fear conditioning (Kenney et al., 2017, Valente et al., 2012) Recently, Yashina and colleagues (2019) have also shown that zebrafish from 3 weeks of age are able to capable of forming spatial memories, by use of a Y-maze task which involved the delivery of a shock in one of the spatial regions of the maze. (Yashina et al., 2019).

1.5.3 Advantages of zebrafish as model organism

In neuroscience, over the past years, zebrafish has become a prominent model organism useful for studies of brain development, function and morphology (Schmidt et al., 2013). Although there are distances in phylogeny between the teleost and mammalian brain, there have been identified homologues for several key brain regions such as the hippocampus and amygdala, as well as the homologous serotonergic system (Wullimann & Mueller, 2004; Parker et al., 2013). Zebrafish are also relatively easy to handle and maintain in captivity over longer time periods and can easily be bred to raise new animals for experiments (see Lawrence, 2007). Due to their small size and rapid development a high throughput can be achieved in various behavioral setups (Valente et al. 2012). Their small size paired with their transparency at early developmental stages allows sequentially for the use of non-invasive imaging. Additionally, the whole zebrafish genome has been sequenced and compared with the human genome, with analyses showing that 69% of human genes have at least one zebrafish orthologue (Howe et al. 2013). Zebrafish are also easily genetically modifiable, as brain development occurs ex-utero, which opens for tools to be used for specifically targeting certain brain areas for techniques such as labeling, laser ablation and optogenetics (Kalueff et al. 2014).

1.5.4 Tools for studying DRN function in CPA

1.5.4.1 Zebrafish lines

To study the neural circuits underlying associative learning, the lab has some experimental tools available to study DRN. These are transgenic zebrafish lines that will be used in a CPA paradigm to study associative learning.

- Tg(TPH2:Gal4)

The Tg(TPH2:Gal4;UAS:NTR-mCherry) zebrafish line drives expression of a fusion protein composed of nitroreductase, acting as the enzymatic active component, and with mCherry as reporter protein. This fusion protein is expressed under the TPH2 (tryptophan hydroxylase 2) promoter, which specifically labels serotonin producing cells in the DRN (Walther et al., 2003; Yokogawa et al., 2012). Previous studies report that in this Tg zebrafish line, some expression is also seen in the spinal cord, but chemogenetic ablation has not led to any apparent damage, changes in survival, or motor deficiencies (Yokogawa et al., 2012).

1.5.4.2 Immunohistochemistry staining (phosphorylated ERK)

To study the neuronal activity underlying associative learning, the lab has developed and optimized a pERK/tERK staining protocol for zebrafish juveniles of 3-4 wpf. This immunohistochemical staining technique allows for the study of neural activity in zebrafish by staining for the kinase ERK, which is involved in a pathway linked to synaptic plasticity. This technique can be applied in the aftermath of fish performing in a CPA paradigm task, as a way of *post-hoc* analysis for studying neuronal activation.

1.6 Background work from host lab in CPA learning for zebrafish

1.6.1 Ontogeny of CPA

In the host lab, previous work has been conducted studying the neural circuits underlying learning, by use of zebrafish as a vertebrate model organism. In the lab, a custom-built behavioral assay for studying CPA has been built and optimized, which allows for the study of associative learning in juvenile zebrafish. Experimental work has shown that juvenile zebrafish of 3-4 weeks post fertilization (wpf) are capable of successfully performing CPA learning, and that the learning performance increases

across performance, by use of this assay (Palumbo et al. 2020, bioRxiv).These findings were in line with similar studies using zebrafish in associative learning tasks (Valente et al., 2012; Yashina et al., 2019), which certifies the applicability of zebrafish juveniles as a model organism for studies of associative learning.

1.6.1.1 dHBl ablation in zebrafish juveniles

Furthermore, experiments performed with transgenic zebrafish juveniles of 3-4 wpf have been done to examine the role of dorsolateral habenula (dHBl) in CPA. Surprisingly, dHBl ablation in zebrafish juveniles lead to an improvement in CPA performance in comparison with control animals and showed a delay in memory extinction (Palumbo et al., 2020, bioRxiv). The dHbl was deemed to be important in the behavioral flexibility of the animal, being highly important for the updating of new information. As zebrafish habenula has receives/projects to DRN, these findings are interesting. A valid step forward from these ablation experiments was to investigate other connected brain structures of the zebrafish, but rather than focusing on areas such as the Dl or Dm which previously have been established in zebrafish to be important for this type of learning, the area of interest was rather to focus on brainstem nuclei. A brainstem nucleus which has connections to the habenula is the DRN, and the various habenulo-raphe circuits have been examined in zebrafish, showing that this circuit is necessary for active avoidance learning, but dispensable for cases of classical fear conditioning (Amo et al., 2014).

2. Thesis objectives

Zebrafish of 3-4 weeks of age have been reported to be capable of performing learning in CPA paradigms. The scope of this master's thesis was to investigate specific brain structures and neural circuitry involved in learning and memory, and to reveal more about how these are implicated in the processes of learning and memory . Both the structure dorsal raphe nucleus (DRN) and its main produced neurotransmitter serotonin (5-HT) have been reported to have an important role in processes of learning, memory and reward, although undefined. We hypothesized that the DRN plays an important role in the learning performance in a CPA task. Further, as a way of assessing the stressful aspect of a CPA task which involves an aversive stimulus, we hypothesized that the treatment of drugs such as Buspirone and Fluoxetine could improve the CPA performance. Investigations into these topics could potentially reveal new knowledge of the phylogenetically conserved serotonergic system, which could be relevant for all species with serotonergic systems, including humans. Additionally, investigations into the role of species-wide neurotransmitter serotonin in learning could reveal more about the function of this signal substance, which could a inspire work for new therapeutical use in use of 5-HT targeting pharmaceuticals that go beyond the current clinical use.

The objectives of this thesis were:

- To study the role of dorsal raphe nucleus (DRN) in learning and memory by use of a CPA paradigm, with transgenic zebrafish lines, by use of chemogenetic ablation.
- To investigate the role of serotonin in processes of learning and memory, using a CPA paradigm, by use of transgenic zebrafish lines. Administering drugs (Buspirone, Fluoxetine) to study the role of Serotonin 5-HT in CPA learning.
- To investigate neuronal activity using a immunohistochemical staining technique for phosphorylated ERK (pERK/tERK) as an indicator for neuronal activity, in transgenic zebrafish lines that have performed in a CPA learning paradigm.

3. Materials and methods

3.1 Zebrafish

3.1.1 Zebrafish maintenance and husbandry

Zebrafish, *danio rerio,* were kept in the main facility in fish tanks of 3.5 liters, at a density of 15-20 fish, dependent on developmental stage. Physiological conditions in Tecniplast ZebTec Multilinking System were kept constant (28 deg. C water temperature, pH 7, 6.0 ppm O2, salinity of 300-1200 uS). The main facility had a 14:10 hour light/dark cycle. For breeding, adult zebrafish were crossed and eggs were collected the following day, and incubated at 28.5 °C in egg water (0.1% methylene blue in artificial fish water). At 5 days post fertilization zebrafish larvae were transferred to the main system.
Zebrafish larvae were kept in larvae nursery tanks under the same conditions until 3-4 weeks post fertilization for experimental use. Zebrafish were fed dry food twice a day (SDS <100 up to 7 dpf, SDS100-200 up to 3 weeks, until up to SDS600 for adult, Sparos LDA, Portugal) according to fish size and developmental stage, as well as Artemia nauplii (GradeO, Great Salt Lakes Utah, USA) once a day. The maintenance and husbandry of zebrafish in the facility was approved by the NFSA (Norwegian Food Safety Authority) and all experimental procedures were performed according to the animal care guidelines.

3.1.2 Zebrafish lines

For different experimental procedures during the work of the master's thesis, several transgenic and genetically modified fish lines were used. In the following sections these fish lines are explained further.

3.1.2.1 Nacre

The fish from the nacre line lack melanophores, which affect their pigmentation and makes their skin more transparent. Mutation in gene encoding in a basic-helix-loop-helix/leucine zipper transcription factor (96). As these fish are transparent, they are extensively used for further crossing of transgenic fish lines, as their transparency gives an advantage for non-invasive in vivo imaging.

3.2. Transgenic zebrafish lines

The transgenic fish lines used are further described in Table 3.1.

Table 3.1 – Summary of transgenic zebrafish lines used in experiments: *Name of line, targeted gene, expression pattern and its reference is listed.*

Fish line	Targeted gene	Expression	Reference
TPH2:Gal4:UAS:NTR_mCherry or **GCAMP6s**	Tryptophan hydroxylase 2	Serotonergic cells in dorsal raphe	Yokogawa, 2012
TPH2:Gal4:UAS:NTR_mCherry x HuC:GCAMP6s	Tryptophan hydroxylase 2	Serotonergic cells in dorsal raphe; neurons of the whole brain.	Yokogawa, 2012; Jiang, 2018

TPH2:Gal4: UAS:NTR_mCherry

Tryptophan hydroxylase 2 (TPH2), an isoform of tryptophan hydroxylase (TPH), is an enzyme involved in the synthesis of serotonin (5-hydroxytryptamine, 5-HT) in the raphe nucleus of the brain (Walther et al., 2003). TPH2 is primarily expressed in the serotonergic neurons in the raphe nuclei, but expression is also seen in neurons in the pretectum (Gaspar, 2012; Lillesaar, 2007). Expression of TPH2 is described to be confined primarily to the dorsal part of the raphe nucleus (Yokogawa, 2012). This zebrafish line is generated using the GAL4;UAS-based gene expression system (Figure 3.2). With activation of the 5-HT-specific promoter TPH2, , GAL4 transcription factors are transcribed, and bind to the upstream activation sequence (UAS) (Kawakami et al., 2016). This mediates the expression of an inserted fusion protein that consists of nitroreductase (NTR) and mCherry (reporter protein).

TPH2:Gal4;UAS:GCaMP6S

This line is similar to the TPH2 specific line described above (TPH2:Gal4:UAS:NTR_mCherry), but instead of the inserted fusion protein (NTR and mCherry) rather drives expression of a transgene encoding a fluorescent calcium

sensor (GCaMP) protein that contains GFP, calcium-binding protein calmodulin (CaM) and CaM-interacting M13 peptide (Jiang, 2018.)

HuC:GCaMP6S

Animals from the HuC:GCaMP6s line contain a transgene encoding a fluorescent calcium sensor (GCaMP) protein consisting of GFP, calcium-binding protein calmodulin (CaM) and CaM-interacting M13 peptide (Jiang, 2018). The HuC (elavl3) promoter (ELAV like neuron-specific RNA binding protein 3) is expressed specifically in neurons of all subtypes, making it useful as a promoter for early marking of neurons (Park, 2000).

TPH2:Gal4:UAS:NTR_mCherry x HuC:GCaMP6s

This line (TPH2:Gal4:UAS:NTR_MCherry) can be used for experimental procedures, or crossed with the HuC:GCaMP6s animals to generate a TPH2:Gal4:UAS:NTR_mCherry x HuC:GCAMP6s transgenic zebrafish line. The animals from this line will both include the expression of the fusion protein consisting of NTR and mCherry in the raphe nucleus, as well as the GCaMP6s expression in the whole brain.

3.3 Immunohistochemistry

3.3.1 pERK/tERK activity staining

Zebrafish larvae of 3-4 weeks of age were euthanized after a behavioral experiment in cold artificial fish water (AFW: 0.0006 mV% marine salt in Reverse Osmosis water (RO H_2O)) and fixed in cooled PFA in 0.25 % PBTx (0.25 % Triton X-100 (PanReac AppliChem) in 1X PBS (Oxoid, Thermofisher)), and stored at 4°C O/N. Fixed animals were washed quickly 3 times in 0.25 % PBTx at RT, and subsequently dissected, which involved decapitation, removal of the skin (dura) and the lower jaw (mandible). The samples were then stored in 0.25% PBTx at 4°C until further use. For permeabilization, larvae were incubated in 0.05% Trypsin-EDTA (Sigma) on ice for 40 minutes, and then washed twice quickly and once over 10 minutes with 0.25% PBTx. For blocking, the larvae were incubated over 1 hour in a blocking solution (2% Normal Goat Serum, 1% BSA, 1% DMSO (Sigma) in 0.25% PBTx) on a shaker at RT. Further, larvae were incubated in primary pERK and tERK antibodies (1:500) (1% BSA, 1% DMSO (Sigma)

in 0.25% PBTx) at 4°C O/N. Larvae were washed 3 times in 0.25% PBTx for 15 minutes and incubated in secondary antibodies (1:200) (2% Normal Goat Serum, 1% BSA, 1% DMSO (Sigma) in 0.25% PBTx at 4°C O/N. Larvae were washed 3 times in 0.25% PBTx for 15 minutes and mounted (figure) in 1.5% LMP agarose and 75% glycerol for imaging by confocal microscopy (Zeiss, LSM 880) using a 20x air objective. Images from the confocal microscope were obtained using ZEN LSM software (Zeiss), analyzed with ZEN Black (Zeiss), and processed further using ImageJ and Adobe Illustrator.

3.3.2 DAPI staining

For DAPI staining, the same procedures of fixation, dissection and permeabilization was performed as in the pERK/tERK staining. Dependent on purpose, DAPI staining was either performed alone, or in combination with pERK/tERK staining. For the combined staining, DAPI staining was conducted after the O/N incubation of the secondary antibody. Larvae were quickly washed and incubated with DAPI (1:1000, 1% DMSO, 0.25% PBTx) for over 2.5 hours, depending on size of samples, at RT, before being mounted for confocal microscopy (Zeiss, LSM 880) using a 20x air objective.

3.4 Confocal microscopy

3.4.1 In vivo confocal imaging

For characterization of the expression profile of transgenic fish lines, in vivo confocal imaging was performed. Juvenile fish of 21-30 days post fertilization (dpf) were anaesthetized with 0.4% tricaine methane sulfonate (MS222, Sigma) and embedded in 2.5% LMP agarose and given supply of AFW. Live, embedded fish were imaged with a Zeiss confocal microscope (LSM 880) using a 20x water objective. Based on excitation/emission spectra of the fluorescent tags of the transgenic lines, the excitation wavelength of the laser was selected (Table xx) Images were acquired with ZEN LSM-software (Zeiss Zen-Black) and processed further using ImageJ and Adobe Illustrator.

Table 3.2 – Overview of fluorescent targets in transgenic lines: *Listed are excitation and emission wavelengths (nm).*

Fluorescent target	Excitation	Emission
GCaMP (GFP)	488	507
mCherry	587	610

3.4.2 Fixed samples

Confocal imaging was performed on fixed brain samples with DAPI staining and pERK/tERK activity staining. Samples were mounted between cover slips in a bottom layer of 1.5 % LMP agarose and a top layer of 75% glycerol. Samples were imaged with a Zeiss confocal microscope (LSM 880) using 10x and 20x air objectives. Excitation wavelengths of the laser were chosen based on the excitation/emission spectra of the fluorescent tags of the antibodies used during the immunohistochemistry staining protocol. Images were acquired with ZEN LSM-software (Zeiss Zen Black) and processed further using ImageJ and Adobe Illustrator.

Table 3.3 – Overview of target protein, primary and secondary antibodies: *Listed are antibody types and excitation and emission wavelengths (nm).*

Target	1st antibody	2nd antibody	Excitation	Emission
Phosphorylated ERK	pERK rabbit antibody (Cell sign. Technology	Alexa Fluor 488 anti-rabbit antibody (Thermofisher)	500	520
Total ERK	tERK mouse antibody (Cell sign. Technology)	Alexa Fluor 408 anti-mouse antibody (Thermofisher)	400	420
Neurons	NISSL	-	488	515-565
AT-rich DNA regions (nuclear counterstaining)	DAPI	-	350	470

3.5 Experimental manipulations

3.5.1 Chemogenetic ablation of dorsal raphe nucleus

Tg(TPH2:Gal4) zebrafish larvae of 3-4 weeks post fertilization were treated with metronidazole (10mM MTZ (Sigma) and 250 ul DMSO) mixed with AFW in petri dishes. Petri dishes were covered with aluminum foil and incubated for 24 hours at 28 °C O/N. The following day MTZ was washed out with and replaced with fresh oxygenated water, and the fish received dry food. The fish were then incubated again O/N at 28 °C. The following morning the larvae were used in the behavioral experiment.

3.5.2 Buspirone treatment experiments

Tg(TPH2:Gal4:NTR_mCherry) zebrafish larvae of 3-4 weeks post fertilization were treated with buspirone (27.5 mg/L, Tocris) mixed with AFW in petri dishes 10 mins prior being used in the behavioral experiment

3.4.3 Fluoxetine treatment experiments

Tg(TPH2:Gal4:NTR_mCherry) zebrafish larvae of 3-4 weeks post fertilization were treated with fluoxetine hydrochloride (100 ug/L, Tocris) mixed with AFW in petri dishes 10 mins prior to being used in the behavioral experiment

3.6 Behavioral setup

3.6.1 Experimental assay

A behavioral setup for studying associative learning has been developed and optimized in the host lab. This setup is amenable for studying CPA learning in zebrafish (Figure 4). The setup has a behavior tracking system software based on algorithms for tracking position of the animals. The setup consists of six Gosselin square Petri dishes of 120mm x 120mm x 15.8mm, adjacently placed on an LCD monitor displaying a visual pattern, which served as arenas for the behavioral experiment. The LCD monitor was installed horizontally under the behavioral arenas and displayed a pattern that marked half of the arenas as red, and the other half as grey. The dishes were covered on their edges to avoid disturbances and social interactions with neighboring fish (figure xx). An Arduino due microcontroller connected to a computer was used for delivery of the aversive stimulus. The arenas were wired with tungsten wires, which served as electrodes for shock delivery through the water in each arena. The electric

shock delivered was of 1 mA intensity and a duration of 10ms, with a frequency of 1.33 Hz. The water in the arenas was 1.2 mg/L Reverse Osmosis H_2O. Water used in the behavioral setup was stored in an incubator holding 28 ⁰C prior to the experiment, to ensure a stable water temperature. Temperature measures in the room and in the water were done both before starting and at the end of the experiments, and either measurement should not be below 25 ⁰C. Additionally, a heater was installed under the behavioral setup to provide stable temperature during the experiment, as well as heat coming from the LCD monitor helped keeping the temperature stable.

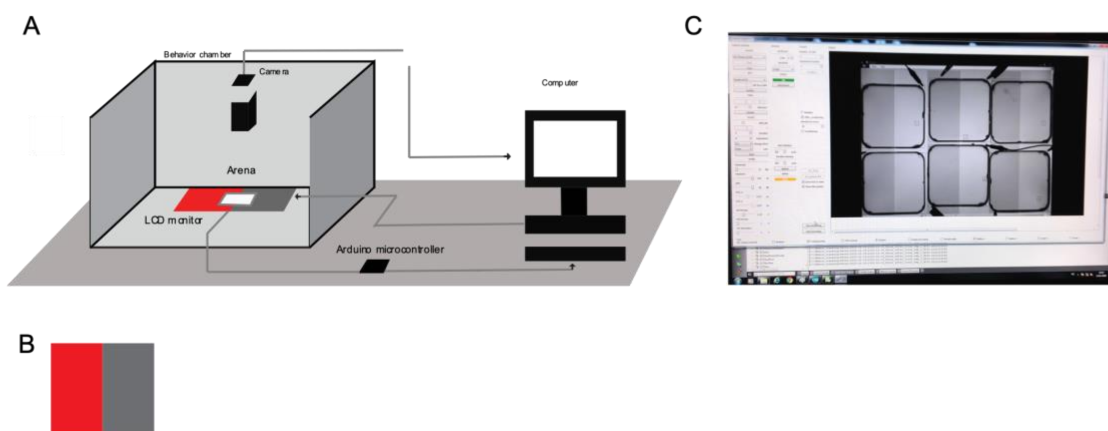

Figure 3.1 – Experimental CPA behavioral assay: *A) Setup overview B) Displayed pattern C) Computer tracking software*

3.6.2 Experimental protocols

3.6.2.1 Basic CPA protocol

All experiments were performed during daytime, and always started before 12:00 PM. To minimize the impact of external influences in the lab, the setup was placed in a separate room. Additionally, the behavior chamber was covered by a black box, to ensure that the only light was coming from the LCD monitor displaying the visual pattern. As the setup contained six dishes, experiments were performed in parallel with six fish at the same time. In Figure 3.2, a basic form of a CPA protocol is shown. The protocol begins with a baseline session lasting 1 hour. The duration of the baseline was determined to be longer than the test sessions for habituation of the animals to the visual stimulus, the wires and electrodes in the arena. Conditioning sessions consisted of four alternating shock and test periods, with shock periods always being

followed by a test period. The shock periods involved the fish receiving the aversive stimulus once they entered the conditioned zone (red compartment), whereas during the test period no shock was delivered; although the visual pattern was still apparent. Conditioning was determined to be split into two shock sessions, rather than one, as a means to maximize learning by repeating the conditions. After the full conditioning session, another test session followed which lasted for 30 minutes, where no shock was delivered, but the visual pattern still apparent. This test session was conducted with the purpose of assessing retainment of memory and extinction of behavior.

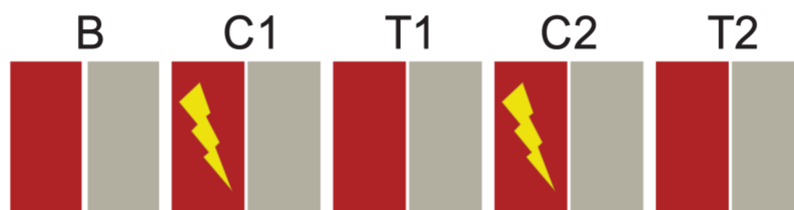

Figure 3.2 – Example of a basic CPA protocol: *B: Baseline. C1: conditioning session 1. T1: Test session 1. C2: conditioning session 2. T2: Test session 2. Protocol involves conditioning sessions followed by test session, where no aversive stimulus is delivered (shock).*

3.6.2.2 Protocol used for final experiments with transgenic fish

This protocol (Figure 6) consisted of a 1 h baseline period, followed by a 2 h conditioning session of alternating shock and test periods, with the red compartment as the conditioned zone, and a subsequent 30 min test session where the fish did not receive a shock. After this, a new 2 h conditioning session followed, but this time the conditioned zone was changed, meaning the fish were rather shocked once they entered the grey compartment; but not the red. After this second conditioning session, a 30 min test session where the fish once again did not receive a shock, which concluded the protocol. The whole protocol lasted for 6 h of recording in total.

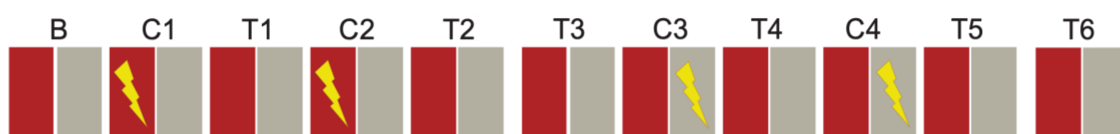

Figure 3.3 – CPA protocol with reversal learning: *Abbreviations: B: Baseline. C1: Conditioning 1. T1: Test 1. C2: Conditioning 2. T2: Test 2. T3: Test session 3 (extinction test). C3: Conditioning 3. T4: Test 4. C4: Conditioning 4. T5: Test 5. T6: Test 6 (extinction). Reversal learning component involved conditioned zone being switched from red compartment to grey compartment (at C3).*

3.6.2.3 Protocol used for experiments followed by pERK/tERK staining, and drug testing experiments

For experiments conducted with the aim of subsequent pERK/tERK activity staining, as well as for testing of drug manipulations, a shortened protocol of was used. This protocol (Figure 7) consisted of a 1 h baseline period, followed by a 1.5h alternating conditioning session. The session was ended during the conditioning period for purposes of assessing neural activity during learning, which was deemed to be most appropriate during a conditioning session, as animals avoid the conditioned arena and learn to associate it with an aversive stimulus.

Figure 3.4 – CPA protocol with 3 sessions: *B: Baseline. C1: conditioning session 1. T1: Test session 1. C2: conditioning session 2.*

3.6.3 Behavioral analysis

To analyze the behavior of the fish from the behavioral experiments, the following statistical parameters were used: fish size (pixels^2), heat maps averaged across all fish of each group, measure of learning performance (time spent in conditioned zone / total session time) with time bins of 2 minutes, velocity (cm/s), freezing (no movement for 2 seconds), distance to midline. For the measures of time spent in conditioned zone, velocity and distance to midline, Wilcoxon sign-rank tests were performed for every part of the protocol for the animal groups in MATLAB (Mathworks). Statistical significance is seen as the p-value is < alpha (alpha = 0.05).

4. Results

4.1 Studying dorsal raphe nucleus function in CPA using transgenic zebrafish

4.1.1 Expression profile of DRN labelled zebrafish line

In advance of performing experiments with chemogenetic ablation of dorsal raphe neurons, the expression profile of the available zebrafish line in the lab had to be examined. Live immobilization was performed on Tg (TPH2:Gal4:UAS:NTR-mCherry) zebrafish juveniles of 21 dpf, before being used for confocal imaging. Images showed the expression of the mCherry protein in the DRN (Figure 4.1) For some animals, expression of the mCherry protein was sometimes seen also in nearby neurons not specifically located in the DRN, which could suggest some unspecific expression.

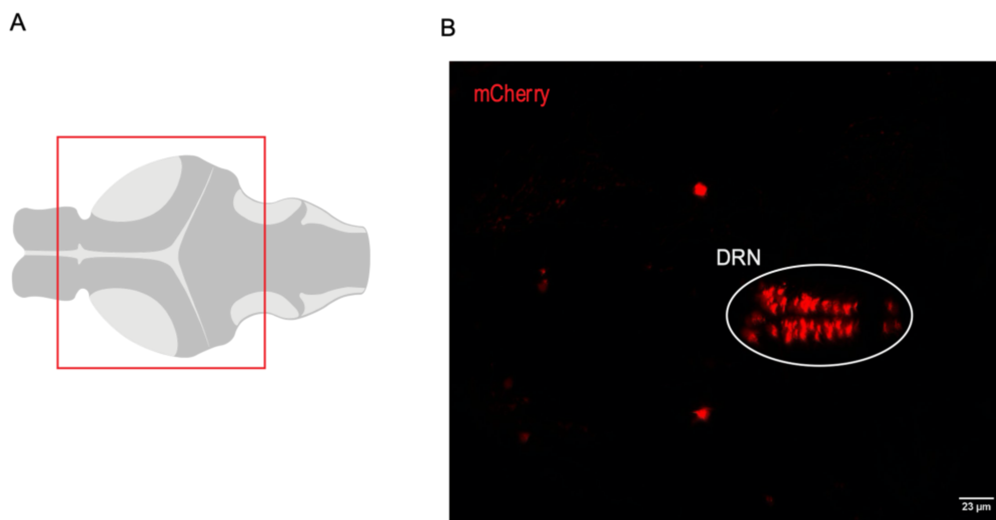

Figure 4.1: Expression profile of TPH2:Gal4:UAS:NTR_mCherry: *A) Illustration of dorsal view of zebrafish forebrain and tectal area. B) Live immobilization maximum intensity projection of confocal microscopy z-stack scan of 21 dpf TPH2 transgenic zebrafish. White circle indicates the DRN. White arrows indicates singular nearby neurons also showing expression of mCherry. Abbreviations: DRN: dorsal raphe nucleus. Scale bar is 25 um.*

4.1.2 Confirming MTZ ablation of DRN neurons

To confirm ablation of DRN, Tg(TPH2:Gal4:UAS:NTR-mCherry) zebrafish were used. After being treated with MTZ (10 mM) for 24h, fish were sacrificed and fixed in PFA,

before being stained with DAPI counterstaining. 5-HT neurons of the DRN are expressing nitroreductase-mCherry fusion protein under a TPH2 promoter. The neurons of the DRN were ablated, characterized by the loss of fluorescence of the mCherry protein in the MTZ treated animals (4.2D). MTZ-treated animals showed either an almost full loss of fluorescence, or that some fluorescent neurons remained. Placebo treated animals showed neurons filled with the mCherry reporter protein, as indicated by Figure 4.2C.

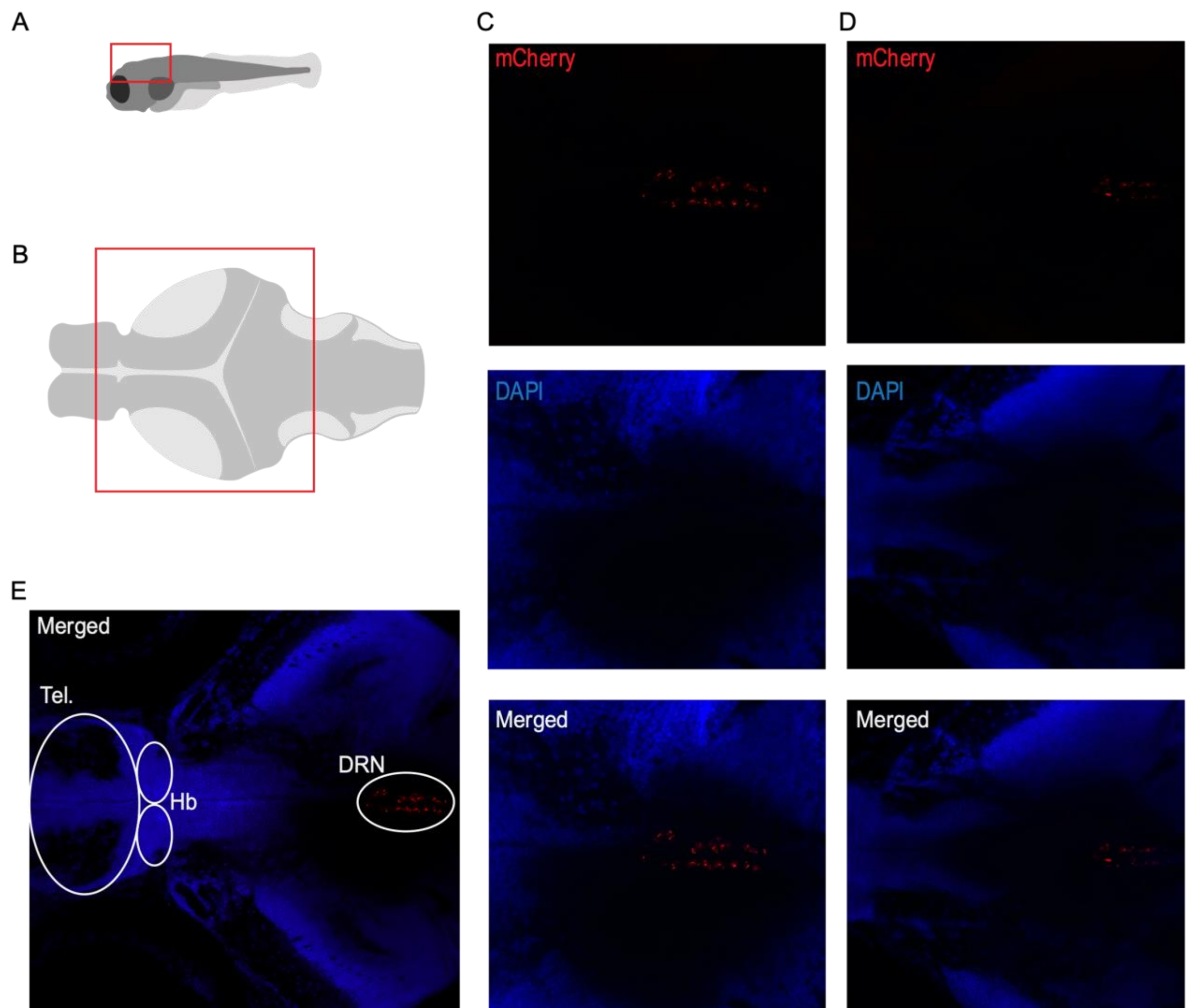

Figure 4.2: DAPI counterstaining MTZ ablation: *A) Side view of zebrafish larvae indicating area where DRN is located. B) Illustration of dorsal view of zebrafish forebrain and tectal area from where scan was performed. C) Confocal z-stack of 21 dpf TPH2 NTR-mCherry zebrafish placebo treated with DAPI counterstaining D) Confocal z-stack scan of 21 dpf*

TPH2 NTR-mCherry zebrafish treated with MTZ (10mM) with DAPI counterstaining E) Repetition of confocal z-stack with white indicators of dorsal telencephalon, habenula and dorsal raphe nucleus. Abbreviations: Tel: telencephalon. Hb: habenula. DRN: dorsal raphe nucleus.

4.1.3 DRN ablated zebrafish in CPA

The role of DRN in learning and CPA is not fully understood. During the work of this master's thesis, the role of DRN was studied by use of chemogenetic ablation of zebrafish 3 to 4-week-old animals. To achieve this, the Tg (TPH2:Gal4:UAS:NTR-mCherry) zebrafish line was used and treated with MTZ, which specifically would ablate neurons in the DRN.

The test group was Tg(TPH2:Gal4:UAS:NTR-mCherry) zebrafish treated with MTZ (10 mM) for 24 hours, followed by a 24 hour washout period, before being used in the behavioral experiment. To circumvent the issue of the chemogenetic ablation ablating more cells than the targeted DRN neurons, due to unspecific expression, fish were pre-screened twice using a screening microscope, at 5 dpf and at 3-4 weeks before being treated with MTZ, to ensure that animals used in the experiment only had expression in the targeted DRN neurons. The control group was Tg(TPH2:Gal4:UAS:GCAMP6s) also treated with MTZ (10 mM) with the same conditions. The control group animals do not have the NTR-component, meaning the MTZ will not lead to neuronal ablation in these animals. There are however studies showing that MTZ regardless of genetic components has a deteriorating effect on zebrafish, which had to be taken into consideration (MTZ reference). MTZ of 10 mM was chosen as similar studies has shown this to be optimal conditions for the use of chemogenetic ablation, without perturbing animal survival or growth (Bram Serneels, unpublished data, Yaksi Lab). The behavioral protocol was based on an already established CPA paradigm developed in the lab, which involved a reversal learning component (see Palumbo et al., 2020). The behavior protocol and results are displayed in Figure 10.

During baseline (B) both groups performed similarly, showing no preference for either compartment of the arena, and no avoidance of either the red or grey color (Figure 4.3A). Both groups preferred the edges of the arena, showing evidence of

thigmotaxis (Schnörr et al., 2011), although the control group showed a small tendency to explore more towards the center of the arena, as seen in the heatmap (Figure 10A). Both groups spent close to the same amount of time in the red zone during the baseline session, at around 40% for the test group across the session, and slightly more for the control group (Figure 4.3B). The average velocity was significantly higher for the DRN ablated animals than the control group from the start of baseline and was the case for the whole protocol (Figure 4.3D).

During the first conditioning session (C1), the control group initially perform better in terms of time spent in conditioned zone (CZ), starting around 20% and the percentage reaching down towards almost 0% by the end of the session (Figure 4.3B). The DRN ablated animals spent more time in the CZ, at around 30% of the time, meaning they received more shocks than the control group, and there was no evident decrease in percentage of time spent for the whole of C1. During the first test session (T1), a gradual increase in time spent in CZ is seen for both groups, with the biggest increase seen for the control group, increasing from just above 0% to over 30% at the end of T1, likely implying the animals started to explore the red compartment of the arena more when no shock was delivered (Figure 4.3B). An increase in time spent in CZ was also apparent for the DRN ablated animals, although not as big an increase as the control group, ending at around 40%. These differences follow as a trend for the whole first conditioning session (C1-T2), with the control group outperforming the test group in terms of learning to avoid the shock. From C3, when the rule was switched and the grey area became the conditioning zone, both groups show a decrease in terms of time spent in CZ during this session (Figure 4.3B), although the control group outperforming. As seen in Figure 10C, both groups keep more distance in the opposite direction when the rule is switched, although the same trend appears also now when the rule is switched, with the DRN ablated animals staying closer to the midline. During T4, a gradual increase is seen again in time spent in CZ for both groups, with each of the groups now spending more time in CZ than during T1 (Figure 4.3B). From the last test session (T6), it is apparent from the heatmap that the DRN ablated animals explore the conditioned zone of the arena when there is no shock (Figure 4.3A). Zebrafish from both groups were of the same size category, and comparable (100-250 px^2) (Figure).

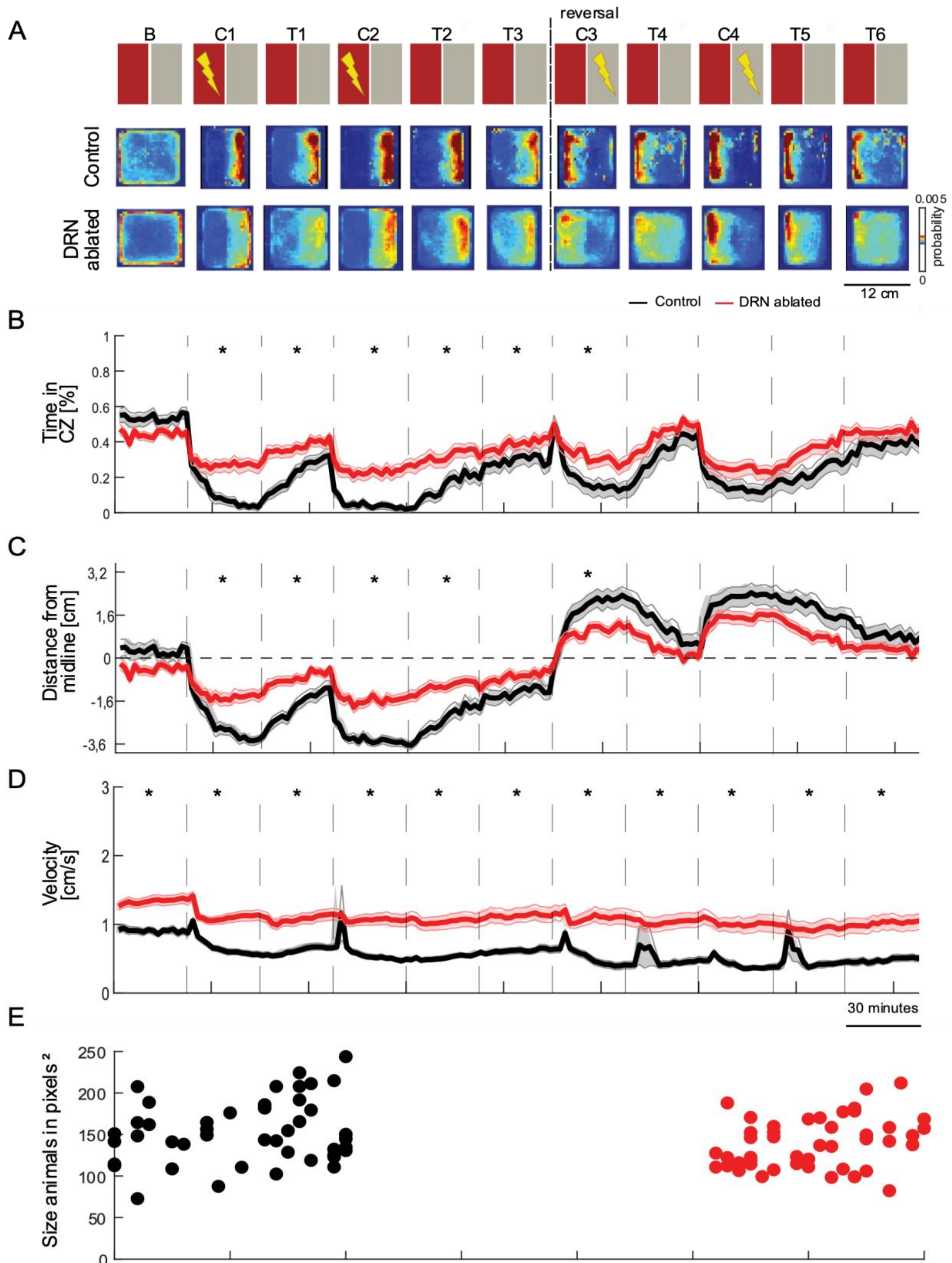

Figure 4.3: CPA protocol of DRN ablated animals (10 mM MTZ) with reversal learning session. *Test group: TPH2 NTR-mCherry transgenic zebrafish MTZ treated (10 mM) (n = 51). Control group: TPH2 GCAMP6s zebrafish MTZ treated (10 mM) (n = 51).* ***(****A) Top row shows a schematic presentation of the behavioral protocol. B: Baseline, C1: Conditioning 1, T1: Test 1,*

C2: Conditioning 2, T2: Test 2, T3: Test 3, C3: Conditioning 3 (reversal learning), T4: Test 4 (reversal learning test), C4: Conditioning 4, T5: Test 5, T6: Test 6. Bottom row shows heat maps of averaged density of position for all zebrafish B) Time spent in the conditioned zone (CZ) of the arena in percentage. C) Average distance from midline in cm. D) Average swim velocity of all zebrafish E) Size plot. Abbreviations: MTZ: metronidazole. Baseline is 1 hour, following sessions are 30 minutes each. *Shadows: S.E.M. Significance ($p < 0.05$) is indicated with an asterisk (*).*

4.1.4 DRN ablated zebrafish treated with Buspirone

The first experiments clearly showed that the DRN ablated animals performed worse than the non-ablated control group animals in terms of avoiding the shock, which suggesting a deficiency in learning (Figure 4.3 A-D). Secondarily, a was the tendency of DRN ablated animals to move and stay closer to the middle of the arena, compared to the control group, which could suggest a more explorative or less defensie behavior in these animals. To assess whether the observed detrimental learning effect of the DRN neuron ablation could be manipulated or altered by targeting 5-HT, the idea of adding a third experimental group was considered. Multiple agonists and antagonists for 5-HT are known and are in clinical use. One idea of testing this that was discussed was to employ a 5-HT agonist. Buspirone is known as a 5-HT agonist, and a full-agonist for the subreceptor 5-HT_{1A} (Loane & Pollitis, 2012, which is known to be involved in learning (Ögren et al., 2008), and for these experiments it was considered the suitable pharmaceutical. As buspirone additionally has shown to have an anxiolytic effect in zebrafish (Maximino et al., 2014), the use of the drug in this CPA assay could potentially highlight the impact of a fear component in this type of learning paradigm. The third experimental group was then to treat DRN ablated animals with buspirone to examine whether this 5-HT agonist is able to rescue the learning performance and potentially compensate for the 5-HT neuron ablation in these animals.

The first test group was Tg(TPH2:Gal4:UAS:NTR-mCherry) zebrafish treated with 10 mM MTZ for 24 hours, followed by 24 hours washout, and the animals in this group was the same as in the test group for the previous experiment. The second test group was Tg(TPH2:Gal4:UAS:NTR-MCherry) zebrafish, treated with MTZ in the same conditions, and also treated with buspirone hydrochloride (27.5 mg/L, Tocris) for 10 minutes before being placed in the behavioral assay. The concentration of buspirone used was based on previous protocols used in the lab for buspirone

treatment, which showed an anxiolytic effect in 3-4-week-old zebrafish in the NTT (Chris Wiest, unpublished data, Yaksi lab). The control group was Tg(TPH2:Gal4:UAS:GCAMP6s) also treated with 10 mM MTZ in the same conditions. The same behavioral protocol for CPA was used, to make comparisons with the previous experiment. The behavior protocol and results are displayed in Figure 4.4.

The treatment of buspirone for DRN ablated animals did not seem to rescue or improve the learning performance (Figure 4.4) The learning performance of the DRN ablated animals compared with the DRN ablated animals treated with Buspirone was similar across the whole protocol. Compared to the control group, neither of the test groups were close in terms of learning performance, spending close to 40% in the conditioned zone during C1 and T2 (Figure 4.4A). Additionally, all groups involved in the experiment showed they were able to learn and adapt to the new rule when the shock was switched (C3, Figure 4.4), showing they were capable of reversal learning. Compared to the control group, neither of the test groups were close in terms of learning performance. In terms of velocity, the control group performed similarly as in the prior experiment (4.3D) and the test groups swam almost twice as fast across the protocol, but the buspirone treatment did not seem to affect the velocity in any significant manner (4.4D). The size of the animals was also for this experiment comparable, and similar across groups (See Appendix figure 2). Overall, the treatment of buspirone did not exert any significant effect on animal performance in this CPA assay, suggesting the observed effect of DRN ablation possibly was not related to 5-HT directly.

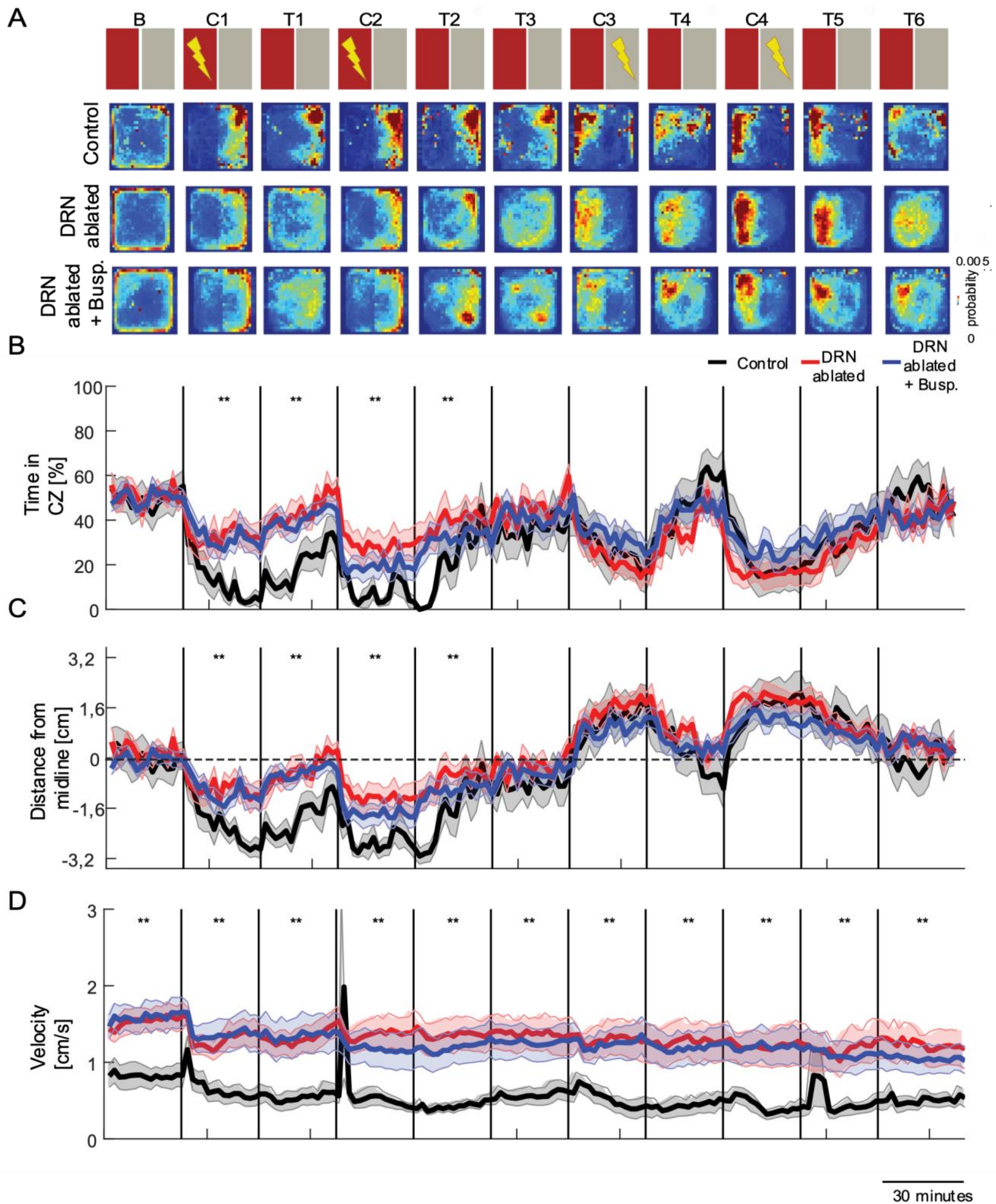

Figure 4.4: Analysis of behavioral experiment for dorsal raphe nucleus ablated animals (10 mM MTZ) and dorsal raphe nucleus ablated Buspirone treated animals. *Test group 1: TPH2 NTR-mCherry transgenic zebrafish MTZ treated n = 11. Test group 2: TPH2 NTR-mCherry transgenic zebrafish MTZ and Buspirone treated n = 17. Control group: TPH2*

GCAMP6s zebrafish MTZ treated n = 14. (A) Top row shows a schematic presentation of the behavioral protocol. Bottom row shows heat maps of averaged density of position for all zebrafish B) Percentage of time spent in the conditioned zone (CZ) of arena C) Distance from midline in cm for animals. Dashed line indicates the midline. D) Average swim velocity of all zebrafish E) Size plot. Abbreviations: MTZ: metronidazole. Shadows: S.E.M. Significance ($p < 0.05$) is indicated with an asterisk, and significance ($p < 0.01$) is indicated by two asterisks (**).

4.1.5 DRN ablated zebrafish treated with Fluoxetine

As the treatment of buspirone did not seem to improve or rescue the learning performance of DRN ablated animals, different pharmaceuticals targeting 5-HT were considered. Another known 5-HT targeting pharmaceutical that has been used in zebrafish and shown an effect, is fluoxetine (Maximino et al., 2014).

Although fluoxetine mainly has been documented to have an anxiolytic effect in zebrafish, as with buspirone, the treatment of this pharmaceutical was assumed to potentially influence the effect of the DRN ablation. Fluoxetine, as an SSRI, exerts it effect by delaying the reuptake of 5-HT (Benfield, Heel & Lewis, 1986), meaning it leaves more 5-HT available to bind to postsynaptic receptors and exerts it effects. As with the buspirone experiments, fluoxetine concentrations were based on a previously used protocol of the lab which showed to have an effect in 3-4 week-old-zebrafish (Chris Wiest, unpublished data, Yaksi Lab). Additionally, as the difference in animal learning performance was evident from the first conditioning sessions in both the prior experiments (4.3A C1-T2, 4.4A C1-T2), the protocol in use was shortened. The session involving the component of reversal learning was removed, as all experimental groups generally seemed to learn the new rule well, and there was not a substantial difference to observe in adopting to the new rule.

The test group and control group were the same as in the experiment shown in Figure 4.4. The second test group added was Tg(TPH2:Gal4:UAS:NTR-MCherry) 3-4 week-old zebrafish treated with MTZ (10 mM) for 24 hours and fluoxetine (100 ug/L) for 48 hours. Treatment of both drugs were started at the same time, but after 24 hours the MTZ washed out, and the water was replaced with fresh, AFW and a new dose of fluoxetine. The behavior protocol and results are displayed in Figure 4.5.

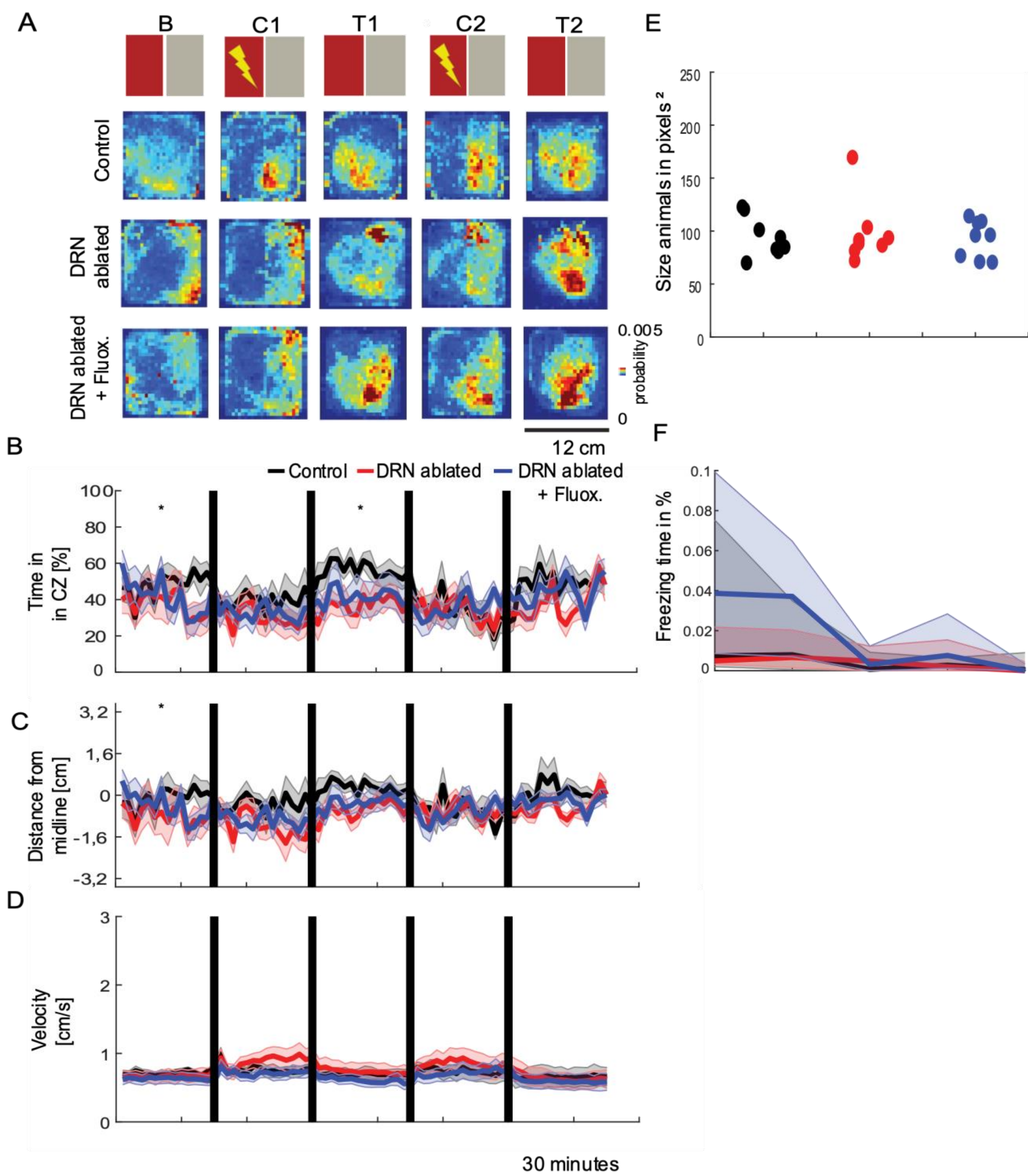

Figure 4.5: Analysis of behavioral experiment for dorsal raphe nucleus ablated animals (10 mM MTZ) and dorsal raphe nucleus ablated Fluoxetine treated animals.

Test group 1: TPH2 NTR-mCherry transgenic zebrafish MTZ treated n = 8. Test group 2: TPH2 NTR-mCherry transgenic zebrafish MTZ and Fluoxetine treated n = 8. Control group: TPH2

GCAMP6s zebrafish MTZ treated n = 8. (A) Top row shows a schematic presentation of the behavioral protocol. Bottom row shows heat maps of averaged density of position for all zebrafish. Baseline is 1 hour, following sessions are 30 minutes each. B) Percentage of time spent in the conditioned zone (CZ) of arena C) Distance from midline in cm for animals. Dashed line indicates the midline. D) Average swim velocity of all zebrafish E) Size plot. F) Freezing behavior with quartiles as error bar. Abbreviations: MTZ: metronidazole. Shadows: S.E.M. Significance (p < 0.05) is indicated with an asterisk (*).

As with the treatment of buspirone, fluoxetine treated animals did not show an improvement in learning performance in this experiment for any of the parameters (Figure 4.5A-C9 and there was not a significant difference between the animals apart from during the baseline session. Additionally, the learning performance of the control group animals was in this experiment worse compared to the prior experiments (4.3B, 4.4B). This could relate to the smaller sample size in this experiment, which was substantially smaller than the two prior experiments. In terms of freezing behavior, which was calculated for animals not moving for 2 seconds and up, there was an increase in freezing for the fluoxetine treated animals.

Overall, neither the treatment of buspirone or fluoxetine in DRN ablated animals seemed to rescue the learning performance. Whether this finding had to do with the was uncertain. A possibility could also be that the MTZ ablation and neuronal loss o 5-HT neurons was too substantial for the 5-HT targeting drugs to alleviate.

4.1.6 Transgenic zebrafish treated with only Fluoxetine and Buspirone

To assess the effect of the drugs buspirone and fluoxetine drugs alone, without the involvement of chemogenetic ablation of 5-HT DRN neurons, animals were considered to be treated with the drugs alone, before being tested in the CPA assay. As background line, the same tg(TPH2:Gal4:UAS:NTR-mCherry) line was used, but this time not treated with MTZ. The same concentrations of buspirone and fluoxetine were used for both these experiments, and the protocol used involved a shorter 2.5-hour protocol. The behavior analysis of the Fluoxetine experiments can be found in Figure 4.6.

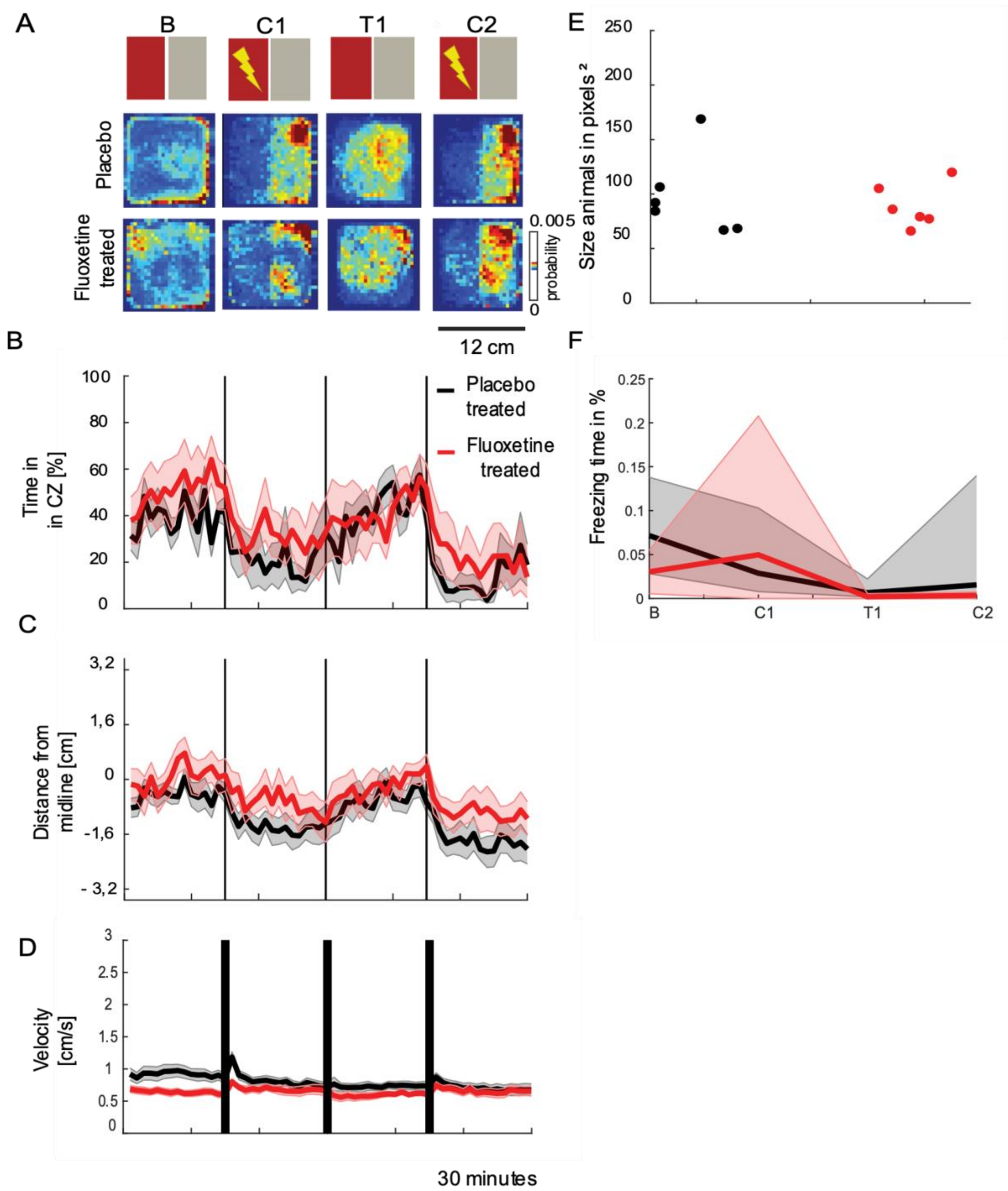

Figure 4.6: Analysis of behavioral experiment for Fluoxetine treatment alone.

*Test group: Tg(TPH2:Gal4:UAS:NTR-mCherry) zebrafish larvae Fluoxetine treated (100ug/L) (n= 6). Control group: Tg(TPH2:Gal4:UAS:NTR-mCherry) zebrafish larvae placebo treated with AFW (n = 6). **(**A) Top row shows a schematic presentation of the behavioral protocol. Bottom row shows heat maps of averaged density of position for all zebrafish. Baseline is 1 hour, following sessions are 30 minutes each. B) Percentage of time spent in the conditioned*

zone (CZ) of arena C) Distance from midline in cm for animals. Dashed line indicates the midline. D) Average swim velocity of all zebrafish E) Size plot. F) Freezing behavior with quartiles as error bar. Abbreviations: MTZ: metronidazole. Shadows: S.E.M. Significance ($p < 0.05$) is indicated with an asterisk (*).

In term of learning performance, the treatment of fluoxetine did not seem to lead to an increase in learning performance (4.6A-C). The only observable difference that the fluoxetine exerted was in terms of velocity, which was slower for the fluoxetine treated animals during baseline and C1 (4.6D B-C1). The overall finding that fluoxetine did not lead to any observable difference could relate to the task and behavioral assay used, and that simply the anxiolytic effect of fluoxetine does not lead to a difference in a CPA assay. Due to a technical issue, and a small sample size, buspirone experiments were not able to be performed analysis on.

4.2 Adopting pERK/tERK staining for studying neural activity during CPA learning

Prior to adopting the pERK/tERK staining for the study of neural activity, a strategy for quantification across aniamls had to be chosen. For the quantification strategy to be reproducible, a clear delineation of the different areas and subdivisions of the zebrafish dorsal pallium had to be performed.

4.2.1 Defining areas of the zebrafish pallium for quantification

To delineate the different areas and subdivisions of the zebrafish pallium, zebrafish of 21 dpf were sacrificed, dissected and stained for DAPI counterstaining, to outline the cells bodies of the pallium. In figure 4.7, the DAPI counterstaining is displayed, indicating the different subdivision of the dorsal zebrafish pallium. The different panels from left to right show selected sections of the dorsal pallium from dorsal to ventral (B -> D). In the most dorsal section, both the dorsolateral (Dl) pallium and dorsomedial (Dm) pallium were visible. In the following section, the Dl was smaller and an area of neuropils, or low amounts of cell bodies, became apparent. In this section, the sulcus ypsyloniformis was also apparent between the Dl and Dm. In the most ventral section, the dorsoposterior (Dp) and dorsocentral (Dc) pallium was visible, as well as the entopeduncular nucleus. Additionally, across all sections the

habenula (Hb) was visible. Based on this, a delineation of the different subdivisions was made as a means for further quantification in the pERK/tERK staining.

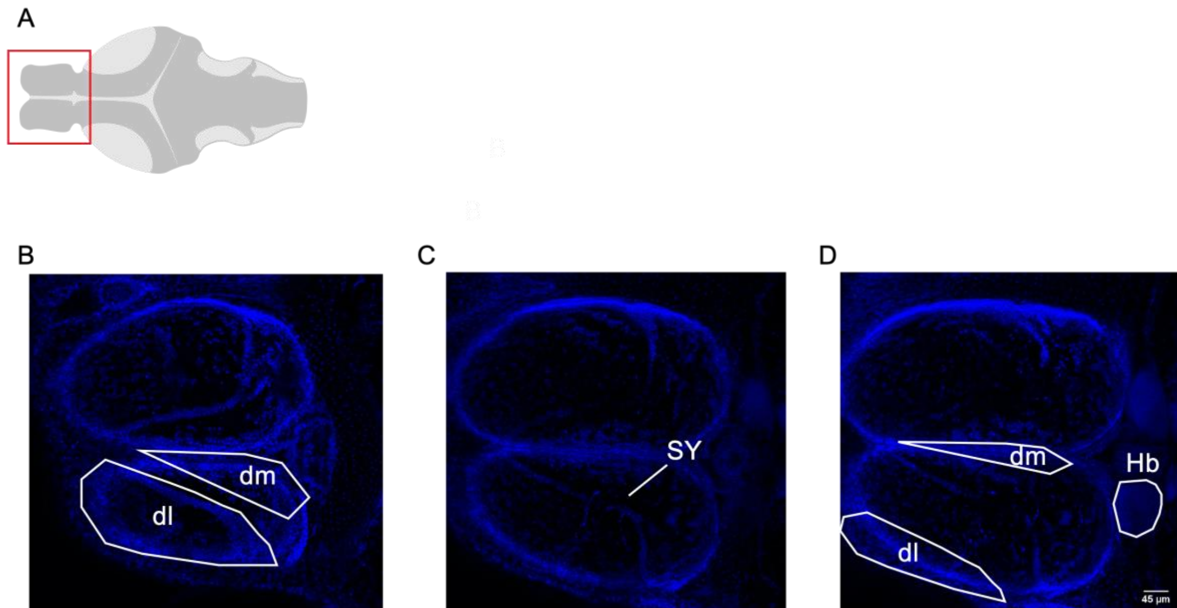

Figure 4.7: Delineation of 21 dpf zebrafish pallium subdivision of confocal z-stack DAPI counterstaining. *A) Illustration of zebrafish with dorsal pallium indicated with the red box (Illustration from Luigi Petrucco, scidraw.io). B) to D): Sections of the dorsal pallium from dorsal to ventral (B->) are displayed. Abbreviations: Dl: dorsolateral, Dm: dorsomedial: Dp: dorsoposterior, Dc: dorsocentral: SY: Sulcus ypsyloniformis: Hb: Habenula: Scale bar: 45 um.*

4.2.2 Testing ERK staining in freely behaving zebrafish

Before using the pERK/tERK staining in CPA, the staining technique had to be tested in a freely behaving fish showing to assess the spontaneous activity. Nacre zebrafish of 21 dpf were sacrificed, dissected and stained for pERK/tERK activity and scanned in a confocal microscope. The animals had only been exposed to daylight prior to being sacrificed. Sections from the confocal stacks can be found in figure 4.8. In red, the activated cells can be seen, which are stained by the pERK secondary antibodies. The patterns of activity seemed to be scattered across the forebrain, with some activity in both the Dl and Dm, as well as in the lateral part of the habenula. The staining of total ERK was mostly seen in areas of neuropils.

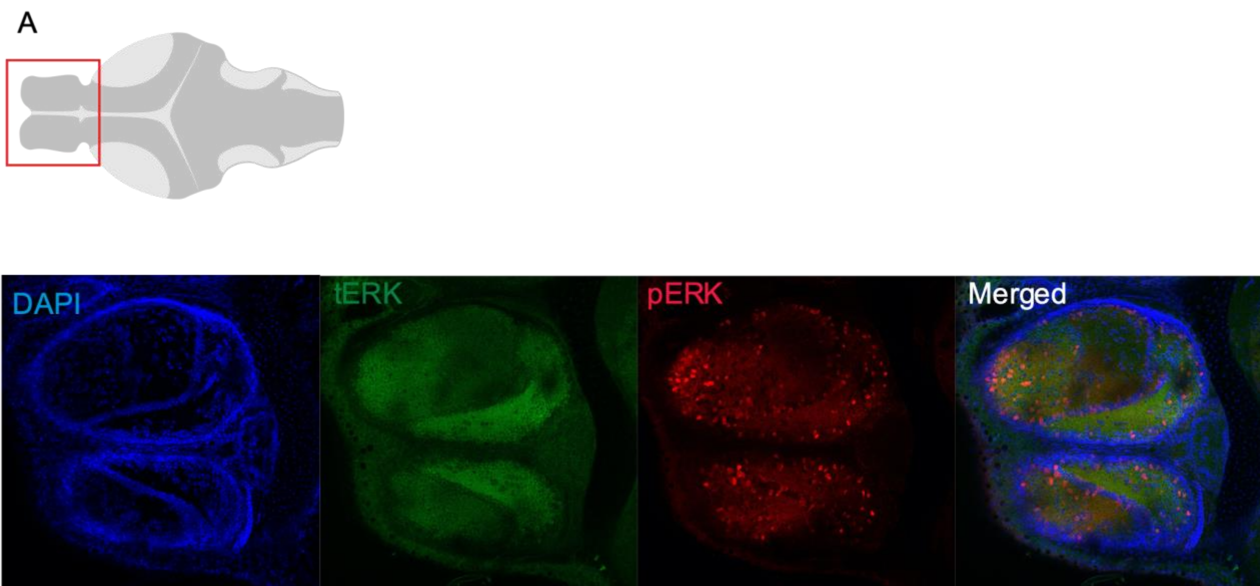

Figure 4.8: Confocal z-stack of 21 dpf freely swimming zebrafish stained for pERK/tERK acitivty. *A) Top row shows illustration of a zebrafish with dorsal pallium indicated with a red box (Illustration adopted from Luigi, scidraw.io). Bottom row from left to right shows confocal images of different markers used for staining cell bodies and ERK. Far right is a merged image. Abbreviations: tERK: total ERK, pERK: phosphorylated ERK.*

4.2.3 CPA protocol with implementation of pERK/tERK staining

To investigate the neural activity of DRN ablated zebrafish, the pERK/tERK immunohistochemical staining method could be utilized. Based on the deficit learning performance seen in DRN ablated animals (Figure 10), an emerging question was what the neural activity looked like in these animals, and whether there was any neural implications of the chemogenetic ablation. Previous work in the host lab using ERK staining following CPA behavior experiments revealed that zebrafish which had a low learning performance had a higher neural activity level in areas of the dorsal telencephalon, specifically the Dm and Dl (Bram Serneels, unpublished data, Yaksi Lab). Based on this, one expectation was that animals that did not learn well would have more neural activity because they receive more shocks and could be unknowing of what to do to avoid the shock. Additionally, given the largest difference was seen in the experiment of Figure 4.3, comparing DRN ablated animals with GCAMP6s controls, the expectation was that these two groups were the most likely to have observable differences in neural activity. As the experiments involving

buspirone and fluoxetine treatments did not show any large differences in animal learning performance (Figure 4.4-4.7).

To implement the pERK/tERK staining following the CPA protocol, some experimental changes had to be considered. Firstly, because the difference in learning performance was evident already from the first conditioning session as seen in Figure 4.3, a shorter protocol was considered as suitable. Secondly, to be able to assess the neural activity of the animals when they were learning, animals were taken out at the end of the second conditioning session (C2). The reasoning behind this was that during the conditioning session, when there is shock delivery in the CZ, the animals are more likely actively associating the shock with the visual pattern, and thereby undergoing learning. Third, a different control group had to be found than the GCAMP6s animals used in the first experiment (Figure 4.3). The reason for this is that GCAMP6s is a green protein, and in the confocal microscope the green would be overlapping with the secondary antibodies used for pERK staining. Therefore, as controls for these experiments, nacre animals treated with MTZ were used. In addition, due to overlap with the mCherry expression, the ERK protocol was changed during the time between the testing of the protocol (4.8) and the use of the staining with CPA protocol. In table 3.2 in the Methods section, the newly established protocol in terms of secondary antibodies can be found. In Figure 4.9 the behavior protocol and analysis can be found, and statistical significance analysis can be found in Supplementary table 2.

During baseline, both groups performed equally, and showed no preference for either compartment of the arena. These findings are similar to those of the previous experiments (Figure 4.3 - 4.7). In most of the parameters shown, DRN ablated animals showed a worse learning performance compared to the control group (4.9, although the difference between both groups was less than in the experiment in Figure 4.3. DRN ablated stay close to 40% of the time in the conditioned zone during C1, with a gradual increase during T1, and a subsequent decline for C2. Both groups did however show evidence of learning by spending less time in the conditioned zone during C2 compared to C1 (Figure 4.9 B), but the Nacre control animals did not achieve similar levels of learning performance as seen with the control animals of Figure 4.3. A striking difference seen in this experiment was the velocity, which in

contrast to the previous experiments was higher for the control animals than the DRN ablated animals (Figure 4.9 D). The size of animals was also similar across group, although on the lower end in terms of size (50-150 $pixels^2$).

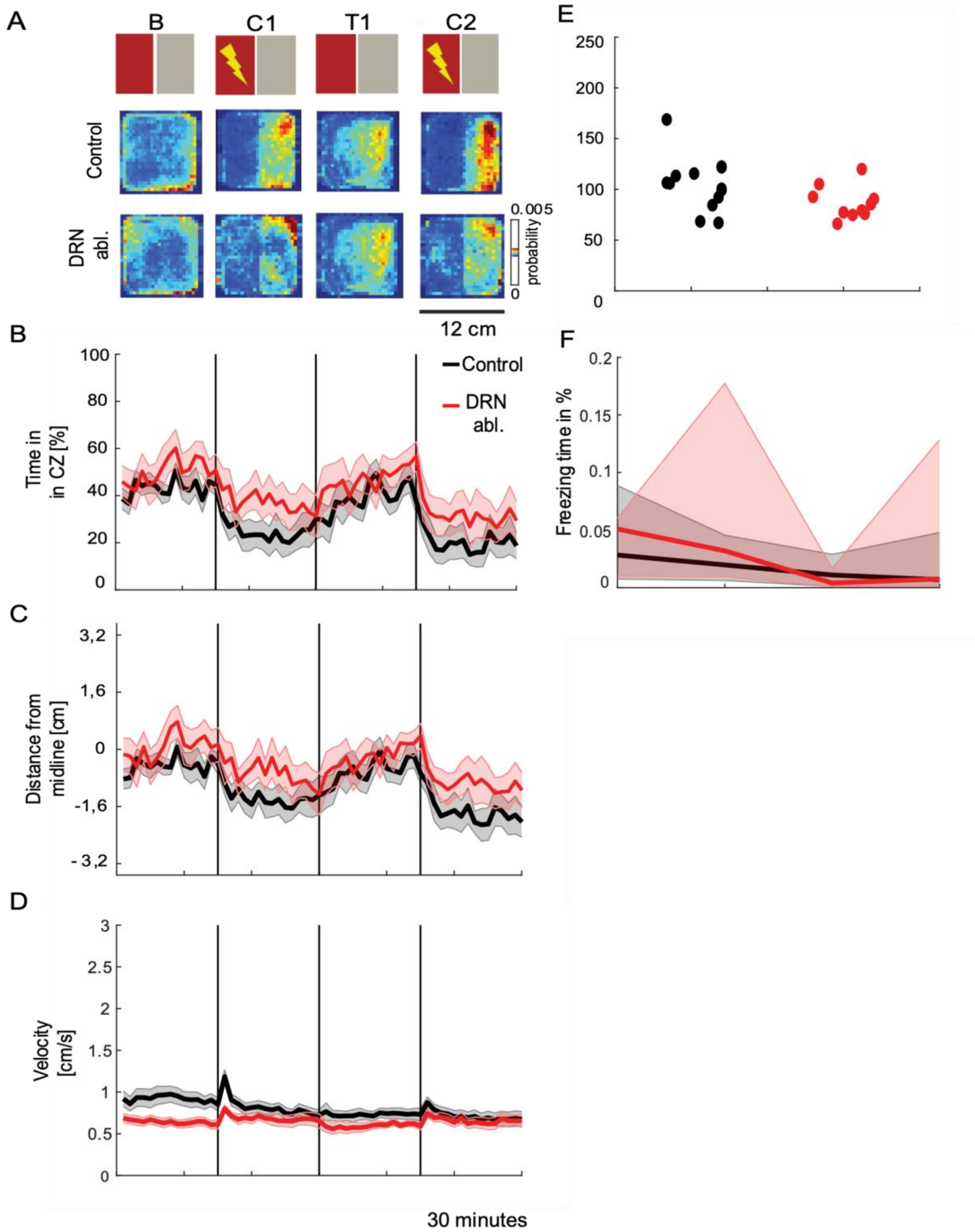

Figure 4.9: Analysis of behavioral experiment for dorsal raphe nucleus ablated animals with shortened protocol. *Test group: TPH2 NTR-mCherry transgenic zebrafish treated with MTZ (10 mM) (n = 12). Control group: Nacre zebrafish treated with MTZ (10 mM) (n = 12). (A) Top row shows schematic presentation of the behavioral protocol. B: baseline, C1: conditioning 1, T1: test 1, C2: conditioning 2. B) Heat maps of averaged density of*

position for all zebrafish C) Percentage of time spent in the conditioned zone (CZ) of arena D) Average swim velocity of all zebrafish E) Size plot in pixels (2). F) Percentage time of animals freezing MTZ: metronidazole. Shadows: S.E.M. Significance ($p < 0.05$) is indicated with an asterisk (*).

4.2.4ERK staining of Nacre animals and DRN ablated animals.

To assess whether the individual ERK activity of DRN ablated animals were noticeably different than control animals in relation to learning performance, each activity pattern from the confocal images were paired together with the individual behavior patterns of heat maps. A z-projection of the top 30 slices of the scanned z-stack was performed, and a median of the projection of the selected slices was performed. By use of the delineations shown in figure 4.7, intensity levels of subdivisions Dl, Dm and the habenula of the dorsal pallium were measured for reasons of comparison. Since phosphorylated ERK only appears to remain in the brain for 10-15 minutes (Randlett et al., 2015), the neural activity will only likely be reflected for the last conditioning session. The ERK activity patterns along with the behavior patterns can be found in figure 4.10, and the session is indicated with a red box (Figure 4.10 A-D).

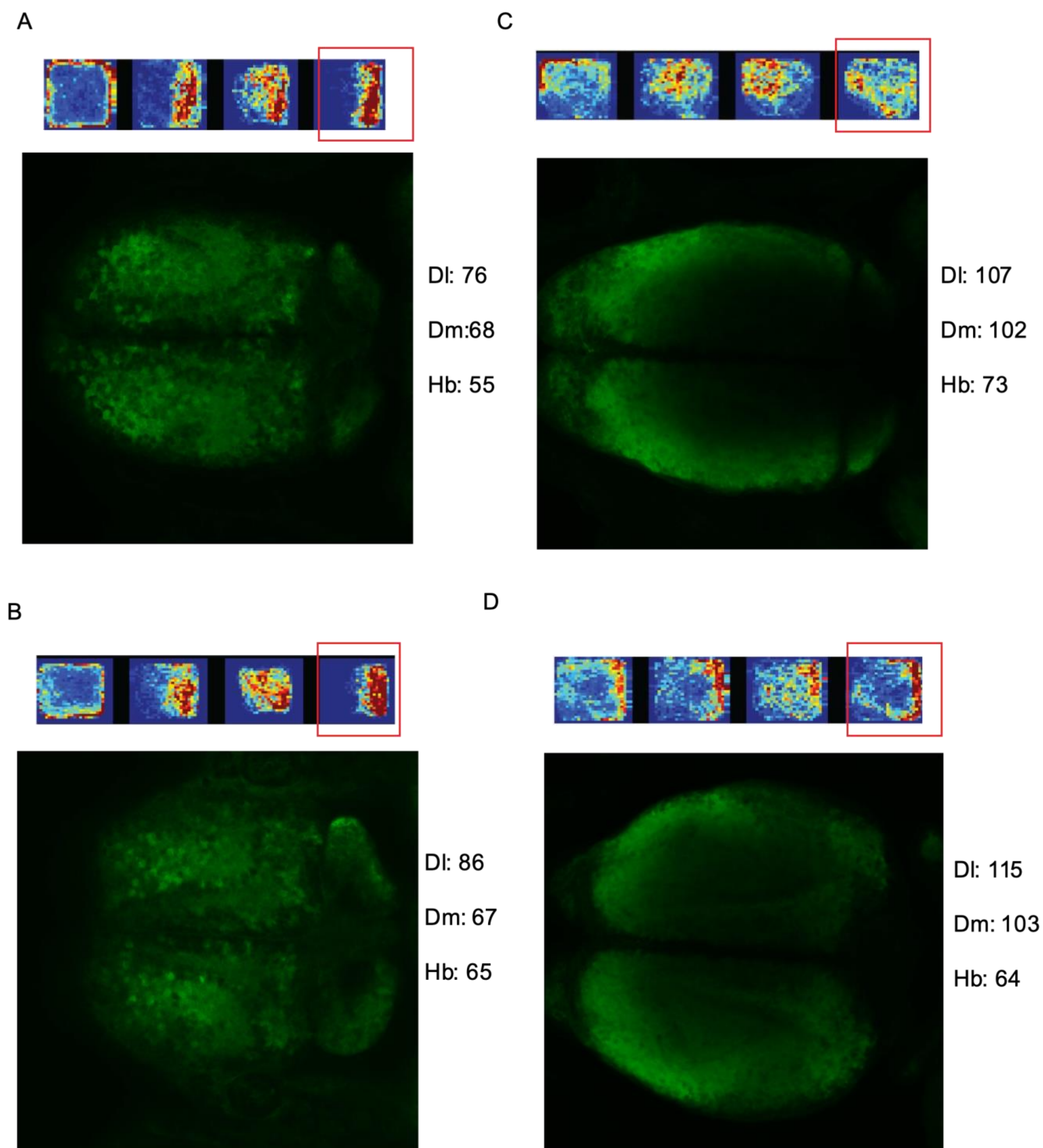

Figure 4.10: pERK activity paired with single plot behavior patterns during CPA protocol of Nacre and DRN ablated animals. *Individual behavior patterns paired with median z-projections of phosphorylated ERK showing neural activity and accompanying*

intensity measurements of selected brain regions in arbitrary units shown on the right hand side. (A.U) for A) Nacre zebrafish B) Nacre zebrafish C) Tg(TPH2:Gal4) DRN ablated zebrafish D) Tg(TPH2:Gal4) DRN ablated zebrafish.

5. Discussion and conclusion

5.1 DRN ablation leads to learning performance impairment in CPA

During the work of this master's thesis, the functional role of the dorsal raphe nucleus (DRN) was examined using transgenic zebrafish. Confocal imaging showed that the Tg (TPH2:Gal4: UAS:NTR-mCherry) has confined expression of the reporter protein in 5-HT neurons of the dorsal part of the raphe nucleus. By treating animals of the transgenic zebrafish line with metronidazole (MTZ), a specific chemogenetic lesion was made in these animals, which successfully ablated neurons of the dorsal raphe nucleus. By testing the 5-HT DRN ablated animals in a CPA paradigm, the learning performance of these animals was assessed. Across different experiments it was shown that 3-4-week-old DRN ablated zebrafish struggled to learn and perform as well as a control group in a CPA assay, and these findings were apparent for multiple behavioral parameters, and consistent across different control groups. The question remains however to what extent the DRN is involved in the learning process on a cellular or molecular level, given its variety of afferent and efferent projections, as well as its variety in neuronal populations employing different neurotransmitters.

For the experiments involving DRN ablated animals, different experimental protocols were used, showing observable differences in learning performance for animals. In terms of the experiments which involved a component of reversal learning, DRN ablated animals did not seem to perform any worse in terms of learning a new rule, or updating previous information, compared to the control groups. Although initially performing worse than control groups, the DRN ablated animals successfully learned to avoid the shock when the conditioned zone switched, suggesting that the involvement in learning for DRN did not directly relate to the integration of new information and coping in stressful situations. This finding opposes the recent findings of dorsolateral habenula (dHBl) in zebrafish (Palumbo et al., 2020), and that the DRN might not be involved in processes of reversal learning.

As evident from most of the experiments, DRN ablated animals showed a deficit in learning performance, by spending more time in the conditioned zone during conditioning sessions (Figure 4.3A, Figure 4.8A). Although this parameter is usually regarded as an indicator for learning performance, and that staying a high

percentage of the time in the conditioned zone would imply the animals receiving more of the shock, the aspect of potential anxiety-like or defensive behavior must be considered. One way to interpret the current findings could be that due to a lack of 5-HT synthesis due to DRN ablation, the overall anxiousness of the animals was reduced. Therefore, the findings of could imply that due to less anxiety-like behavior, the animals experience less fear of the conditioned zone and explore more of the arena towards the midline, as seen in the parameters of time spent in conditioned zone, distance to midline, as well as the heat maps (Figure 4.3A-C). This is evident from the parameter of distance to midline in particular (4.3), which for the DRN ablated animals was apparent that they stayed much closer to the midline and the zone where the shock was received. The current CPA assay does however lack specific measures for examining anxiety-like or defensive behavior in zebrafish directly, compared to tests such as the novel tank test (NTT) or light-dark test (Maximino et al., 2013; Maximino et al., 2014). Therefore, the potential anxiety-like or defensive behavior component cannot be assessed fully in these experiments. Further analysis could however examine behavioral parameters aimed at anxiety-like behavior in particular, to investigate whether the observed learning deficit could relate to anxiety levels. Another possibility as to why the DRN ablated animals spend more time in the conditioning zone could relate to the aversive stimulus used in this CPA assay, and the involvement of 5-HT in pain perception modulation.

Since the role of serotonin in pain perception is contrasted and assumed to be both involved in the inhibition and promotion of pain (Telner, Lepore & Guillemot, 1979; Marks et al., 2009), it can be difficult to assess whether the chemogenetic ablation of 5-HT DRN neurons in the animals in these experiment actually had an impact on this sensation, and whether it had any influence the learning performance. One expectation could be that if the DRN ablation leads to an inhibition in pain perception, the ablated animals would explore more of the conditioned zone and act less aversive in response to the shock, because they experience the pain from the aversive stimulus as less painful. Due to the conflicting evidence and unclarity into the exact role of serotonin in these pain perception processes however; these questions remain unanswered. A possible procedure for investigating this could be to assess the behavior of animals right after the shock is received, in terms of velocity, to assess whether there is a difference in behavior in response to delivered shocks.

Attempts of such analysis were performed, which initially showed signs of DRN ablated animals to swim slower in response to the shock compared to control animals, which could imply a lowered sensitivity or perception of pain (See Supplementary figure 1) .These analyses were not thoroughly examined though, and a suitable way of quantifying the difference in velocity between animals was not found during the limited time frame of this thesis. Further analysis into these parameters could contribute to a better understanding of whether pain perception explains some of the observed deficits in learning performance as seen in DRN ablated animals.

Considering the neuronal population of the DRN is vast, and multiple neuronal populations exists that are GABAergic, glutamatergic and peptidergic neuronal, the the 5-HT neuronal population represents only a minority (Jacobs & Azmitia, 1992; Dorocic et al., 2014; Lillesaar et al., 2009; Lillesaar, 2011). Although the use of TPH2 is a serotonergic specific marker, and the use of this transcription factor would only target 5-HT neurons, there is a possibility that the observed learning deficit does not relate to the 5-HT DRN neurons directly, but rather the serotonergic projections they provide to areas such as the forebrain, or its involvement in the interconnected reward circuitry of the brain (Liu et al., 2014). Additionally, as 5-HT levels have been implicated to be elevated in the amygdala during fear conditioning (Bauer et al., 2014), a potential role of the DRN in learning could relate to this, and the serotonergic projections this structure provides to the amygdala and its related structures.

5.2 Targeting serotonin by use of pharmaceuticals buspirone and fluoxetine does not lead to a substantial increase in learning performance in CPA

The involvement of serotonin in learning in a CPA paradigm was investigated in two ways: 1) by treatment of DRN ablated animals with 5-HT targeting pharmaceuticals, thereunder buspirone and fluoxetine, and 2) treatment of non-ablated transgenic zebrafish with buspirone and fluoxetine, compared to placebo treated animals. Neither of these experiments showed a substantial effect, or an improvement in learning in the CPA paradigm, suggesting the two pharmaceuticals in question does not have an impact on the type of learning. It was clear that neither of the drugs had

any impact on any of the behavioral parameters that are used for assessing learning, and the only observable and consistent difference seen with the treatment was for velocity of animals. The velocity of treated animals with both buspirone and fluoxetine was slower compared to placebo treated and control groups, suggesting the animals were more relaxed and exerted less anxiety-like behavior, which is in line with the previous findings of fluoxetine and buspirone treatment in zebrafish (Maximino et al., 2013). Although our expectations were that the use of a 5-HT_{1A} agonist could potentially compensate for the ablation of 5-HT, previous studies performed in mice have implicated that the use of buspirone actually disrupts the fear conditioning, rather than enhancing it (Quartermain, Clemente & Shemer, 1993). This was shown in a fear conditioning test involving the pairing of a tone and an electrical shock, and the effect was only apparent when buspirone was given before the conditioning session, but not before the test trial. These findings, although performed in rats, would be in line with the findings of this thesis, suggesting that buspirone does not lead to a beneficial effect in terms of learning in a fear conditioning CPA paradigm.

Similarly, as the experiments involving fluoxetine neither exerted a substantial effect in terms of learning performance, recent experiments involving fluoxetine treatments in fear conditioning in rats have shown similar effects. It was shown that fluoxetine reduced conditioned fear expression, and it was suggested that fluoxetine might be effective in reducing conditioned fear expression when administered immediately or after aversive experiences (Graham, Dong & Richardson, 2018). Overall, in consideration the overall anxiolytic function, it is possible to assume that the treatment of fluoxetine and buspirone in theory should not lead to an improvement in learning performance in a fear conditioning paradigm. A less anxious or less defensive animal would not stay more in the safe zone, and would rather be expected to explore more of the conditioned “danger” zone, and thereby would show as in the parameters of learning performance. Although our expectations were that these drugs could rescue the learning performance in DRN ablated animals, by modulating 5-HT levels, further details and examinations into how these pharmaceuticals actually function in fear conditioning reveal that the effect might be opposite of our expectations

5.3 ***Immunohistochemical staining for pERK/tERK activity shows elevated activity in DRN ablated animals***

An immunohistochemical staining technique staining for phosphorylated ERK, as a marker for synaptic plasticity and neuronal activity, was adopted as a post-hoc analysis method for examining neural activity following a CPA paradigm. Analyses showed that transgenic DRN ablated animals, who performed worse in terms of learning performance, had heightened levels of activity compared to nacre control animals, who performed better in terms of learning. Delineations and intensity measures of areas in the dorsal pallium showed that this activity was in the areas of the dorsolateral (Dl) and dorsomedial pallium (Dm) areas Dl and Dm in DRN ablated animals compared to nacre controls. These findings can be interpreted in multiple ways, but one possible explanation for the elevated activity in the animals which performed worse could be that these animals received more shocks and were not able to associate the visual stimulus with the shock, which lead to a heightened neuronal activity. Further experiments with different conditions in the CPA protocol could reveal more as to what causes this neural difference, and whether the perception of the electrical shock can explain these differences.

The MAPK/ERK pathway, which is the targeted pathway of synaptic plasticity in this immunohistochemical technique, does not only stain neurons, but also glial cells (Gao, 2009, Suo et al., 2019). A neuronal staining was included in the current ERK protocol (Nissl stain), but to the current point a suitable method for taking use of this for segregating out neurons from glial cells have not been found.

During the recent months, a method of alignment of ERK activity patterns of zebrafish has been implemented in the host lab as a quantification strategy for analyzing multiple scans of zebrafish brain, to better quantify and see activity across groups of animals. This strategy involves the use of aligning z-stacks onto a reference brain, based on a ratio of pERK divided by tERK. The use of this as a quantification strategy, as well as obtaining a larger sample size of the test and control groups used in the experiments of this thesis, could be a valid step forward.

5.4 Experimental influences

5.4.1 Effects of the developmental stage of animals

During all of the experiments involved in the work of this master's thesis, zebrafish of 3-4 weeks-old were used. Although animals of this age have been shown to be capable of cognitive tasks such as learning (Valente et al., 2012; Yashina et al., 2019; Palumbo et al., 2020), there can be large variability from animal to animal in this developmental range. At the stage of 3-4 weeks old, zebrafish undergo metamorphosis from larva to juvenile (Schmidt et al., 2013), and large differences can be seen in terms of velocity between larva and juvenile zebrafish. In terms of developmental stage, the cohort of animals used in the work of this thesis showed some variability in terms of size. As seen in the size plots of some of the behavioral experiments, a range of different sizes was apparent. Efforts were made to ensure that selected fish were comparable in terms of size prior to selection for the experiments, although

5.4.2 Regenerative capacity of zebrafish

Zebrafish are known to have a large regenerative capacity and are known to be able to regenerate their heart, as well as having neurogenesis (Gemberling et al., 2013). Additionally, at the 3-4 week stage of development, zebrafish go under metamorphosis from larvae to juvenile stage (Schmidt et al., 2013), which also leads to brain development and neurogenesis. Because of this, it is possible that some neurogenesis happens in response to the chemogenetic ablation, although there have not been examinations of this during the work of this thesis.

5.4.3 Effect of water and room temperature

Water temperature and room temperature is known to have an effect on animal comfort and behavior. As experiments were performed both during winter and spring/summer periods, climatization and temperatures could be different for the different experiments, which could influence the behavior of animals across all parameters. Different efforts made for stabilizing temperatures, such as installing a heater and ensuring regular temperature on the water used in the behavioral arenas suggest against the effect of water temperature on the behavior of the animals.

5.5 Conclusion

Throughout this master's thesis, the role of DRN and 5-HT in learning has been investigated. Along with experiments involving a reversal learning component, DRN is implicated as involved in fear learning in a CPA paradigm, showing across multiple behavioral parameters and experiments. Chemogenetic ablation of 5-HT neurons in the DRN showed that animals struggled with learning but were able to update a previously learned rule over the course of the CPA paradigm. Further, by use of an immunohistochemical staining technique (pERK/tERK) performed for assessing neuronal activity, DRN ablated animals showed heightened levels of activity overall, and specifically in dorsolateral and dorsomedial subdivisions of the dorsal pallium. The findings of this thesis can be used to inspire novel investigations into the serotonergic system and its involvement in learning, which could inspire novel treatments of mental illnesses related to this, such as anxiety and depression

5.6 Future perspectives

During the work of the thesis, the behavioral experiments conducted have only been analyzed to a limited extent, and by only a certain number of behavioral parameters. Parameters such as number of shocks delivered, animals' response after receiving the shock, midline approaching behavior, decision making during conditioning, as well as avoidance behavior of the shock could be examined. These parameters can be investigated to assess whether the learning DRN ablated animals differ in the number of shocks they receive, or whether there's a difference in the response to shock, and if any of these parameters relate to the deficit in learning performance. As DRN ablated animals in the experiments performed in this thesis only were subject to experiments once before being sacrifice, one strategy could be to perform multi-day training as a means for assessing long-lasting memory, and whether animal learning performance improves over time. However, given the learning effect was mostly present from the initial sessions of the behavioral protocol, the findings reveal that DRN more likely is involved in learning processes, rather than long-term memory. Given the complex function of DRN and 5-HT in the brain, and the known involvement in anxiety-like or defensive behavior, one strategy could be to segregate the anxiety component, by altering the conditions for the shock delivery, and thereby investigating whether the detrimental learning effect can be related to levels of

anxiety or defensive behavior. As experiments were performed in sequence and during different time periods, the sample sizes of these experiments were not held consistent across experiments. Further experiments could be performed in the same condition to increase the sample size to a similar extent as in the first behavioral experiments. Finally, as the ERK staining in the lab recently has been optimized and found useful for neuronal activity staining, this method can be expanded and utilized further.

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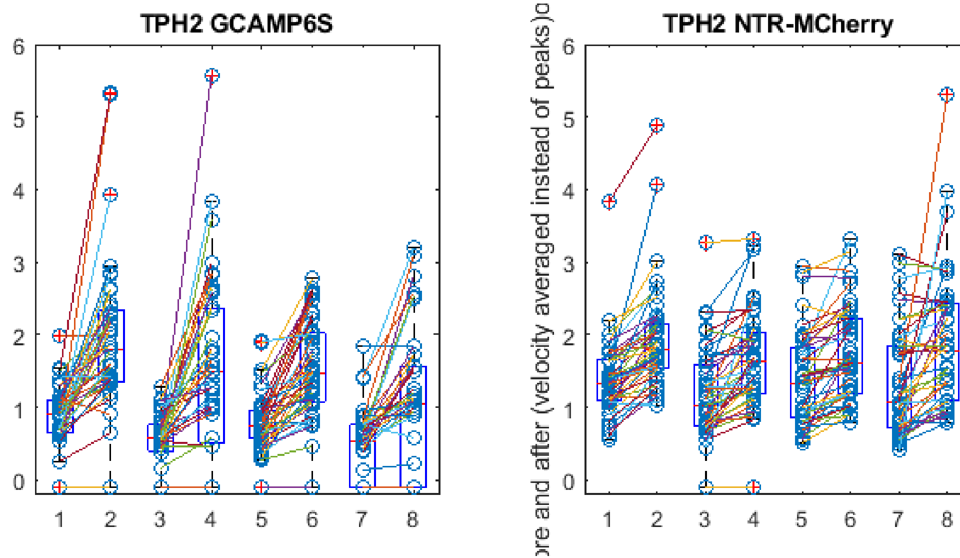
Appendix: Supplementary figures



Supplementary figure 1 showing heightened in experiment of figure 4.3.

Supplementary table 1

Statistical analysis of experiment with buspirone treatment alone	time red	velocity
Baseline	0.000*	0.000
Conditioning 1	0.4180	0.000
Test 1	0.0211*	0.000
Conditioning 1 shock 2	0.2576	0.0158*
Statistical analysis of experiment of MTZ vs non-mtz		
Baseline	0.3091	0.5489
Conditioning 1	0.000***	0.000***
Conditioning 1 test 1	0.002	0.000***
Conditioning 1 shock 2	0.000***	0.000***
Statistical analysis of experiment of DRN ablated vs. GCAMP6S control		
Baseline	0.37	0.00
Conditioning 1	0.000	0.00
Test 1	0.000	0.00
Conditioning 1 shock 2	0.00	0.00
Test 2	0.00	0.00
Test 3	0.00	0.00
Conditioning 2 shock 1	0.00	0.00
Test 4	0.00	0.00
Conditioning 2 shock 2	0.22	0.00

Test 5	0.21	0.00
Test	0.24	0.00
Conditioning 1 shock 2	0.18	0.00