The role of central amygdala neuronal types in drug-related and appetitive behaviors

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CHAPTER 1. INTRODUCTION

Drug abuse and obesity are two major health problems the world faces today. According to World Health Organization, 35 million people worldwide are suffering from drug abuse, while 650 million people are categorized as obese [1,2]. These extensive numbers paint a dire picture, and we need more understanding of the underlying mechanisms causing these diseases, so that we can attack these health problems. At first sight, drug abuse and obesity might not seem similar, but both are heavily dependent on disordered rewarding and appetitive brain circuitry [3,4]. The central amygdala is a brain area that is involved in both rewarding and appetitive behaviors. This thesis tries to elucidate the mechanisms underlying these behaviors a bit more, while mainly focusing on the role of the central amygdala, and some of its major neuronal types.

1.1 Central amygdala

The central amygdala (CeA) is a striatum-like structure located in the brain and has been implicated in a variety of behaviors. Traditionally, the CeA is thought to be an output nucleus responsible for fear-related behaviors and in particular fear conditioning. Early lesion and electrical stimulation studies have shown that manipulation of the CeA can alter the expression of learned defensive behaviors [5]. These studies lead to the conclusion that the CeA is the output, or an exit station for fear conditioning, rather than being involved in the acquisition and storage of fear memories. However, recent studies have demonstrated a role for the CeA in the acquisition and consolidation of defense behaviors [6–8]. In addition, the CeA started to be implicated in more diverse behaviors than fear conditioning. Based on these studies, the CeA has emerged as an integrative hub that combines relevant sensory information about the external and internal environment and translates this into appropriate behaviors. These behaviors have included, but are not limited to, defensive, anxiety, rewarding, appetitive and addictive and drug-related behaviors [9–14]. With such a diverse set of behaviors, it is thought that genetically distinct CeA neuronal types, and variation in the output of these different neurons, might be responsible for these different behaviors. This thesis aims to provide a deeper understanding of the role of different CeA neuronal types in several understudied behaviors, primarily drug-related and appetitive behaviors.

1.2 Connection and composition of the central amygdala

The CeA is part of the amygdalae, which are two almond shaped areas located in the temporal lobe. The amygdala can be divided into two main areas, the basolateral amygdala (BLA), which consists of the lateral amygdala (LA) and basal amygdala (BA), and the central amygdala (CeA), which consists of the capsular (CeC), lateral (CeL) and medial (CeM) central amygdala. The genetic composition of BLA and CeA neurons is rather distinct [15]. In addition, the BA and LA differ from the CeA in embryological origin, and also in morphology. While the BLA consists of mainly excitatory pyramidal neurons, the CeA comprises predominantly GABAergic neurons. The differences between the different subnuclei can point towards different roles of these nuclei in behaviors. This thesis focuses on the role of the CeA in several types of behaviors

1.2.1 Cell types within the central amygdala

The majority of neurons in the CeA are GABAergic neurons, and they express a wide variety of different genetic or neurochemical markers. The two major cell types that have been studied in the CeA, are the Prkcd-expressing and SST-expressing neurons [16]. In addition to these two markers, more genetic markers are being identified. Newer markers include, but are not limited to, corticotropin releasing hormone (CRH), neurotensin (NTS), calcitonin receptor-like (Calcrl), tachykinin 2 (Tac2), serotonin receptor 2a (Htr2a) and vasoactive intestinal peptide 2 (VIPR2) [17,18]. Prkcd-expressing neurons and SST-expressing neurons make up approximately 80-90% of the neurons in the CeA, and they are largely non-overlapping [16]. CRH, Tac2 and NTS seem to be a largely similar population, and they make up a sub-population of SST-expressing neurons, while having close to no overlap with Prkcd-expressing neurons [17,18]. In addition, the Calcrl population shows most overlap with Prkcd-expressing neurons, while having little overlap with SST-expressing neurons [19]. The Htr2a population seems to be a very heterogeneous populous, having overlap with SST, Prkcd, CRH, NTS and Tac2 [17]. The VIPR2 population has been largely unstudied in the central amygdala, but preliminary data from our lab shows that it is likely a sub-population of SST-expressing neurons and only shows little overlap with Prkcd-expressing neurons.

1.2.2 Central amygdala connectivity

Apart from their local inhibitory connections within the CeA, the CeA also has a wide variety of inputs and outputs, with different functionalities [20]. The CeA receives inputs from the medial prefrontal cortex, insular cortex, sensory cortex, hippocampus, bed nucleus of the stria terminalis, parabrachial nucleus, substantia nigra pars compacta, the ventral tegmental area, raphe nucleus, intercalated cells, paraventricular nucleus and the basolateral amygdala. In addition, the CeA sends outputs to the dorsal motor nucleus of the nervus vagus, the nucleus tractus solitaries, the locus coeruleus, the periaqueductal grey, the hypothalamus, the basal forebrain and the globus pallidus. This wide variety in interconnectivity makes it possible for the CeA to be involved in a diverse range of behaviors.

1.3 Central amygdala in various behaviors

The extensive connections that the central amygdala has with different brain areas provides an optimal situation for the central amygdala to be involved in different behaviors. Over the years, the central amygdala has been implicated in numerous behaviors, although it's role in aversive behaviors, and in particular fear conditioning, has been most extensively studied[21]. Apart from fear conditioning, the central amygdala has also been shown to be involved in anxiety, fear, rewarding, addictive and appetitive behaviors, amongst others [20]. This chapter aims to give an overview of the knowledge available about the role of the central amygdala in these behaviors, and to point out what areas and questions remain to be researched and answered.

1.3.1 Central amygdala in defensive behaviors

Animals and humans alike are able to connect a dangerous or aversive stimulus with cues in the environment that might have predictive value to the dangerous or aversive stimulus. The presentation of these environmental cues in subsequent exposures can then elicit a defensive response. This type of conditioning is called Pavlovian fear conditioning and has been a very commonly used paradigm to study the acquisition and expression of defensive

memories. In Pavlovian fear conditioning, an initially neutral stimulus (the conditioned stimulus; CS) is paired with an innate aversive stimulus (the unconditioned stimulus; US). After training, the CS is able to elicit defensive responses in the absence of the US. Traditionally, lesion studies and pharmacological studies were used to elucidate the role of certain brain areas in behaviors, and early studies showed that the central amygdala might have been involved in the expression of fear memories. It was thought that the lateral part of the amygdala received input from the US and CS, causing synaptic alterations in the lateral amygdala [22]. Subsequent exposure to the CS then elicits a response in the lateral amygdala, which activates the central amygdala and causes downstream targets in the hypothalamus and areas in the brainstem to control defensive behaviors. In this model, the central amygdala is seen as an 'output' station for these memories, rather than being involved in the acquisition of fear memories [23,24].

However, more recent studies have shown that the central amygdala is also involved in the acquisition of fear memories after all. Through pharmacological inactivation of the central amygdala, combined with optogenetic stimulation and electrophysiological measures, it was shown that the central amygdala was necessary for the formation of fear memories [6,7,9,25]. In addition, research has uncovered the role of CeA neuronal types in defensive behaviors. Using electrophysiological in vitro recordings, it was shown that fear conditioning could induce a robust plasticity onto SST-expressing neurons in the CeA, which was required for the formation of a fear memory [9,26,27]. In vivo imaging demonstrates a similar picture, in which SST-expressing CeA neurons are activated during recall of a fear memory [28]. In addition, optogenetic activation of SST-expressing CeA neurons elicits freezing behavior [28,29]. These studies together show that SST-expressing CeA neurons play an important role in the formation and expression of fear memories and defensive behaviors.

1.3.2 Central amygdala in anxiety

Even though fear and anxiety have overlapping components, fear is mostly described to be in response to an imminent or immediate threat, while anxiety is used when the source of the anxiety is uncertain or distant in either space or time [30]. Anxiety disorders are incredibly common in our population and a lot of research has been done with human subjects to identify brain areas that are involved in anxiety disorders. Several studies over the years have demonstrated with neuroimaging that individuals with post-traumatic stress disorder and generalized anxiety disorder have a hyperactive or hyperreactive amygdala compared to healthy subjects [31–34]. Animal studies have provided more insight into the involvement of specific sub-nuclei of the amygdala, as well as different cell types and the circuitry involved in anxiety behaviors. Primate studies have shown that excitotoxic lesions of the bilateral CeA in monkeys lead to reduced anxiety upon exposure to innately fearful stimuli [35]. Regarding the role of neuronal types in the CeA in anxiety, there have been different studies showing that both SST-expressing neurons and Prkcd-expressing neurons might play a role in mediating anxiety. Using electrophysiology, it was shown that an increase in the excitatory input onto SST-expressing neurons caused heightened anxiety, a phenomena that was also demonstrated in stressed animals [36]. In addition, activation of Prkcd-expressing CeA neurons causes an increase in anxiety levels as measured on several anxiety paradigms, and an inhibition of these same neurons leads to a decrease in anxiety, pointing to a bidirectional control of anxiety for Prkcd-expressing CeA neurons [37]. These studies show that there is still a lot unknown about the role of the CeA and its neuronal subtypes in anxiety related behaviors.

1.3.3 Central amygdala in appetitive behaviors

At the beginning of the 21st century, research into the role of the central amygdala in rewarding behaviors, in addition to aversive behaviors, started to pick up steam. However, a lot remains unknown about the role of the central amygdala in rewarding behaviors, and in particular the role of different neuronal subtypes in these behaviors. The reward system in the brain is evolutionarily optimized and makes sure that animals will adapt to changing environments and make effort to pursue rewarding stimuli and avoid aversive stimuli [38]. The reward circuitry in the brain involves several cortical and sub-cortical brain areas, and the neurotransmitter dopamine plays an important role in this circuit. The medial prefrontal cortex, the orbitofrontal cortex, the nucleus accumbens, the ventral pallidum, the amygdala, and the ventral tegmental area, among others, are connected together and give rise to a complex system that underlies reward processing. They together comprise the psychological components of reward: liking, wanting and reward learning [39,40].

In this thesis we are mainly focusing on the role of the CeA specifically in appetitive behaviors. Several studies have shown that the CeA plays a role in these behaviors, including reward processing and reward seeking, and drug-related behaviors [14,41–46]. For example, optogenetic activation of the CeA paired with the delivery of a sucrose reward, can increase the motivation to seek this reward over another identical reward without the optogenetic activation [14]. Interestingly, the optogenetic activation alone is not rewarding. In addition to a sucrose reward, the same methods were used to show that CeA activation could also increase the motivation of an animal to obtain a cocaine reward. This shows that the CeA might be involved in the motivation to pursue rewards, regardless of which type of reward this is [44]. These results have been replicated and the preference for a CeA stimulation is shown to be robust enough to persist even in the presence of a negative stimulus [47]. Other studies have looked at projection-specific inputs or outputs from the CeA and found similar results. Activation of projections from the CeA to the ventromedial PFC could produce a similar increased motivation to obtain a reward, without the stimulation itself having inherent positive value [41]. In addition, optogenetic stimulation of CeA NTS neurons projecting to the parabrachial nucleus, a nucleus that receives a variety of sensory inputs and is involved in maintaining homeostasis of the body, has rewarding properties and increases intake of both sugar water and ethanol [46]. In addition, ablation of these neurons inhibits ethanol intake, pointing towards a role of these neurons in reward-seeking behaviors.

1.3.4 Central amygdala in consummatory behaviors

Apart from the role of the CeA in rewarding and drug-related behaviors, the CeA has also been implicated in consummatory, or food-related, behaviors [42,48,49]. Studies have used c-fos expression to demonstrate that the CeA is activated during feeding behaviors, both in a high-fat diet environment, during fasting, and feeding after fasting [48,50,51]. When the CeA was activated as a whole, a reduction was seen in food intake, and inhibition of food intake was seen after being presented with an aversive cue, pointing towards a role for the CeA in feeding control and inhibition [52,53]. However, other studies have shown that activation of the CeA could increase food intake [42,49]. Thus, the exact role of the CeA in consummatory behaviors is not known, with studies showing CeA activation associated with positive feeding behavior, but also negative feeding behavior. These differences in effects could be explained by different neuronal types, or by different input or outputs to and from the CeA. When the CeA was activated through opioid receptor signaling, it could cause an increase in consummatory behaviors directed at a stimulus previously associated with a sucrose reward, while inhibition of the CeA through muscimol could reduce consummatory behavior [42]. In addition, similar effects were seen on baseline food intake, with an increase in food

intake after CeA opioid receptor activation, and a decrease after muscimol injections [42]. When looking at cell-type specificity, it was shown that Htr2a-expressing CeA neurons, a heterogeneous group of cells that has overlap with SST, CRH, NTA and Tac2, were active while animals are feeding, and artificial activation of these neurons causes an increase in food intake [49]. In addition, Prkcd-expressing CeA neurons have been implicated in negative feeding control, and can bidirectionally modulate food intake. Activation of these neurons reduces food intake, while inhibition can increase food intake, even when animals are sated or presented with toxic-like tastants [54]. These studies show that there has been some progress been made in pinning down the CeA circuitry underlying food intake, but more has to be done to understand the exact circuits and neuronal players involved in consummatory behaviors.

1.4 Sex differences

Over the years, more and more attention has been brought to the fact that biological differences between males and females can give rise to differences in a wide variety of behaviors. Sex difference in the dopaminergic system have been documented, which can give rise to sex differences in reward-related behavior [55]. This shows that it is of great importance to understand the differences between males and females and to include both sexes in research, especially when studying reward-related behaviors, to get the complete picture.

1.4.1 Sex differences in appetitive and consummatory behaviors

Sex-differences in the patterns of drug use and abuse have been well documented. On average, females escalate drug use quicker than males, and have a higher chance to relapse [56]. One of the reasons for these differences can be found in hormonal levels. Females go through cycles in which the levels of their hormones, in particular estradiol, vary. A number of studies have shown that the presence of estradiol can affect dopamine signaling, and therefore alter reward-related behaviors [57–59]. The presence of estradiol can increase the rewarding effects, causing reinforcing behaviors to be more rewarding [55]. Not surprisingly, this can affect how females and males differ in appetitive and consummatory behaviors. When females are in the part of their cycle that is characterized by high estradiol levels, dopamine neurons in the VTA are more active, and there seems to be reduction in the sensitivity of the feedback loop, together leading to an increase in dopamine release [60]. During this period, females are more susceptible to drug-reward and cue-reward associations [60]. Related from sex differences in addiction and reward-processing, it has also been known that there are sex differences in consummatory behaviors, which also rely partly on dopamine signaling. Human studies have demonstrated differences in the prevalence of obesity, with women having higher levels of obesity than men [61]. It is not far-fetched to consider sex differences in consummatory behavior in animals. Taken these studies together, it is important to separate males and females in research analyses and to be mindful of potential sex differences.

1.5 Synaptic plasticity in the central amygdala

Since this study focuses on the role of different CeA neuronal types, and its roles in different behaviors, it can be interesting to look at the changes in synaptic plasticity that are underlying these specific behaviors. Some studies have focused on the alterations in synaptic activity and synaptic strength in the CeA in relation to drug use. It is important to understand the acute effects of drugs on the brain, since single drug exposures can already cause

neural adaptations, leading to progressively more adaptations during subsequent drug use [62]. Most drugs exert their effects through an increase in dopamine, norepinephrine, and serotonin, which in turn can affect glutamate receptors on a synaptic level, including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) [63,64].

1.5.1 AMPAR plasticity

AMPARs can consist out of combinations of four different subunits: GluA1, GluA2, GluA3 and GluA4 [65–67]. The two most common types of AMPARs in the brain are heterodimers of GluA1/2 and GluA2/3 [68,69]. GluA1-containing AMPARs have been implicated in a wide variety of different behaviors, and GluA1-containing AMPAR plasticity has been well documented [70]. During learning, GluA1-containing AMPARs are trafficked into the synapse, a process that underlies the formation of memories [71]. This process can be facilitated through a rise in norepinephrine (NE), which phosphorylates the GluA1 subunit, causing an increase in GluA1-containing AMPAR trafficking [72]. The role of NE in the central amygdala has not been extensively studied, but the central amygdala does receive projections from the locus coeruleus, the major source of NE in the brain [73].

1.6 Scope of the thesis

This thesis aims to provide a better understanding of the role of different CeA neuronal types in appetitive and drug-related behaviors. In **Chapter 2**, we examine the neuronal and synaptic effects of a psychostimulant, methamphetamine, on CeA neuronal types. We use *in vivo* single-cell calcium imaging to show that SST-expressing CeA neurons are robustly activated by a single methamphetamine dose in males, and neuronal activity goes back to baseline afterwards. To follow up, we look at the changes in synaptic activity in both SST-expressing and Prkcd-expressing CeA neurons after a single dose of methamphetamine. Interestingly, we find that synaptic activity is altered in females, with Prkcd-expressing CeA neurons being affected 30 min after a methamphetamine exposure, whereas SST-expressing CeA neurons show a change 24 hours after methamphetamine exposure. These effects are only seen in females. This shows that methamphetamine can affect neuronal and synaptic activity in a sex-dependent manner.

In **Chapter 3**, we hypothesize that the CeA neuronal activation we observe after methamphetamine exposure could mediate some of the acute behavioral effects of methamphetamine. We manipulate different CeA neuronal subtypes while measuring the rewarding, hyperactive and anxiogenic properties of methamphetamine. We find that SST-expressing, Prkcd-expressing and VIPR2-expressing CeA neurons *do not* mediate any of these acute behavioral effects from methamphetamine.

In **Chapter 4**, we look at the role of VIPR2-expressing CeA neurons in feeding and homeostatic behaviors. We find that inhibition of VIPR2-expressing CeA neurons could increase feeding behaviors slightly in males only. No effects are found on body weight or locomotor activity. We next identified a strong projection from VIPR2-expressing CeA neurons to the lateral parabrachial nucleus, and hypothesize that this projection could be responsible for the behavioral effect of VIPR2-expressing CeA neuron inhibition.

In **Chapter 5** we examine AMPAR plasticity in the CeA, as this could be a potential mechanism through which psychostimulants could affect CeA neurons. We found that an increase in intracellular cyclic adenosine monophosphate (cAMP) could induce synaptic potentiation in CeA neurons, and this potentiation is not dependent on GluA3-containing AMPARs, and might be independent of AMPAR subunit composition.

Lastly, **Chapter 6** provides general conclusions, perspectives and future directions of the findings in this thesis. **Chapter 7** presents a detailed description of all experimental methods and materials that were used in the work described in this thesis.