Valence in the Nucleus Accumbens - Identifying Neural Populations in Appetitive and Aversive Responses

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Escola de Medicina

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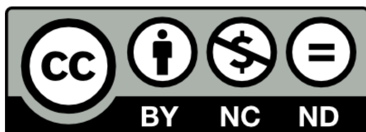

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STATEMENT OF INTEGRITY

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Resumo

O núcleo accumbens (NAc) é reconhecido como um componente essencial do circuito de recompensa, estando associado ao processamento de eventos recompensadores e aversivos e contribuindo para comportamentos motivados. O NAc é uma "interface límbico-motora", e estudos mostram o seu envolvimento em codificar valência - o valor intrínseco de uma certa experiência e consequentes respostas emocionais e motivacionais. O NAc é constituído maioritariamente por neurónios espinhosos médios GABAérgicos (MSNs), divididos naqueles que expressam o recetor de dopamina D1 (D1-MSNs) e nos que expressam o recetor de dopamina D2 (D2-MSNs). Estas populações foram tradicionalmente segregadas anatomicamente (*via direta* vs *indireta*) e funcionalmente (*recompensa e valência positiva* vs *aversão e valência negativa*). No entanto, muitos estudos recentes desafiaram esta segregação simplista. Porém, é ainda incerto quais as populações neuronais que codificam valência no NAc, e esta é uma questão crucial na compreensão de distúrbios com défices emocionais.

Nesta dissertação, avaliámos os níveis de ativação neuronal associados com estímulos de valência negativa (choque na pata) ou positiva (cocaína) no NAc, usando a amígdala basolateral (BLA) e central (CeA) como regiões controlo, usadas por codificarem valência. Também caracterizámos um vetor viral controlado por c-fos para estabelecer uma estratégia de marcação de ativação neuronal para trabalho futuro. Além disso, efetuámos ativação optogenética de neurónios responsivos a estímulos na BLA, usando o mesmo vetor, para induzir comportamentos de valência e validar a nossa metodologia.

Os nossos dados mostram que o NAc core (NAcc) e a BLA contêm populações neuronais que respondem ao choque, medido por uma maior densidade de células c-fos^{+} em comparação os controlos. Porém, a cocaína não induziu alterações significativas de ativação neuronal. Quanto ao vetor (conduzindo expressão de *channelrhodopsin*-eYFP), determinámos que 16h pós-exposição a estímulo seria o período mais adequado para observar marcação viral. Ao usar este vetor e expor murganhos a estímulos positivos ou negativos, dados de densitometria de fluorescência mostraram apenas uma tendência para maior ativação neuronal na BLA após o choque na pata, sem efeitos no NAc, em comparação com animais controlo; sem efeitos devido à cocaína. Por último, ativação optogenética de neurónios responsivos a choque na pata da BLA induziu uma tendência de evasão num teste de preferência de lugar em tempo real (RTPP), contudo essa mesma ativação não induziu preferência de lugar condicionada (CPP).

Embora os nossos dados mostrem ativação neuronal no NAcc e BLA em resposta a choque, a falta de diferenças na exposição a cocaína, junto com dados de densitometria e optogenética, indicam a necessidade de desenvolver novas ferramentas para marcar neurónios que codifiquem valência no NAc.

Palavras-chave: ativação neuronal, choque na pata, cocaína, nucleus accumbens, valência

Abstract

The nucleus accumbens (NAc) is recognized as an essential component of the reward circuit, being associated with processing of both rewarding and aversive events, and contributing for motivated behaviours. The NAc is a "limbic-motor interface", and evidence shows its involvement in valence encoding – the intrinsic value of a given experience and consequent emotional and motivational responses. The NAc is mainly constituted by GABAergic medium spiny neurons (MSNs), divided into those expressing dopamine receptor D1 (D1-MSNs) and those expressing dopamine receptor D2 (D2-MSNs). These populations have been traditionally segregated anatomically (*direct* vs *indirect pathway*) and functionally (*reward and positive valence* vs *aversion and negative valence*). However, many recent studies have challenged this simplistic segregation. Yet, it is still uncertain which neuronal populations encode valence in the NAc, and this is a crucial question in the understanding of disorders with emotional deficits.

In this thesis work we evaluated the neuronal activation levels associated with negative- (footshock) or positive-valence stimuli (cocaine) in the NAc, using the basolateral (BLA) and central (CeA) amygdala as control regions, used because they encode valence. We also characterized a c-fos-driven viral vector in order to establish a neuronal activation labelling strategy for future work. Furthermore, we performed optogenetic activation of stimulus-responsive neurons in the BLA, using the same vector, to induce valence-specific behavioural responses and validate our methodology.

Our data shows that the NAc core (NAcc) and the BLA contain neuronal populations that respond to shock, measured by a higher c-fos^{+} cell density in comparison to controls. However, cocaine induced no significant changes in neuronal activation. Regarding the vector (driving channelrhodopsin-eYFP expression), we found that 16h-post stimulus exposure would be the more adequate timeframe to observe neuronal labelling. When using this vector and exposing mice to positive or negative stimuli, densitometry fluorescence data showed only a tendency for higher neuronal activation in the BLA after footshock, with no effects in the NAc, in comparison with control animals; with no effects due to cocaine. Lastly, optogenetic activation of footshock-responsive neurons in the BLA induced a tendency for avoidance in a real time place preference test (RTPP), yet this same activation did not induce conditioned place preference (CPP).

While our data show neuronal activation in the NAcc and BLA in response to shock, the lack of differences with cocaine exposure together with densitometry and optogenetics data, indicates the need to develop new tools to label valence encoding neurons in the NAc.

Key words: cocaine, footshock, neuronal activation, nucleus accumbens, valence

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List of abbreviations and acronyms

AAV: adeno-associated virus

AAV2: adeno-associated virus serotype 2

AAV5: adeno-associated virus serotype 5

AC: anterior commissure

Act-seq: activated cell population sequencing

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AmpR: ampicillin resistance gene

AP: anteroposterior

ARC: activity-regulated cytoskeleton-associated protein

ArchT: archaerhodopsin

BLA: basolateral nucleus of the amygdala

BNST: bed nucleus of the stria terminalis

Ca^{2+}: calcium ions

CamKII: calmodulin-dependent protein kinase

CCK: cholecystokinin-octapeptide

CeA: central nucleus of the amygdala

ChR2: channelrhodopsin-2

Co: cortical nucleus of the amygdala

CPA: conditioned place aversion

CPP: conditioned place preference

Cre (or CreER): Cre recombinase enzyme

CRH: corticotropin-releasing hormone

CRLR: calcitonin receptor-like receptor

CS: conditioned stimulus

D1-MSN: medium spiny neuron expressing dopamine receptor D1

D1R (or DR1, D1, Drd1): dopamine receptor D1

D2-MSN: medium spiny neuron expressing dopamine receptor D2

D2R (or DR2, D2, Drd2): dopamine receptor D2

DGAV: *Direção-Geral de Alimentação e Veterinária*

DNQX: 6,7-dinitroquinoxaline-2,3-dione

DOX: doxycycline

DR: dopamine receptor

DREADDs: designer receptors exclusively activated by designer drugs

DRN: dorsal raphe nucleus

DTR: diphtheria toxin receptor

DV: dorsoventral

DYN: dynorphin

E. coli: *Escherichia coli*

eYFP: enhanced yellow fluorescent protein

FBS: fetal bovine serum

FELASA: Federation for Laboratory Animal Science Associations

FR: fixed-ratio

GABA: *gamma*-aminobutyric acid

GFP: green fluorescent protein

HPC: hippocampus

HTR2A: serotonin receptor 5-hydroxytryptamine 2A

I.P.: intraperitoneal

ICSS: Intracranial self-stimulation

ICVS: *Instituto de Investigação em Ciências da Vida e Saúde*/ Life and Health Sciences Research Institute

IEG: immediate early gene

IF: immunofluorescence

LA: lateral nucleus of the amygdala

LB: lysogeny broth

LDT: laterodorsal tegmental nucleus

LH: lateral hypothalamus

LHb: lateral habenula

M: medial nucleus of the amygdala

Met-ENK: methionine-enkephalin

MGV: mean grey value

ML: mediolateral

mRNA: messenger ribonucleic acid

MSNs: medium spiny neurons

NAc: nucleus accumbens

NAcc: nucleus accumbens core

NaCl: sodium chloride

NAcs: nucleus accumbens shell

NMDAR: N-methyl-D-aspartate receptor

NpHR: halorhodopsin

OFC: orbitofrontal cortex

pAAV: adeno-associated virus plasmid

PAG: periaqueductal grey

PBS: phosphate buffered saline

PBS-T: phosphate buffered saline with 0.3% triton x-100

PFA: paraformaldehyde

PFC: prefrontal cortex

PIT: Pavlovian-to-instrumental transfer

PKC-δ (or PRKCD): protein kinase c-δ

PNOC: prepronociceptin

PPP1R1B: protein phosphatase 1 regulatory subunit 1B

PR: progressive-ratio

PVT: paraventricular nucleus of the thalamus

rAAV: recombinant adeno-associated virus

RNB: reversible neurotransmission blocking

rpm: rotations per minute

RSPO2: roof plate-specific spondin-2

RT: room temperature

RTPP: real time place preference

SCH23390: 7-chloro-3-methyl-1-phenyl-1,2,4,5-tetrahydro-3-benzazepin-8-ol

scRNA-seq: single-cell RNA sequencing

SEM: standard error of mean

SKF81297: 6-chloro-2,3,4,5-tetrahydro-1-phenyl-1H-3-benzazepine-7,8-diol

SN: *substantia nigra*

SOC: super optimal broth with catabolite repression

TN: tetanus toxin

TRAP: targeted recombination in active populations

US: unconditioned stimulus

VIP: vasoactive intestinal polypeptide

VP: ventral pallidum

VTA: ventral tegmental area

WPRE: woodchuck hepatitis virus posttranscriptional regulatory element

ZIF268: zinc finger protein 268

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CHAPTER 1 – Introduction

1. Introduction

Daily, individuals have to filter information and focus on emotionally-relevant stimuli, and respond in an adequate manner. Affective valence is a component that includes both the pleasure-displeasure value inherently linked with a given stimulus, and any kind of behavioural, physiological and emotional consequences it induces in an individual (Berridge, 2019). Considering that valence aligns experiences along a positive-neutral-negative axis, this concept is heavily associated with reward and aversion, such that appetitive/rewarding circumstances have a positive valence, while aversive circumstances have a negative valence. Some stimuli are innately rewarding or aversive to the individual, without requiring any type of learning such as the unconditioned approach to female odour by male rats (positive valence) or unconditioned avoidance in the presence of cat (any predator) odour. However, most stimuli are initially neutral and require learning to form emotional associations between these and the motivationally relevant outcome (Pavlov, 2010).

The study of brain circuits and regions involved in valence is especially pertinent to study the pathophysiology of affective disorders with deficits in reward and aversion processing, such as depression and addiction (Cooper et al., 2017; Dichter et al., 2012; Russo & Nestler, 2013).

To study valence encoding, researchers have been characterizing neuronal activity patterns in response to positive or negative stimuli or cues predicting those stimuli. In the latest years, the development of optogenetics/chemogenetics and new transgenic models, allowed to causally link the activity of specific neuronal ensembles with rewarding or aversive responses. Various brain regions, such as the nucleus accumbens (NAc), the basolateral amygdala (BLA) and the ventral tegmental area (VTA), amongst others, have been shown to be involved in the reward system and/or valence encoding, (reviewed in (Balleine & Killcross, 2006; Berridge & Kringelbach, 2015; Kelley & Berridge, 2002; Namburi et al., 2016; Richard et al., 2013; Russo & Nestler, 2013; Sesack & Grace, 2010).

The NAc has been implicated in motivated behaviours, and in aversive and appetitive responses, for both natural and drug rewards (Berridge & Kringelbach, 2015; Carlezon & Thomas, 2009; Roitman et al., 2005). The majority of NAc neurons are GABAergic medium spiny neurons (MSNs) (Gerfen, 1992), divided into those expressing dopamine receptor D1 (DR1; D1-MSNs) or dopamine receptor D2 (DR2; D2-MSNs). For decades these neuronal populations have been considered to be segregated both anatomically and functionally. Conventionally, D1- and D2-MSNs were considered as comprising the direct and indirect striatal pathways, respectively, which was later reconsidered since D1-MSNs also integrate the indirect pathway (Kupchik et al., 2015). On a functional level, studies have associated D1-MSNs with reward and positive reinforcement (positive valence) and D2-MSNs with aversion and punishment

(negative valence) (Hikida et al., 2010, 2016; Kravitz et al., 2012; Lobo et al., 2010; Volman et al., 2013). Nevertheless, recent studies have revealed this model to be overly simplistic. For instance, optogenetic stimulation of either D1- or D2-MSNs supports self-stimulation (Cole et al., 2018). Other works, including from our team, have shown that both populations can drive reward and aversion ((Golden et al., 2019; Namvar et al., 2019; Natsubori et al., 2017; Soares-Cunha et al., 2016a, 2018, 2020; Steinberg et al., 2014) – further exploration of this topic will be shown in a later section).

In the following sections, we focus on valence encoding in different brain regions, emphasising the NAc. We will explore electrophysiological studies regarding valence encoding in the NAc and studies that link the NAc with rewarding and aversive behaviours, as well as presenting parallel relevant information for the BLA and the central amygdala (CeA) subnuclei.

1.1. Affective valence

One of the earlier works on emotion was devised by Darwin (1872). He proposed that, to some extent, emotions existed equally throughout species, and animal emotions were homologues to human (basic) ones. Emotions would be linked with visible actions, which would be selected throughout evolution, and recognizable across species.

A common theme in emotion theory is the association of emotions with internal and external changes in organisms (*e.g.* observable behaviours). In humans, a subjective component can be assessed (Anderson & Adolphs, 2014). However, objective and subjective levels of affective responses are not necessarily concurrent, conveying the same information, nor doing so to the same degree (Berridge & Kringelbach, 2015).

Dimensional emotional theories attempt to integrate emotional states into various dimensions (contrasting with discrete theories; (Mendl et al., 2010)), and affective valence is a repeatedly exhibited criteria (*e.g.* (Russell, 1980; Russell & Barrett, 1999; Watson & Tellegen, 1985)). We highlight the Circumplex Model of Affect (Russell, 1980; Russell & Barrett, 1999) as a contextualizing example for the scope of this dissertation. This model aligns emotional neurophysiological states by combining two axes, arousal (engagement level) and pleasure (affective valence) ("core affect"; (Russell, 2003)) (**Figure 1**).

Nevertheless, valence must be distinguished from other concepts such as salience and arousal, also associated with stimuli. Salience is considered as the level of perception of a stimulus (*i.e.* capacity of being recognized and induce behavioural changes) (Uddin, 2015), while arousal is linked with increased activation and awareness to a given situation (Critchley et al., 2013), being able to impact

salience (Lee et al., 2020). Neurons may, at times, appear to process valence, but instead encode the salience or arousal of a stimulus, independently from the positive or negative value.

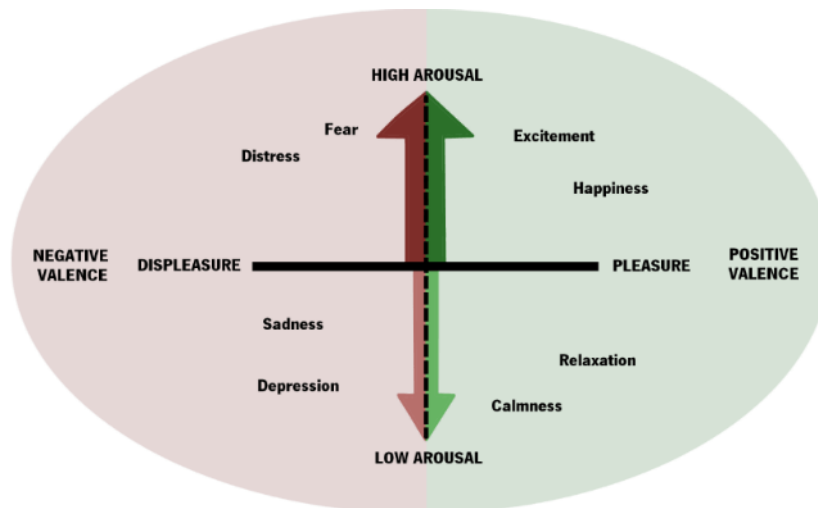


Figure 1 - Core affect represented in the Circumplex Model of Affect model. a) Alignment of emotional states in two dimensions, arousal (from high activation to low arousal) and affective valence (from pleasant to unpleasant). Positive affective descriptors (right) are opposed to negative affective descriptors (left). High arousal states are usually associated with motivation to either avoid threats (top left) or to seek reward (top right). Adapted from (Russell, 1980) and (Russell & Barrett, 1999).

Briefly, affective valence has been defined as not only the intrinsic value of a certain experience, but also the resultant hedonic, emotional, and motivational responses (Berridge, 2019). On one end of the spectrum, we have stimuli with a positive valence, meaning they are intrinsically pleasant and originate appetitive reactions, while negative valence stimuli are intrinsically unpleasant, initiating aversive reactions (**Figure 1**). Specifically, the stimuli are considered positive or negative unconditioned stimuli (US), respectively, because they elicit such responses innately (Pavlov, 2010). When a previously neutral stimulus (*e.g.* a sound, an odour) is presented prior to an US, it attains a particular valence, being now considered a conditioned stimulus (CS) (Pavlov, 2010). Freezing due to a footshock-paired tone (negative CS) or licking in response to a light combined with a sucrose reward presentation (positive CS), for example, are such cases, being cases of Pavlovian conditioning.

For example, regarding associated reactions, US tasting stimuli such as sucrose or quinine elicit quite robust and evolutionarily conserved responses. Sucrose, a positive valence tasting stimulus, elicits lip licking, tongue protrusions and, exclusively in hominoids, a relaxed, smile-like expression. Negative responses like gaping, headshaking and, in hominoids only, nose-wrinkling, and grimacing expressions are seen in response to quinine (Berridge, 2000, 2019; Grill & Norgren, 1978; Steiner et al., 2001).

As stated, while from a more psychological standpoint affective valence is often considered and assessed in a subjective manner, the more objective reactions that can derive from a specific valence

encoding include physiological and/or behavioural effects which can for the most part be measured, and therefore, studied.

1.2. Valence neurocircuitry

Valence has been associated with varied brain regions. For instance, the mesolimbic brain reward system includes brain areas implicated in valence encoding and in rewarding and aversive responses (Namburi et al., 2016).

A variety of studies have contributed to expand the knowledge on the involvement of distinct brain areas in valence (presented in this section), namely, NAc (Berridge & Kringelbach, 2013; Knowland & Lim, 2018; Namburi et al., 2016; Nieh et al., 2013), the VTA (Namburi et al., 2016; Nieh et al., 2013), the BLA (Balleine & Killcross, 2006; Namburi et al., 2016; O'Neill et al., 2018; Šimić et al., 2021), the CeA (Balleine & Killcross, 2006; Šimić et al., 2021) and the hippocampus (Namburi et al., 2016; Nieh et al., 2013).

The NAc has been particularly studied, being a recognized central region of the mesocorticolimbic reward circuit, heavily engaged in rewarding and motivated behaviours, processing both natural rewards and drugs of abuse (such as food and cocaine), along with encoding aversive conditions (Berridge & Kringelbach, 2015; Carlezon & Thomas, 2009). Having inputs from an assortment of cortical, sub-cortical and limbic regions and outputs to other parts of the basal ganglia, sections of the cortex and some thalamic regions (Heimer & Alheid, 1991; Sesack & Grace, 2010), it has been proposed as a major interface of limbic processing and motivation into motor effects (Mogenson et al., 1980).

The amygdala nuclei, such as the BLA and CeA, are also a focal point of study in emotional processing. The BLA is known to react to stimuli, including valence processing of both negative and positive contexts, being also associated with fear processing, as well as reward-related mechanisms (*e.g.* reward learning and motivation) (Balleine & Killcross, 2006; Sah et al., 2003; Šimić et al., 2021). The CeA is implicated in fear mechanisms (including physiological responses), along with rewarding and aversive learning (Balleine & Killcross, 2006; Sah et al., 2003; Šimić et al., 2021).

Other regions that were, at least to some extent, linked with valence (or reward and/or aversion processing) include the laterodorsal tegmental nucleus (LDT; (Nieh et al., 2013)), the ventral pallidum (VP; (Berridge & Kringelbach, 2013; Knowland & Lim, 2018)), the lateral hypothalamus (LH; (Namburi et al., 2016)), the lateral habenula (LHb; (Knowland & Lim, 2018; Nieh et al., 2013)), the *locus coeruleus* (Namburi et al., 2016), the paraventricular nucleus of the thalamus (PVT; (Barson et al., 2020; Kirouac,

2015)), the bed nucleus of the *stria terminalis* (BNST; (Lebow & Chen, 2016)) and the auditory cortex (Concina et al., 2019).

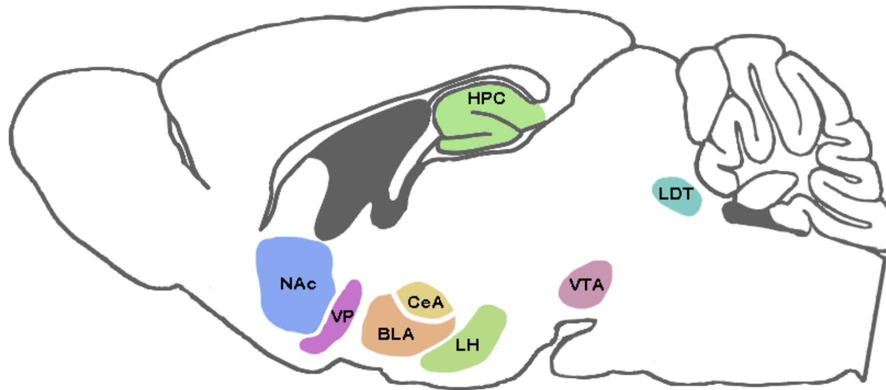


Figure 2 – Simplified valence circuitry. a) Schematic sagittal representation of the main regions involved in valence and reward/aversion. Localization across the mediolateral axis, size and shape are adjusted for simplicity and representation purposes. Brain ventricles shown in black. NAc – Nucleus Accumbens; VP – Ventral Pallidum; BLA – Basolateral Amygdala; CeA – Central Amygdala; LH – Lateral Hypothalamus; VTA – Ventral Tegmental Area; LDT - Laterodorsal Tegmental Nucleus; HPC – Hippocampus. Adapted from (Paxinos & Franklin, 2001).

It is essential to note that neuronal populations, even within one particular brain region, do not necessarily encode valence in the same manner. As distinctly explained by Namburi et al. (2015), neuronal subpopulations can, for instance, be classified in a functional manner (*i.e.* activity changes in response to stimuli) and by genetic profile and/or anatomical characteristics (*i.e.* diverging inputs or outputs). Following this logic, many studies have defined valence encoding subpopulations. For example, Kim et al. (2016), showed two genetically distinct neuronal subpopulations in the BLA, the RSPO2+ neurons (shock-responsive that drive aversion) and the PPP1R1B+ neurons (water reward-responsive that drive place preference). Regarding anatomically distinct subpopulations, neurons projecting from the prelimbic cortex to the BLA, or from the BLA to the CeA, have been shown to promote avoidance (Huang et al., 2020; Namburi et al., 2015). Occasionally, studies may combine these concepts, as seen in Stuber et al. (2011), who examined glutamatergic NAc-targeted BLA neurons, found to drive appetitive behaviours. Lastly, even the same neuronal populations might be subject to plastic changes due to learning after experiences with a specific valence (for instance, increasing synaptic strength of fear-processing neurons during fear conditioning), which may influence behavioural changes that occur with said stimuli. For example, a study has described plasticity alterations induced by fear and reward conditioning in BLA-NAc (which can induce positive reinforcement) and BLA-CeA (which can induce negative reinforcement) neurons (Namburi et al., 2015). Fear conditioning lowered the AMPAR/NMDAR ratio (a measure of the strength of glutamatergic synapses) in the internal capsule afferents of BLA-NAc

neurons, and enhanced the AMPAR/NMDAR ratio in the internal capsule afferents of BLA-CeA neurons, while the reverse occurred for reward conditioning (Namburi et al., 2015).

Furthermore, valence is not an inflexible quality by any means, depending on many factors beyond the salience and value of a stimulus. For one, valence can be innate or learned. Innate valence can be studied when presenting a stimulus without any prior exposure, being inherently appetitive or aversive, and induce responses in individuals accordingly (*e.g.* (Grill & Norgren, 1978; Steiner et al., 2001)). Valence can also be assigned to previously neutral circumstances via conditioning – in these cases, neutral stimuli turn into conditioned stimuli when a learning association with a valence-stimulus is created, as explained in the previous section (*e.g.* (Gore et al., 2015a; Roitman et al., 2005)). Similarly, even appetitive stimuli can be considered aversive when consistently preceding a negative-valence stimulus (Roitman et al., 2010). The same stimulus can also have opposing valences depending on the internal state of an individual – typically aversive stimuli can be considered rewarding if there is a physiological lack of that component (*e.g.* neurons in salt-deprived animals respond to NaCl, usually aversive, as a reward) (Loriaux et al., 2011).

Additionally, valence is encoded differentially depending on anatomic location. For instance, an "affective keyboard" has been described in the NAc shell, with a rostrocaudal pattern from positive- to negative-valence responses, with the more rostral area being associated with appetitive behaviours and the most caudal NAc shell being associated with fear-related behaviours (Berridge & Kringelbach, 2015; Faure et al., 2010; Reynolds & Berridge, 2001, 2002; Richard & Berridge, 2011). Even more interestingly, this keyboard can vary with the setting, with the fear-inducing area being much more caudally restricted, in a home-like environment, and much more prominent in a stressful environment, with the reverse occurring for the positively-associated area (Berridge & Kringelbach, 2015; Reynolds & Berridge, 2008). Further detailing of some of the topics mentioned here will be presented in later sections.

1.3. Neuroanatomy and neuronal populations of the nucleus accumbens

The NAc is deemed an integral part of the rodent ventral striatum (Zahm & Brog, 1992), having been for long divided into core and shell subregions (Záborszky et al., 1985). The same occurs for the human ventral striatum, similarly split into core and shell (Voorn et al., 1996). For the majority of the NAc anterior-posterior axis progression, the anterior commissure (AC) is a clear structural reference point, especially in the coronal plane (Paxinos & Franklin, 2001; Paxinos & Watson, 2007), being enveloped by the NAc core (NAcc), which is in turn ventrally, medially, and laterally enveloped by the NAc shell (NAcs) (Záborszky et al., 1985).

The NAc gathers inputs from varied limbic, cortical, and sub-cortical areas, being a known converging region of the basal ganglia (Heimer & Alheid, 1991; Sesack & Grace, 2010). Afferent projections are diverse, including glutamatergic inputs from the amygdala (Fuller et al., 1987; Heidbreder & Groenewegen, 2003; Mcdonald, 1991; Morgane et al., 2005; Phillipson & Griffiths, 1985; Stuber et al., 2011), thalamus (Fuller et al., 1987; Lanciego et al., 2004; Phillipson & Griffiths, 1985), hippocampus (Heidbreder & Groenewegen, 2003; Kelley & Domesick, 1982; Morgane et al., 2005), cortical regions such as the prefrontal cortex (PFC) (Fuller et al., 1987; Mcdonald, 1991; Phillipson & Griffiths, 1985; Yin & Knowlton, 2006) and its specific subregions such as the prelimbic and infralimbic cortices (Lanciego et al., 2004; Yin & Knowlton, 2006); cholinergic inputs from the LDT (Dautan et al., 2014) and dopaminergic inputs from the VTA (Hnasko et al., 2012; Phillipson & Griffiths, 1985; Tritsch et al., 2012).

Regarding efferents, the NAc primarily projects via GABAergic neurons to regions of the basal ganglia and some thalamic and cortical areas (Heimer & Alheid, 1991; Sesack & Grace, 2010). Major outputs comprise projections to the VP (Groenewegen & Russchen, 1984; Heimer et al., 1991; Lu et al., 1997; Nauta et al., 1978; Záborsky & Cullinan, 1992; Zhou et al., 2003), the VTA (Groenewegen & Russchen, 1984; Heimer et al., 1991; Lu et al., 1997; Nauta et al., 1978; Zhou et al., 2003) and diencephalon structures such as the thalamus and the lateral hypothalamus (Groenewegen & Russchen, 1984; Heimer et al., 1991; Mogenson et al., 1983; Nauta et al., 1978; Williams et al., 1977). There is also evidence of efferents to other areas such as the amygdala, globus pallidus, *substantia nigra*, LHb, BNST, *substantia innominata* and septum (Groenewegen & Russchen, 1984; Heimer et al., 1991; Mogenson et al., 1983; Nauta et al., 1978; Williams et al., 1977; Zhou et al., 2003). A schematic outline of the above-mentioned connections is presented in **Figure 3**.

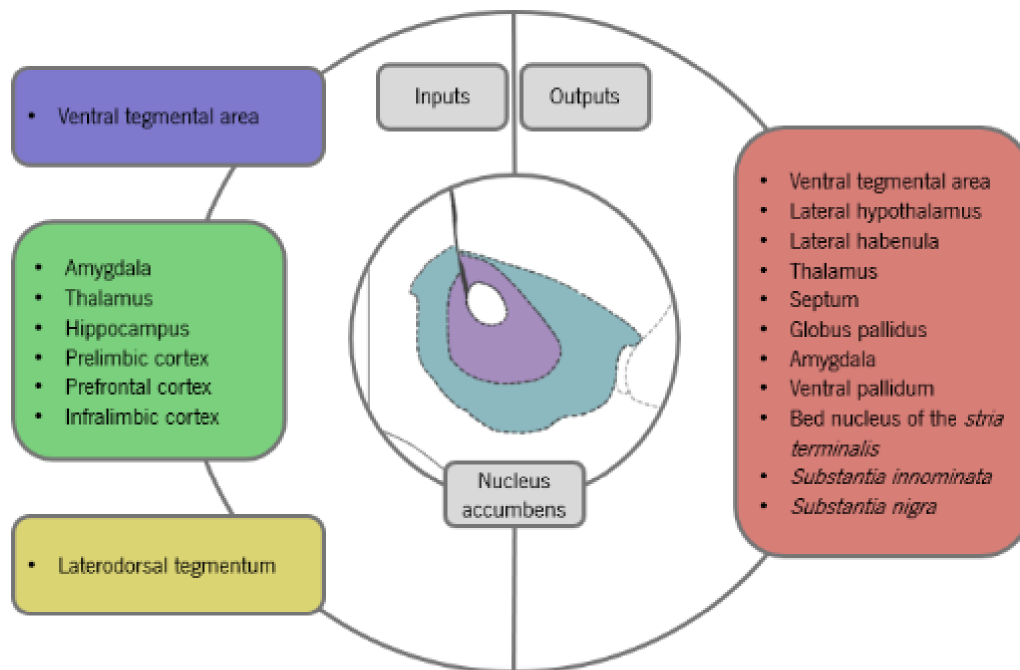


Figure 3 - Inputs and outputs of the nucleus accumbens. a) Simplified and non-comprehensive schematic representation of NAc input and output regions. Afferents: glutamatergic inputs from the amygdala, thalamus, hippocampus, prefrontal, prelimbic and infralimbic cortices (left, green); cholinergic inputs from the laterodorsal tegmentum (left, gold) and dopaminergic inputs from the ventral tegmental area (left, blue). Efferents: GABAergic projections to regions as the ventral pallidum, ventral tegmental area, thalamus, lateral hypothalamus, amygdala, globus pallidus, *substantia nigra*, lateral habenula, bed nucleus of the *stria terminalis, substantia innominata* and septum (right, red).

Around 95% of striatal neurons are GABAergic medium spiny neurons (MSNs) (Gerfen, 1992), being commonly divided into D1-MSNs or D2-MSNs, and the remaining neurons (≈5%) are interneurons (Graveland & Difiglia, 1985).

These populations have differential projection patterns regarding ventral striatum outputs. D1-MSNs comprise direct projections to output regions of the basal ganglia, such as the VTA (direct pathway; **Figure 4a**), while both subpopulations project indirectly to these areas through the VP (indirect pathway; **Figure 4b**) (Kupchik et al., 2015).

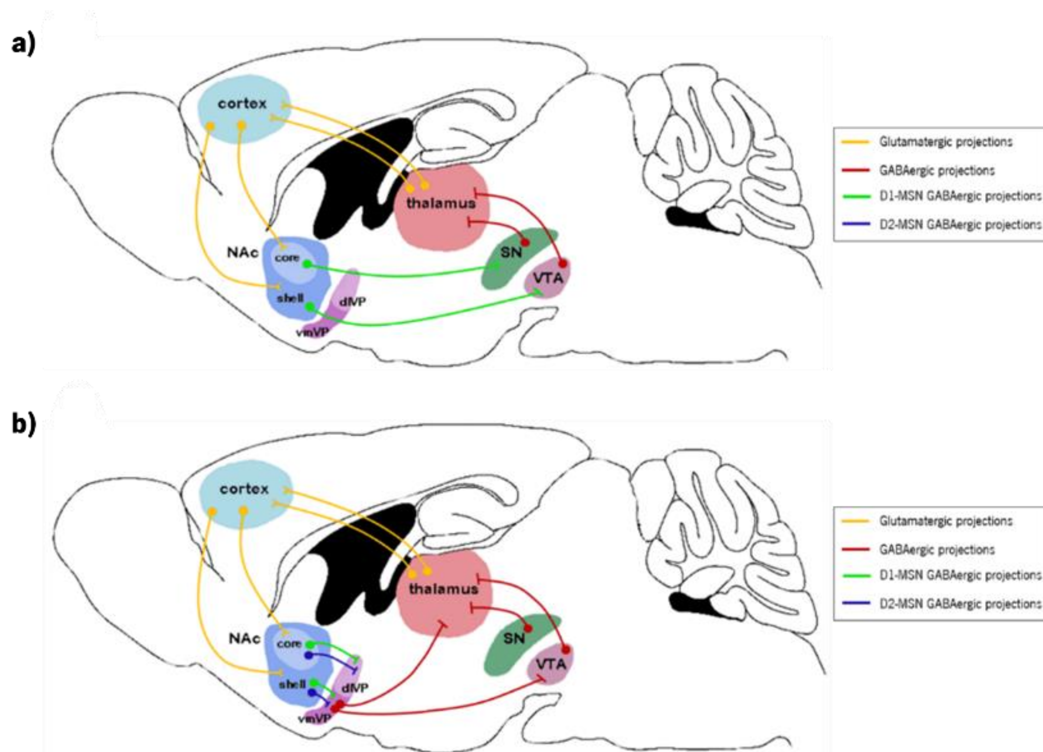


Figure 4 – Direct and indirect ventral striatal pathways. a) The direct pathway in the ventral striatum includes D1-MSNs projections from the NAc to the VTA/SN, which project to the mediodorsal thalamus. **b)** The indirect pathway involves both D1-/D2-MSNs projections from the NAcc to the dlVP and from the NAcs to the vmVP, which in turn projects to the VTA. Localization across the mediolateral axis, size and shape are adjusted for simplicity and representation purposes. Brain ventricles shown in black. NAc – Nucleus Accumbens; VP – Ventral Pallidum; dlVP – dorsolateral pallidum; vmVP – ventromedial pallidum; VTA – Ventral Tegmental Area; SN – substantia nigra. Adapted from (Paxinos & Franklin, 2001; Soares-Cunha et al., 2016b).

Concerning spatial distribution, both D1-MSNs and D2-MSNs seem randomly distributed across the NAc core, which does not occur for the NAc shell, in which D2-MSNs were demonstrated to have a nonuniform distribution throughout shell subregions (Gangarossa et al., 2013).

Additionally, D1-MSNs and D2-MSNs have distinctive neurochemical properties, with D1-MSNs shown to express substance P and dynorphin, whereas VP-projecting D2-MSNs express enkephalin (Gerfen, 1992; Gertler et al., 2008; Lu et al., 1997). NAc subregions also diverge regarding neurochemical markers. The NAc core presents greater levels of preproenkephalin (PPE, enkephalin precursor) (Rogard et al., 1993), calbindin (Prensa et al., 2003), $GABA_A$ receptors (Churchill et al., 1992) and limbic system-associated membrane protein (LAMP) (Prensa et al., 2003), and lower levels of substance P (Prensa et al., 2003), calretinin (Prensa et al., 2003), μ-opioid receptors (Churchill et al., 1992), serotonin ((Deutch & Cameron, 1992), serotonin receptors (Patel et al., 1995) and dopamine (Deutch & Cameron, 1992), while the reverse occurs for the NAc shell. Glutamate decarboxylase has

higher mRNA levels in the shell, but the effect was ascribed to higher cell density, due to comparable amounts of neurons considered positive (Rogard et al., 1993).

The striatal division can also be defined in terms of compartments, due to its patch-matrix mosaic organization. Patches are typically high in μ-opioid receptor binding, while the matrix is characterized by elevated cholinergic markers and calcium-binding protein immunoreactivity (Graybiel & Ragsdale, 1978; Zahm & Brog, 1992). Neuropeptides such as enkephalin, dynorphin and substance P are expressed in both patches and matrix (50-65% of each compartments' neurons), with substance P presenting lower density in the ventromedial striatum and dynorphin and enkephalin being evenly scattered across the striatum (Gerfen & Young, 1988).

1.4. Functional evidence on valence encoding in the nucleus accumbens

Electrophysiological studies

The NAc has been demonstrated as selectively encoding appetitive taste stimuli, leading to a predominant inhibitory response in a primarily (but not exclusively) sucrose-responsive population in an unconditioned presentation context (innate response) ((Roitman et al., 2005); see **Figure 5a**). Similarly, this mainly inhibitory effect has been observed with other studies focusing on sucrose and other rewards (saccharin, (Wheeler et al., 2008; Wilson & Bowman, 2004); sucrose, (Nicola et al., 2004b; Roitman et al., 2010); reviewed in (Wheeler & Carelli, 2009)).

Interestingly, some NAc populations seem to encode not only absolute hedonic characteristics, but also relative reward value (Taha & Fields, 2005; Wheeler et al., 2005). After intraoral administration of sucrose solutions of different concentrations, distinct neuronal populations were observed (Wheeler et al., 2005), with no inhibition predominance. This shows that, while it commonly seems to be the case, a decrease in NAc activity is not a ubiquitous effect. Some populations ("inhibitory" and "excitatory") had, for the low concentration when alternating with the high concentration, reduced activity responses (*i.e.* a lesser decrease/increase in firing rate after reward, respectively), when compared to isolated administration of the low concentration. In the reverse scenario, these neurons had increased activity responses (*i.e.* a greater decrease/increase in firing rate after reward, respectively).

Regarding aversion, in exposures to unpleasant taste stimuli (specifically, quinine), delivered without prior exposure (innate reaction), a mainly excitatory effect was observed in the NAc in a neuronal population almost solely responsive to quinine ((Roitman et al., 2005); see **Figure 5a**). In line with this,

varied aversive stimuli presented to cats (*e.g.* air puffs) induced increases in NAc firing rates (Yanagimoto & Maeda, 2003).

The majority of studies have taken more complex approaches, utilizing diverse behavioural tests, in which there are CS-US pairings. Subsets of NAc neurons are known to process predictive cues, associated with reward presentation (learned response), and most of these responses develop when the association between cue (CS) and appetitive stimuli (US) is learned (Setlow et al., 2003).

Early electrophysiology studies in primates have demonstrated an increase in activity after cue-reward associations (juice and cocaine; (Bowman et al., 1996); juices of varied flavours (Cromwell & Schultz, 2003; Hassani et al., 2001)). This was likewise demonstrated using rodents in a pure Pavlovian conditioning context, where the majority of cells responding phasically to a sucrose-predicting cue had an increase in activity (Roitman et al., 2005). For all sucrose-responsive cells, activity alterations due to a quinine-predicting cue were never equivalent (Roitman et al., 2005). A CS predicting saccharin has originated a similar response (Wilson & Bowman, 2005). In addition, when assessing Pavlovian approach, cue presentation prior to a sucrose delivery led to phasic changes in activity with 53% of transient inhibitions and 47% of transient excitations. The presentation of a no-reward cue elicited, for these two subpopulations respectively, smaller excitations and no significant inhibition (Day et al., 2006). Similarly, smaller increases or decreases in activity after no-reward cue exposure have been found in the NAc shell (but not in the NAc core) (Ghitza et al., 2003).

Neurons in the NAc also encode stimuli that predict delivery of non-natural rewards like cocaine, with a mainly excitatory response to the reward-predicting cue, for up to a month of abstinence (Ghitza et al., 2003; Hollander & Carelli, 2007), with NAc core activation after the cocaine-cue being much higher when compared with short-term abstinence (Hollander & Carelli, 2007).

Other factors that affect the level of cue-related increases in activity include the relative expected rewarding value (Hassani et al., 2001), size of the expected reward (Cromwell & Schultz, 2003), time until reward presentation (Bowman et al., 1996; Shidara et al., 1998) and certainty of the expected reward (Fiorillo et al., 2003).

There are also neuronal subsets known for responding to aversion-predictive cues (learned reaction) in the NAc (Setlow et al., 2003). Comparably to rewarding outcomes, these changes in activity arise with the learned link between cue and stimulus (Setlow et al., 2003). This is the case when the majority of neurons respond phasically to a quinine-predicting cue with an increase in activity (Pavlovian conditioning) (Roitman et al., 2005).

Transitory inhibition of NAc neurons has also been shown in behavioural paradigms such as self-administration of varied natural and drug rewards (cocaine, (Chang et al., 1998; Peoples et al., 1998; Peoples & West, 1996); heroin, (Chang et al., 1997, 1998); sucrose, (Nicola et al., 2004a); water, (Carelli et al., 2000); food, (Carelli, 2002; Carelli et al., 2000); ethanol, (Janak et al., 1999)).

Moreover, varied neuronal populations seem to have specific (phasic) patterns of inhibitions and/or excitations associated with stages of operant behaviour, such as approach or reward consumption (water, (Carelli et al., 2000; Carelli & Deadwyler, 1994; Carelli & Wondolowski, 2003); cocaine, (Carelli et al., 2000; Carelli & Deadwyler, 1994; Carelli & Wondolowski, 2003; Chang et al., 1998; Peoples et al., 1998); heroin, (Chang et al., 1998); food, (Carelli et al., 2000); sucrose, (Kravitz et al., 2006; Nicola et al., 2004a)). Some NAc activity modulations also occur on a session-long (tonic) level (sucrose, (Kravitz et al., 2006); cocaine, (Chang et al., 1998; Ghitza et al., 2006; Peoples et al., 1998)).

Regarding tasks with water and food reinforcement and cocaine self-administration, some populations present specific activity changes around the operant response. Pertinent to valence encoding, some cells display an increase and others a decrease in firing upon reinforced lever pressing. Remarkably, a distinct population showing excitations pre- and post-response, occurred only for cocaine (Carelli et al., 2000; Carelli & Deadwyler, 1994; Carelli & Wondolowski, 2003). Interestingly, firing rate was (for the most part) higher for water-reactive cells than cocaine-reactive cells (Carelli et al., 2000; Carelli & Deadwyler, 1994). The number of cocaine-reactive cells also seems to track self-administration learning (Carelli & Wondolowski, 2003). Others (cocaine self-administration, FR1 (fixed-ratio 1)) have shown that some neurons (with tonic changes in activity) presented a phasic inhibitory response a couple of minutes after lever pressing (Peoples et al., 1998). In another study (FR1 for sucrose), some neurons showed phasic increases and decreases in firing concurrent with the operant response, with some maintaining these activity changes throughout consumption (Kravitz et al., 2006).

Additionally, a distinction must be made between natural and drug rewards. While the types of responsive populations were similar to what was established in earlier work (water *vs* cocaine, (Carelli & Deadwyler, 1994)), the overlap in the aforementioned activity patterns (including those less relevant for valence) was substantial between water- and food-reactive cells and non-coinciding between natural reinforcers and drug self-administration (with water-, food- and cocaine-reactive cells) (water *vs* cocaine; (Carelli & Wondolowski, 2003); water *vs* food *vs* cocaine; (Carelli et al., 2000); also reviewed in (Carelli, 2002)). Nonetheless, subpopulations of neurons appear to also distinguish qualitatively different appetitive reinforcers (cocaine *vs* heroin, (Chang et al., 1998); water *vs* sucrose, (Roop et al., 2002)), not

only following reward, but also other behavioural stages such as reward-predicting cue presentation in a conditioned context (juices of varied flavours; (Hassani et al., 2001)).

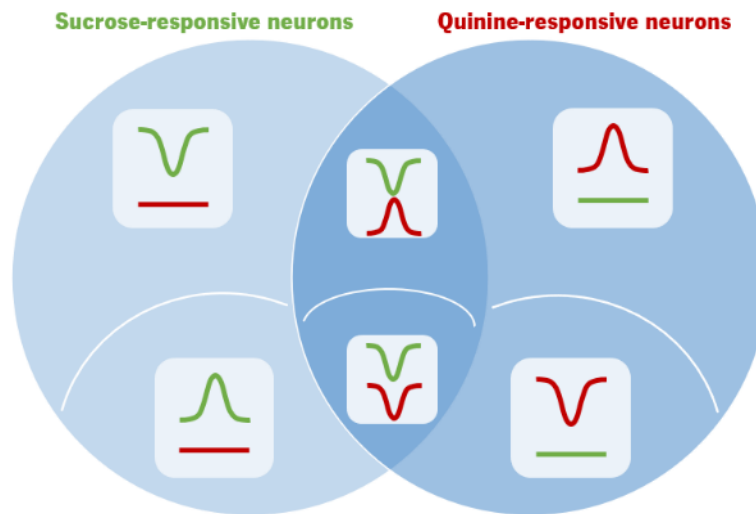


Figure 5 – Main activity variations after primary rewarding tastant (sucrose) and aversive tastant (quinine) in phasically-responding neurons of the NAc. a) The majority of NAc neurons responding to sucrose and quinine are valence-specific (no activity changes in the opposing valence stimulus), with a prevalence of inhibitions for sucrose-only neurons and excitations for quinine-only neurons. From the relatively fewer neurons responding to both tastants, most exhibit opposing activity changes. Based on the electrophysiological recordings from (Roitman et al., 2005).

Valence plasticity in the NAc

NAc neuronal activity changes can vary with a shift in the rewarding value of a stimulus, the motivational state of the animals and more intricate conditioning associations with different stimuli, which is explored below.

For example, when sucrose delivery is conditioned with lithium chloride (aversive), neurons that typically respond to reward with inhibition pattern change to an excitatory pattern after sucrose intake (Roitman et al., 2010). A highly concentrated sodium chloride (NaCl) solution induces inhibitions in salt-responsive populations when an animal is sodium deprived, which would typically lead to excitations due to the aversive quality of saturated salt solutions in a standard non-motivated status (Loriaux et al., 2011).

In addition, cocaine is known to induce a negative affective state, which is associated with natural reward devaluation (Volkow et al., 2004). When a flavoured saccharin solution paired with saline delivery is presented, NAc neurons have a predominant transitory decrease in firing rate (comparable to the effect after an appetitive stimuli). When a distinctly flavoured saccharin solution is given, paired with cocaine delivery, the response is akin to the reaction to an aversive stimulus (prevalent transitory excitations) ((Carelli & West, 2014; Wheeler et al., 2008); reviewed in (Wheeler & Carelli, 2009)). Although particular

neuronal subpopulations are not specified, these findings show the flexibility of valence encoding in this brain region.

In sum, while considering potential confounding issues mainly related with the diverse methodologies chosen across literature, these findings have shown that neuronal activity in the NAc exhibits some general valence activity patterns. For the most part, transitory inhibitions after reward and excitations after aversive circumstances are observed. Regarding post-cue responses, excitations after both rewarding and aversive conditioning are more prevalent, though not universal. Furthermore, it is important to refer that NAc activity can also be characterized by complex patterned variations across behavioural stages (*e.g.* reward approach or lever pressing), and can differ with the context of stimulus presentation, the motivational state of animals and behavioural paradigms, amongst other factors.

Though different NAc neuronal ensembles that encode valence have been identified, one common limitation was the absence of tools to mark these neurons and posteriorly characterize them at a molecular level. So, several questions in the field remain: "Are positive valence neurons D1-MSNs, D2-MSNs, interneurons, or are they intermingled?". Nowadays, with the development of optogenetics that allows optical tagging of neuronal populations during *in vivo* electrophysiological recordings, and neuronal activity-driven rodent transgenic lines, this opens a new array of opportunities to characterize these neuronal ensembles in detail and develop new tools to manipulate them.

1.4.1. From valence to behaviour

Many brain regions are known to encode valence and being involved in associated responses. Nonetheless, a great deal of attention has been given to the striatum, particularly the NAc, since it has been deeply implicated in both rewarding and aversive learning (Hikida et al., 2013; Parkinson et al., 1999; Setlow et al., 2003); reinforcement and punishment (Kravitz et al., 2012); rewarding and aversive-motivated behaviours (Day et al., 2006; Parkinson et al., 1999; Salamone, 1994; Young, 2004); reward prediction (Hollerman et al., 1998; Schultz et al., 1997) and encoding relative reward value (Wheeler et al., 2005). Because of its broad function, the NAc is regarded as a major relay region in the basal ganglia, acting as a limbic-motor interface (Mogenson et al., 1980; Soares-Cunha et al., 2016b).

Additionally, the two NAc subregions have been (not exclusively) implicated in many functions. The NAc shell has been, by way of example, linked with reinforcement (Parkinson et al., 1999), valence-related motivation and novelty (Bassareo et al., 2002; Parkinson et al., 1999) and Pavlovian incentive learning (Corbit et al., 2001); while the NAc core functional involvement includes, non-comprehensively, Pavlovian responses (Cardinal et al., 2002; Parkinson et al., 1999, 2000), instrumental conditioning

(Corbit et al., 2001), cue-reward associative learning (Day et al., 2007) and reward seeking for both natural and drug rewards (Bobadilla et al., 2020; Ito et al., 2004). Many functions tend to be common to both subregions. For instance, the NAc shell is also important in reward seeking (Anderson et al., 2003; Schmidt et al., 2006), and both subregions are implicated for aversive learning (Managò et al., 2009).

Studies with higher anatomical comprehension also show that NAc function can vary within its subregions. For example, when injecting muscimol ($GABA_A$ receptor agonist) on opposite ends of the NAc shell, distinct parallel behaviours were noted. Caudal injection led to conditioned place avoidance, aversive responses to both rewarding and aversive stimuli, and defensive burying behaviour (characteristic rodents' reaction to threats), whereas rostral administration led to increased feeding and appetitive responses to reward and conditioned place preference (Reynolds & Berridge, 2001, 2002).

Place preference tests are typical paradigms used to evaluate the rewarding or aversive properties of stimuli. The conditioned place preference test (CPP) is based on passive Pavlovian conditioning, in which animals learn to associate a specific chamber with a previous emotionally relevant stimuli, such as a stimulus delivery or a pharmacological or optogenetic manipulation. On the other hand, the real-time place preference (RTPP) paradigm also measures place preference, but on an immediate timeframe, potentially showing the role of specific neuronal populations in approach/avoidance behaviours.

Projections from the hippocampus to the NAc shell are needed to induce conditioned place preference after a natural reward (LeGates et al., 2018), and activating projections from the LDT to the NAc can augment motivation, induce place preference and positive reinforcement, with the opposite being true when the projections were inhibited (Coimbra et al., 2019). Likewise, the NAc shell was shown to be essential for CPP establishment after exposure to a rewarding drug (morphine) (Li et al., 2015).

The NAc is also necessary for acquired active avoidance behaviours, as eliminating the activity of the NAc shell, disrupts these behaviours (Ramirez et al., 2015). Additionally, this region is critical for choosing cued-based approach or avoidance, including in a conflicting context. In particular, the caudal NAc core appears to enable approach and restrict avoidance, and inactivation of this subregion ($GABA_A$ and $GABA_B$ receptor agonism - muscimol and baclofen) resulted in a formerly non-existent preference for a neutral cue-space over simultaneous opposing cues (Hamel et al., 2017). Interestingly, another study found that NAc shell inactivation (via baclofen and muscimol) increases operant responding in a potentially punishing ("conflict") context, while lower lever pressing occurred in both conflicting and non-conflicting contexts if the NAc core was inactivated (Piantadosi et al., 2017).

Pertaining to aversion, the benefit of preserving these responses on an evolutionary level is quite evident. Prey organisms tend to react to threats by a set of defensive mechanisms. Freezing (*i.e.* a

condition of increased sensory processing and suppression of movement) is one of the most frequent, having been shown to happen due to a rapid learning association between contextual cues and exposure to aversive stimuli (Fanselow, 2018; Fanselow & Lester, 1988; Lang & Davis, 2006). Cholinergic action in the NAc affects fear conditioning, having been observed after delivery of carbachol (cholinergic agonist) in the NAc, which interrupted acquisition and retrieval of conditioned fear, abolishing freezing responses (Schwienbacher et al., 2006).

Once salience reaches a certain level (*i.e.* impending predatory approach), the motivation is to avoid the threatening situation (flight response) (Fanselow, 1994; Lang & Davis, 2006). Prey may also engage in more aggressive behaviours, used to escape and/or temporarily incapacitate predators. This is the case of defensive treading (*i.e.* defensive burying behaviour) (Rodgers et al., 1997), in which the NAc has been implicated. Importantly, this defensive behaviour takes place after GABA agonism (muscimol) (Reynolds & Berridge, 2001) and glutamate antagonism (6,7-dinitroquinoxaline-2,3-dione; DNQX) in the NAc caudal shell (Faure et al., 2010). With NAc shell caudal inactivation, other common behaviours, such as distressed noises and escape attempts, were also noted (Reynolds & Berridge, 2002).

1.5. The role of D1-MSNs and D2-MSNs in rewarding and aversive responses - What is known and what is (still) unknown

Evidence so far strongly suggests that NAc is involved in the processing of both rewarding and aversive events. To truly understand how this region encodes both positive and negative valence, one must reflect about the different neuronal populations that constitute this nucleus. As mentioned, ≈95% of NAc neurons are GABAergic MSNs, generally separated into D1-MSNs and D2-MSNs. Conventionally, these subpopulations have been assigned two opposite functions: D1-MSNs were associated with reward (positive valence) and D2-MSNs with aversion (negative valence). Indeed, seminal studies have shown that the direct pathway/D1-MSNs are essential in reward learning and reinforcement, while the indirect pathway/D2-MSNs influence aversive learning and punishment (Hikida et al., 2010, 2016; Kravitz et al., 2012; Volman et al., 2013). However, recent studies have shown that both populations can be involved in reward and aversion, as described below and included for better understanding in **Table 1**.

1.5.1. Optogenetic and Chemogenetic studies

In this context, optogenetics has shown to be a great tool to investigate the influence of D1-MSNs and D2-MSNs in rewarding and aversive contexts, allowing for selective manipulation of said neurons to explore their role in valence-related processes.

A seminal work has shown that optogenetic activation of D1-MSNs increased cocaine conditioning, while D2-MSN activation reduced it (Lobo et al., 2010). Analogous results were achieved when using morphine (Koo et al., 2014). This, together with studies showing that activating D1- and D2-MSNs (in the dorsal striatum) generates continual reinforcement and transitory punishment, correspondingly (Kravitz et al., 2012), led to the general assumption that these two subpopulations had opposing roles in valence associated behaviour.

In line with a pro-reward role, optogenetic inhibition of D1 neurons represses cocaine sensitivity (Chandra et al., 2013), while activation (using Drd1a-iCre mice and designer receptors exclusively activated by designer drugs; DREADDs) increases alcohol consumption (Strong et al., 2020). Moreover, modulation of social behaviour was observed by optogenetically activating VTA-NAc projections, and D1-MSNs were proven essential for this prosocial modulation. Also, activation of NAc D1-MSNs themselves augments social interaction (Gunaydin et al., 2014).

However, a recent study showed an increase in motivation after briefly optogenetically stimulating either D1-MSNs or D2-MSNs (concurrent with cue-reward exposure) in mice (Soares-Cunha et al., 2016a). The same outcome was observed with D2-MSN activation in rats, while optogenetic inhibition (with halorhodopsin; NpHR) led to a reduction in motivation. Activating D2-MSNs rescued motivational deficits in a model with disruption of such mechanisms (Soares-Cunha et al., 2016a). Optogenetic activation of D2-MSNs was again found to mediate motivation, and D1R and D2R signalling was necessary for rewarding outcomes (Soares-Cunha et al., 2018). In accordance, brief optogenetic activation of both subpopulations prompted increased preference for cocaine-paired context and positive reinforcement. When the optogenetic approach was prolonged, the stimulation became aversive and, for D2-MSNs, cocaine-paired place preference was reduced (Soares-Cunha et al., 2020). These findings show that, depending on the pattern of neuronal activation, both subpopulations can encode reward or aversion. Others have demonstrated that, while optogenetic stimulation of D2-MSNs does not support self-stimulation in some situations (passive location-based self-stimulation), this is not always the case, as activating both subpopulations can strongly support this behaviour in a spout self-stimulation context (Cole et al., 2018), indicating that both populations can be concurrently pro-rewarding (even if dissimilar in strength).

In agreement with potential pro-rewarding roles, D1- and D2-MSNs in the ventrolateral striatum encode goal-directed behaviour for food at different behavioural stages, and do not appear to have antagonistic roles. Optogenetic inactivation of either population at trial start attenuates motivated performance (Natsubori et al., 2017). However, if inactivation is used during lever pressing, only D1-MSN inhibition would result in motivation attenuation (Natsubori et al., 2017).

Interestingly, photostimulating D2-MSNs does not modify cocaine behavioural sensitization, except if performed during withdrawal, which diminishes cocaine-based sensitization (Song et al., 2014).

Furthermore, optogenetic activation of dynorphin-expressing neurons (dyn; expressed in D1-MSNs) in the dorsal NAc shell induced place preference, whilst in the ventral NAc shell induced place aversion (Al-Hasani et al., 2015). Therefore, anatomical location of neuronal populations is, as has been shown frequently, a factor to consider.

Another experiment noted that inhibition of D2-MSNs (via DREADDs) increased cocaine motivation and optogenetic stimulation inhibited cocaine self-administration (Bock et al., 2013), hinting at a role of these neurons in limiting drug reinforcement. Inhibition of D2-MSNs (using Drd2a-iCre mice and DREADDs) also increases alcohol consumption (Strong et al., 2020).

D1-MSNs also appear to have some influence in aversive processing. D1-MSNs are involved in aggression mechanisms, since chemogenetic inhibition of D1R leads to lower levels of aggression-seeking and aggression self-administration (Golden et al., 2019).

1.5.2. Pharmacological studies

Pharmacological studies have also expanded the roles of D1-MSNs and D2-MSNs, largely by manipulating D1R and D2R activity through local administration of respective antagonists and agonists.

For instance, reward learning acquisition is diminished both when the direct pathway is inhibited using a bilateral blockade (tetanus toxin) and when SCH23390 (D1R antagonist) is injected in the intact side of D-aRNB (asymmetric blockade) mice. Intriguingly, activating D2 neurons in the NAc (quinpirole, D2R agonist) of I-aRNB mice disrupts reward learning switch, as did the bilateral blockade of the indirect pathway, showing that D2 neuron inactivity is needed in some aspects of reward learning (Yawata et al., 2012). Applying similar techniques for targeting D2-MSNs (agonism) in the intact side of I-aRNB mice disrupted aversive avoidance learning, showing that D2R inhibition can be essential for this process (Hikida et al., 2013).

In addition, activity of D2 neurons in the NAc core and shell, as well as D1 neuronal activity in the core, are important for aversive memory acquisition (Managò et al., 2009). In fact, D1R inactivation

(intra-NAc) reduced avoidance reactions (Wietzikoski et al., 2012), as did using D2R pharmacological inactivation (Boschen et al., 2011). D2-MSNs (NAc core) have also been implicated in reacting to "less appetitive" situations, following delivery of quinpirole (D2R agonist), since the typical inclination towards a reward lever (*vs* a non-reward lever) was not acquired throughout unexpected reward omission (Porter-Stransky et al., 2013). Additionally, intra-NAc D1R inactivation is associated with a compromised acquisition of place preference for cocaine (Baker et al., 1998) and ethanol (Young et al., 2014).

Again, suggesting parallel functions, cooperation between D1- and D2-MSNs is necessary for intracranial self-stimulation (facilitated by VTA-NAc dopamine projections), since antagonism of either D1R (SCH23390) or D2R (raclopride) in the NAc mitigates this behaviour (Steinberg et al., 2014).

Antagonism of both D1R and D2R lessened performance in a Pavlovian-to-instrumental transfer task (PIT), showing that both neuron types are processing appetitive salience (Lex & Hauber, 2008). Inactivation of both D1 and D2 neurons reduced the extinction stage after morphine-based CPP, indicating that both populations are at least partaking in encoding appetitive attributes of stimuli throughout extinction (Namvar et al., 2019).

Likewise, blockade of D1R and D2R via antagonism in the NAc abolished acquisition of amphetamine-based place preference (Liao, 2008) and inactivating both receptors affected the level of cue response negatively, but leaves motivation to wait (with no energy spending) unaltered (Wakabayashi et al., 2004). Inactivating both receptors (intra-NAc) reduces the quantity of feeding behaviour and enhances feeding period, without changing total food intake (Baldo et al., 2002) and inactivation in the core and shell reduces lever pressing without altering acquisition itself and even increasing food intake as well (Nowend et al., 2001).

Interestingly, ablation of striatopallidal neurons expressing D2 receptor (using Cre-mediated expression of diphtheria toxin receptor, DTR) enhanced amphetamine place preference, suggesting, similarly to previously mentioned studies, a drug reward-limiting effect (Durieux et al., 2009).

Activation of D1 and D2 neurons (intra-NAc) is reinforcing in a CPP context (White et al., 1991) and usage of D1R and D2R agonists (SKF81297 and quinpirole, respectively) in the NAc shell appears to re-establish cocaine-seeking (Schmidt et al., 2006). Comparably, simultaneous agonism of D1R and D2R in the NAc shell is reinforcing, causing intracranial self-administration (Ikemoto et al., 1997), and D1R inactivation in the NAc shell reduces reinstatement of drug seeking (Anderson et al., 2003).

D2 neurons also seem to limit aversive reactions in some contexts, since activating this population (intra-NAc, D2 agonism) in addicted rats inhibited physical symptoms of opiate withdrawal (Harris & Aston-Jones, 1994).

In addition (and again showing the importance of anatomic location), while only D1-MSNs were shown to be critical for glutamate antagonism-induced changes in feeding behavior (rostral NAc shell), the production of aversive responses after glutamate antagonism required the two subpopulations (caudal NAc shell) (Richard & Berridge, 2011). Furthermore, dopamine antagonism in the core abolished caffeine-based place aversion, while the same strategy in the shell abolished caffeine-based place preference (Yee et al., 2020).

Table 1 - Summary of main findings implicating striatal neurons (mainly NAc D1-MSNs and D2-MSNs) in valence encoding.

Neuron Type (NAc, unless specified)	**Methodology**	**Behavioural Task**	**Valence-related effects**	**Reference**
		Optogenetic and Chemogenetic studies		
D1-MSNs	ChR2	Cocaine-based CPP	Increased preference	(Lobo et al., 2010)
D2-MSNs			Reduced preference	
D1-MSNs	ChR2	Morphine-based CPP	Increased preference	(Koo et al., 2014)
D2-MSNs			Reduced preference	
D1-MSNs	ChR2	Stimulation-paired capacitive trigger	Continued reinforcement	(Kravitz et al., 2012)
D2-MSNs			Transitory punishment	(dorsal striatum)
D1-MSNs	NpHR	Open Field + cocaine	Reduced cocaine locomotor sensitization	(Chandra et al., 2013)
D1-MSNs	Activation via DREADDs	Intermittent access to 20% alcohol in a 2-bottle choice test	Increased alcohol consumption	(Strong et al., 2020)
D2-MSNs	Inhibition via DREADDs		Increased alcohol consumption	
D1-MSNs	ChR2	Social Interaction Test	Increased social interaction	(Gunaydin et al., 2014)
D1-MSNs	ChR2	Progressive-Ratio task (PR) / PIT task	Increased motivation	(Soares-Cunha et al., 2016a)
D2-MSNs			Increased motivation / Rescued motivation deficits	
D2-MSNs	NpHR		Reduced motivation	
D2-MSNs	ChR2	PR task	Increased motivation	(Soares-Cunha et al., 2018)
D1-MSNs/D2-MSNs	D1R, D2R antagonism		Reduced motivation	
D1-MSNs /D2-MSNs	ChR2 (brief stimulation)	Stimulation-based CPP	Induced preference	(Soares-Cunha et al., 2020)
D1-MSNs /D2-MSNs	NpHR		Induced aversion	
D1-MSNs /D2-MSNs	ChR2 (prolonged stimulation)		Induced aversion	
D1-MSNs /D2-MSNs		RTPP	Induced aversion	
D1-MSNs /D2-MSNs	ChR2 (brief stimulation)	Cocaine-based CPP	Increased preference	
D2-MSNs	ChR2 (prolonged stimulation)		Reduced preference	
D1-MSNs	ChR2	Passive location-based self-stimulation	Supported self-stimulation	(Cole et al., 2018)
D1-MSNs /D2-MSNs		Spout self-stimulation	Supported self-stimulation	

D1-MSNs /D2-MSNs D1-MSNs	ArchT (at trial start) ArchT (during lever pressing)	PR task	Reduced motivation Reduced motivation	(Natsubori et al., 2017) (ventrolateral striatum)
D2-MSNs	ChR2 (during withdrawal)	Open Field + cocaine	Reduced cocaine locomotor sensitization	(Song et al., 2014)
Dyn$^+$ (dorsal shell) Dyn$^+$ (ventral shell)	ChR2	CPP (Y-maze) / RTPP / ICSS	Increased preference Reduced preference	(Al-Hasani et al., 2015)
D2-MSNs D2-MSNs	Inhibition via DREADDs ChR2	Cocaine self-administration (PR sessions) Cocaine self-administration	Increased motivation Reduced motivation	(Bock et al., 2013)
D1-MSNs	Inhibition via DREADDs	Aggression-seeking test Aggression self-administration test	Reduced aggression-seeking Reduced aggression self-stimulation	(Golden et al., 2019)
		Pharmacological studies		
Striatonigral D1-MSNs Striatopallidal D2-MSNs	Tetanus toxin (TN) + DOX blockade	Standard Food *vs* Chocolate-based CPP One-trial inhibitory avoidance	Disrupted reward learning Disrupted aversive learning	(Hikida et al., 2010)
Striatonigral D1-MSNs Striatopallidal D2-MSNs	TN + DOX blockade TN + DOX asymmetric blockade + D1R antagonism (contralateral) TN + DOX blockade TN + DOX asymmetric blockade + D2R agonism (contralateral)	Visual Cue Task (VCT), Response-Direction Task (RDT)	Disrupted reward learning acquisition Disrupted reward learning acquisition Disrupted reward learning switch Disrupted reward learning switch	(Yawata et al., 2012)
Striatopallidal D2-MSNs	TN + DOX asymmetric blockade + D2R agonism (contralateral)	One-Trial Inhibitory Avoidance Task	Disrupted aversive learning	(Hikida et al., 2013)
D1-MSNs (core) D2-MSNs	D1R antagonism D2R antagonism	One-Trial Inhibitory Avoidance Task	Reduced avoidance Reduced avoidance	(Managò et al., 2009)
D1-MSNs	D1R antagonism	Two-way active avoidance	Reduced avoidance	(Wietzikoski et al., 2012)
D2-MSNs	D2R antagonism D2R agonism (post-training)	Two-way active avoidance	Reduced avoidance / Increased escape failures Reduced avoidance	(Boschen et al., 2011)
D2-MSNs (core)	D2R agonism	Appetitive operant paradigm ("foraging" preference)	Reduced preference for reward during unexpected reward omission	(Porter-Stransky et al., 2013)
D1-MSNs	D1R antagonism	Cocaine-based CPP	Reduced preference	(Baker et al., 1998)
D1-MSNs	D1R antagonism	Ethanol-based CPP	Reduced preference	(Young et al., 2014)

VTA-NAc dopaminergic	ChR2 (+ D1R/D2R antagonism)	Intracranial self-stimulation (ICSS)	Reduced ICSS	(Steinberg et al., 2014)
D1-MSNs	D1R antagonism	PIT task	Reduced motivation	(Lex & Hauber, 2008)
D2-MSNs	D2R antagonism		Reduced motivation	
D1-MSNs	D1R antagonism	Morphine-based CPP	Reduced extinction stage	(Namvar et al., 2019)
D2-MSNs	D2R antagonism		Reduced extinction stage	
D1-MSNs	D1R antagonism	Amphetamine-based CPP	Reduced preference	(Liao, 2008)
D2-MSNs	D2R antagonism		Reduced preference	
D1-MSNs	D1R antagonism	Progressive Delay Task	Reduced cue responding	(Wakabayashi et al., 2004)
D2-MSNs	D2R antagonism		Reduced cue responding	
D1-MSNs	D1R antagonism	''Free-feeding'' Test	Reduced feeding behaviour / Increased feeding period	(Baldo et al., 2002)
D2-MSNs	D2R antagonism		Reduced feeding behaviour / Increased feeding period	
D1-MSNs	D1R antagonism	Fixed-Ratio 5 (FR5) Schedule of Reinforcement / Choice Procedure Session	Reduced lever pressing / Increased chow intake	(Nowend et al., 2001)
D2-MSNs	D2R antagonism		Reduced lever pressing / Increased chow intake	
Striatopallidal D2-MSNs	Diphtheria toxin receptor-based blockade	Amphetamine-based CPP	Increased preference	(Durieux et al., 2009)
D1-MSNs	D1R agonism	Agonist-based CPP	Increased preference	(White et al., 1991)
D2-MSNs	D2R agonism		Increased preference	
D1-MSNs (shell)	D1R agonism	FR5 Schedule of Reinforcement	Reinstatement of cocaine-seeking	(Schmidt et al., 2006)
D2-MSNs (shell)	D2R agonism		Reinstatement of cocaine-seeking	
D1-MSNs + D2-MSNs (shell)	D1R agonism + D2R agonism	Intracranial Self-Administration (ICSA)	Increased ICSA	(Ikemoto et al., 1997)
D1-MSNs (shell)	D1R antagonism	FR5 Schedule of Reinforcement	Reduced reinstatement of cocaine-seeking	(Anderson et al., 2003)
D2-MSNs	D2R agonism	Morphine + Scoring of Withdrawal Symptoms	Reduced opiate withdrawal physical symptoms	(Harris & Aston-Jones, 1994)
D1-MSNs (rostral shell)	D1R antagonism	Tests of Spontaneous Motivated Behaviours	Abolished glutamate disruption-based enhanced feeding	(Richard & Berridge, 2011)
D1-MSNs (caudal shell)	D1R antagonism		Abolished glutamate disruption-based defensive behaviours	
D2-MSNs (caudal shell)	D2R antagonism			
NAc DR$^+$ (core)	Dopamine antagonism	Caffeine-based CPP	Reduced aversion	(Yee et al., 2020)
NAc DR$^+$ (shell)			Reduced preference	

1.6. Neuroanatomy and neuronal populations of the amygdala: basolateral and central nuclei

The amygdala is an almond-shaped brain region that arises in both hemispheres, found medial relative to the temporal lobe in humans, and medial and dorsal relative to olfactory areas in rodents (Paxinos & Franklin, 2001; Paxinos & Watson, 2007; Sah et al., 2003). Various anatomical characterizations subdivide the amygdala in 13 nuclei and cortical areas, with considerable similarities between rodents, humans and other primates. These nuclei include the basolateral nucleus (BLA), the lateral nucleus (LA), the central nucleus (CeA), the medial nucleus (M) and the cortical nucleus (Co) (Chareyron et al., 2011; Sah et al., 2003). The BLA and lateral nucleus are subdivisions of the basolateral complex, being surrounded by the CeA, the caudate putamen and the external capsule in rodents. In turn, the CeA also edges with the medial nucleus and with the caudate putamen, as well as the globus pallidus (Sah et al., 2003).

Regarding afferent connections, the BLA receives projections from the LA, a connection that is associated with sensory information input and processing (Babaev et al., 2018; Duvarci & Pare, 2014; Šimić et al., 2021; Tovote et al., 2015). Other input regions encompass the orbitofrontal cortex (OFC), the basal forebrain, the dorsal raphe nucleus (DRN), the infralimbic cortex and the VTA (Correia & Goosens, 2016; Huang et al., 2020; Saddoris et al., 2005).

BLA outputs are varied, including cortical, subcortical and limbic regions (Price, 2003; Sah et al., 2003), such as the OFC and the medial PFC (Laviolette & Grace, 2006; Saddoris et al., 2005), the hippocampus (Felix-Ortiz & Tye, 2014), the *substantia innominata* (Krettek & Price, 1978a, 1978b), the BNST (Krettek & Price, 1978a, 1978b), the dorsal striatum (Bourgeais et al., 2001), the NAc (Bourgeais et al., 2001; Krettek & Price, 1978a, 1978b), the hypothalamus (Krettek & Price, 1978a, 1978b), but also the lateral amygdala and CeA (Babaev et al., 2018; Duvarci & Pare, 2014; Šimić et al., 2021; Tovote et al., 2015; Tye et al., 2011).

In turn, the CeA projects to a vast array of areas (Price, 2003; Sah et al., 2003; Šimić et al., 2021; Tovote et al., 2015), such as the periaqueductal grey (PAG) (Babaev et al., 2018; LeDoux et al., 1988), the hypothalamus (Babaev et al., 2018; Bourgeais et al., 2001; Krettek & Price, 1978a, 1978b; LeDoux et al., 1988), the *substantia innominata* (Bourgeais et al., 2001), the BNST (Bourgeais et al., 2001; Krettek & Price, 1978a, 1978b; LeDoux et al., 1988) and effector regions, such as, for example, the brainstem (Veening et al., 1984). It should be noted that a considerable number of connections with multiple amygdala subnuclei are bidirectional (Mcdonald, 1998; Price, 2003).

Concerning neuronal populations, the BLA is mostly constituted by large spiny pyramidal cells, which are glutamatergic in nature and have many structural similarities with pyramidal cortical cells, while the remaining cells are GABAergic interneurons (Braak & Braak, 1983; Sah et al., 2003; Spampanato et al., 2011). The CeA is largely formed by GABAergic striatal-like cells, being for that considered a more "striatal" segment of the amygdala (Sah et al., 2003; Swanson & Petrovich, 1998).

Neuropeptides are also a point of divergence between the BLA and CeA. The BLA contains cells positive for somatostatin, protein phosphatase 1 regulatory subunit 1B (PPP1R1B), roof plate-specific spondin-2 (RSPO2), vasoactive intestinal polypeptide (VIP) and cholecystokinin-octapeptide (CCK). The CeA includes cells expressing methionine-enkephalin (MET-ENK), somatostatin, protein kinase c-δ (PKC-δ, PRKCD), corticotropin-releasing hormone (CRH), neurotensin, tachykinin 2, CCK, dopamine receptor D2, substance P, calcitonin receptor-like receptor (CRLR) and serotonin receptor 5-hydroxytryptamine 2A (HTR2A), with some peptides overlapping in expression within the region (Kim et al., 2016, 2017; McCullough et al., 2018; Roberts et al., 1982). Neurochemical markers in the central nucleus are unequally distributed across different subnuclei (Kim et al., 2017; McCullough et al., 2018).

1.7. Functional evidence on valence encoding in the basolateral and central nuclei of the amygdala

The amygdala, as a whole, processes both reward- and aversion-related mechanisms, something which has been explored in electrophysiological studies. For example, neurons in this brain area are implicated in encoding appetitive value, with a frequently excitatory profile that appears to track rewards with different magnitudes (Bermudez & Schultz, 2010). A primate study depicted diverse amygdala populations, including neurons encoding unexpected water reward (and expected reward, with lower firing rates); responding preferentially to unexpected aversion (*i.e.* air-puffs); and processing both unexpected reward and aversion ("non-valenced" specificity) (Belova et al., 2007). Amygdala also encodes olfactory, auditory, visual, and somatosensory stimuli, with multimodal-responsive cells occurring specially in the BLA and CeA (Uwano et al., 1995). This brain region encodes reward acquisition and extinction, with subpopulations processing opposing valences (Livneh & Paz, 2012), and processing positive and negative value of visual stimuli throughout learning (Paton et al., 2006).

BLA cells react to primary rewards. From glucose-responding neurons, a sizable number of cells exhibited excitations, similarly to the population encoding the glucose-predicting cue (Muramoto et al., 1993). Additionally, it was frequent (but not universal) that activity changes (*i.e.* excitations or inhibitions) to reward matched the ones originated by the predicting cue (Muramoto et al., 1993). In a primate study

using different reward schedules with visual cues, a considerable number of BLA cells responded to cues (often excitatory activity), while some reacted to reward delivery (often an activity increase) (Sugase-Miyamoto & Richmond, 2005). Furthermore, cue-reactive cells (excitatory effect) were also noted in an operant task in which animals received a cue and sucrose if they executed a nosepoke. Some BLA neurons responded to cues only if a port entry for sucrose occurred, while another subset sustained cue-response during reinstatement, even if no port entry occurred (Tye & Janak, 2007). Using a reward extinction paradigm with sucrose, Tye et al. (2010) found varied BLA populations with phasic activity changes. One of these populations initially responded to port entries only if sucrose was delivered, but during extinction responded to unrewarded entries (mainly inhibitory in both instances, but not only) (Tye et al., 2010). Some responded to empty port entries only in extinction, others responded to port entries with sucrose delivered only and others responded to all port entries with a predominant decrease in firing (Tye et al., 2010). This links this brain area with processing reward outcome and availability, reinforcement and motivational cues in general. Lastly, other study found that BLA projecting neurons respond with increases in activity practically exclusively to reward-predictive cues (Lee et al., 2016). Curiously, the subpopulation with significant excitations throughout cue-reward presentations appears to depend on the learned responding behaviour (Lee et al., 2016). The CeA also responds to at least some reward-related mechanisms, such as feeding, seeing that high-fat diet has an effect in excitatory synaptic function in *Pnoc* (prepronociceptin) neurons of this subnucleus (Hardaway et al., 2019).

The BLA also encodes primary aversive stimuli. After footshock exposure, responsive neurons in the BLA presented similar levels of excitations and inhibitions, as did the shock-predicting cue (Muramoto et al., 1993). Moreover, BLA subpopulations have been shown to process valence using a fear paradigm and comparing neuronal activity during cue exposure with delivery of distinct fluids (one aversive and one rewarding) (Shabel et al., 2011). In this study, neuronal activity variations in a subset of neurons were found to be more comparable in two aversive situations of different modalities (fear *vs* unpalatable taste) than when comparing fear with a palatable taste (Shabel et al., 2011).

Both regions seem to encode cue exposures in aversive learning. Aversion-predictive cues induced excitations in some BLA projection neurons, a subset with almost no overlap with the cue-reward-responsive population (Lee et al., 2016). In another conditioned approach, when saccharin was used by Yasoshima et al. (1995) as a cue for delivery of lithium chloride, both BLA and CeA neurons responded strongly to the cue, once taste aversion has been established. In the BLA, a considerable population increased spiking in response to saccharin (Yasoshima et al., 1995). In contrast, the main effect detected in the CeA was inhibitory, being also observed due to exposure to other unpalatable stimuli even before

conditioning, which did not happen in the BLA (Yasoshima et al., 1995). However, these results are not unanimous, as Kim et al. (2010) noted inhibitions in the BLA when saccharin was used as a cue when establishing taste aversion.

Intriguingly, the BLA appears to be engaged in predator expectation, with a frequently inhibitory outcome (only 4.5% of cells were activated) (Amir et al., 2019). However, the authors question if these neurons are activated by the predator itself or associated cues, since from the few activated cells, many respond to non-threatening stimuli (Amir et al., 2019).

Anatomical localization is a factor that should also be taken into account. Indeed, while overall more units present excitations to cues (reward and aversive collectively), the BLA presents interesting variations across the anteroposterior, medial lateral, and dorsal ventral axes (Beyeler et al., 2018). Both increases and decreases in firing rate occur after a cue-sucrose or a cue-quinine, and excitations (collectively) arise preferentially in more dorsal, lateral and posterior locations, and more dorsally for the cue-quinine (Beyeler et al., 2018). Inhibitions appear to present a trend for favouring more ventral coordinates for cue-quinine, relative to the cue-sucrose cells (Beyeler et al., 2018).

1.7.1. From valence to behaviour and implications of the basolateral and central nuclei of the amygdala

The BLA and CeA nuclei are regions involved in both reward and aversion, being for that implicated in valence encoding (Balleine & Killcross, 2006; Namburi et al., 2016; O'Neill et al., 2018; Sah et al., 2003; Šimić et al., 2021; Tovote et al., 2015). BLA is heavily linked with aversive learning (Balleine & Killcross, 2006), especially fear acquisition and responding (Duvarci & Pare, 2014; Tovote et al., 2015). Other functions include appetitive learning (Balleine & Killcross, 2006; Wassum & Izquierdo, 2015), devaluation of reinforcers (Hatfield et al., 1996; Holland & Gallagher, 2004), motivated behaviours (Correia & Goosens, 2016), processing reward cost-and-benefit and goal directed-behaviour (Wassum & Izquierdo, 2015), pain-associated processing (Neugebauer, 2015; Neugebauer et al., 2020), anxiety-like behaviour (Tovote et al., 2015; Tye et al., 2011), memory reconsolidation (Wu et al., 2014) and social behaviour (Felix-Ortiz & Tye, 2014; Huang et al., 2020). Similarly, the CeA is involved in varied mechanisms, comprising fear processing and responding (Duvarci & Pare, 2014), anxiety processing (Tovote et al., 2015; Tye et al., 2011), pain processing (Neugebauer, 2015; Neugebauer et al., 2020), emotional discrimination (Ferretti et al., 2019) and aversive and rewarding conditioning (Balleine & Killcross, 2006). The amygdala also has a significant role in motivation-related behaviours, including approach relative to appetitive contexts (motivation to seek reward) and avoidance due to aversive

contexts (motivation to avoid painful, fear-inducing and/or anxiogenic stimuli) (Šimić et al., 2021; Tovote et al., 2015).

Basolateral amygdala studies

There is evidence implicating the BLA in approach behaviour. When using a Pavlovian odour task, in which an odour was presented together with optogenetic activation of nicotine-induced neurons (positive value), the animals spent more time in the odour chamber (Gore et al., 2015a). Optogenetic stimulation of BLA neurons (cue-paired) enhances cue-based reward-approach behaviour for water (Servonnet et al., 2020). Additionally, in a PIT paradigm, excitatory glutamatergic activity in the BLA was found important for outcome-directed reward-seeking behaviour, since AMPA receptor antagonism reduced cue-based response enhancement (Malvaez et al., 2015).

Some BLA populations are involved in encoding aversion. In a Pavlovian paradigm, mice learned to avoid a chamber that was previously paired with the activation of shock-responsive cells, to evade the aversive association ((Gore al., 2015a); similar avoidance results with an optogenetic approach with fear conditioning in (Redondo et al., 2014)). Another study highlighted that BLA-centromedial amygdala projections can drive place avoidance when optogenetically activated (Namburi et al., 2015). Prelimbic cortex-BLA neurons promote place avoidance when stimulated via ChR2, while also inducing social deficits (Huang et al., 2020). Inputs from the insular cortex to the BLA are required to drive conditioned taste aversion, both at the acquisition and retrieval levels (Kayyal et al., 2019).

In the work of Tanimoto et al. (2003), a conditioned place aversion (CPA) paradigm was performed with formalin (intraplantar) and acetic acid (intraperitoneal) injections serving as conditioning noxious stimuli, with both compounds inducing place aversion. Formalin-conditioned avoidance was eradicated after lesion in the BLA (Tanimoto et al., 2003). In another Pavlovian paradigm with an aversive stimulus exposure associated with an operant response, the BLA was found to actively influence shifting lever pressing towards the neutral lever (avoiding the aversive experience) (Killcross et al., 1997).

Regarding motivation, the importance of the BLA has been noted, as optogenetic activation of glutamatergic BLA-NAc cells enhances appetitive seeking behaviours, and inactivation reduces reward responding (Stuber et al., 2011). The BLA, and in particular its prelimbic PFC- and NAc core-directed outputs, is also required for cocaine-seeking (conditioned reinstatement) (Stefanik & Kalivas, 2013). This region is also pertinent in limiting reward-seeking behaviours if reward is linked with a potential aversive experience, as shown in the enhanced operant responding during a conflict stage when the BLA was

inactivated (Piantadosi et al., 2017). The BLA is also necessary for conditioned feeding ((Holland & Gallagher, 2003); PIT only in (Hall et al., 2001)).

Interestingly, specific and distinct BLA populations that clearly encode positive (nicotine) or negative (footshock) valence unconditioned stimuli have been described by Gore et al. (2015a). In more detail, when optogenetically activating BLA stimuli-reactive neurons, typical physiological and behavioural reactions were observed. These responses included, for shock-specific reactivation, reduced heart/respiration rate and increased freezing (a defensive behaviour), and, for nicotine-specific reactivation, elevated heart/respiration rate. Freezing was also noted at cue presentations, after conditioning with stimulation of shock-responsive cells (Gore et al., 2015a). Accordingly, if excytotoxic lesions are performed in the BLA post-training in a conditioned fear context, cue-related freezing levels were considerably diminished (Anglada-Figueroa & Quirk, 2005). Chemogenetic inhibition of the BLA reduces escape to an impending threat (active response), while enhancing freezing (passive response), demonstrating a role in encoding a switch into active defensive behaviour (Terburg et al., 2018).

Response to aversive stimuli such as a shock can likewise be observed in BLA via calcium (Ca^{2+}) activity (Sengupta et al., 2018). Interestingly, this study also showed that the BLA is required for fear learning (measured as suppression of operant responding) and safety behaviours (Sengupta et al., 2018).

Kim et al. (2016) also described two genetically distinct populations in the BLA that encode valence in an opposed and rather antagonistic manner. $RSPO2^{+}$ cells respond to shock, can drive aversion and negatively affect reward-seeking and reward conditioning, while $PPP1R1B^{+}$ cells are activated by a water reward, support place preference and can negatively alter freezing after shock exposure, as well as aversive conditioning (Kim et al., 2016).

Central amygdala studies

In line with the notion of being a major amygdala output region, the CeA has also been linked with both avoidance and approach behaviours. Chemogenetic inhibition of CeA projections to the lateral hypothalamus (via DREADDs) alleviated CPA, and activation induced avoidance in otherwise naïve animals (Weera et al., 2021). Dopamine agonism (quinpirole) in the CeA enhances morphine CPP on lower morphine dosages, whilst reducing CPP when a higher dose was used (Rezayof et al., 2002). Dopamine antagonism (sulpiride) lowers CPP acquisition (Rezayof et al., 2002). Avoidance behaviour due acetic acid and formalin (aversive) conditioning can be eradicated by CeA excytotoxic lesions (CPA, (Tanimoto et al., 2003)). The CeA was also found necessary for curbing aversive lever pressing (Killcross et al., 1997). Some of the aforementioned studies (Killcross et al., 1997; Tanimoto et al., 2003) appear

to indicate a similar, yet not necessarily equal, role in avoidance behaviour relative to the BLA (see previous subsection).

Specific genetically-defined populations of the CeA are also known to encode rewarding behaviours like self-stimulation (*e.g.* neurotensin^{+} neurons in the medial nucleus) (Kim et al., 2017). CeA function is also required for establishing PIT responding for food ((Holland & Gallagher, 2003); PIT only in (Hall et al., 2001)). Additionally, the CeA is involved in feeding behaviour, considering that muscimol ($GABA_A$ agonism) decreases feeding in sated and hungry rats, and bicuculline ($GABA_A$ antagonism) can alleviate it for some muscimol concentrations (Miñano et al., 1992). Moreover, GABAergic HTR2A^{+} CeA cells modulate eating behaviour – activation via DREADDs promotes feeding in both satiated mice and in contexts in which feeding would be aversive (Douglass et al., 2017).

Again, consistent with relaying affective information to downstream regions, the CeA processes unconditioned stimuli. This was shown, for example, by Steinberg et al. (2020), in which CeA-lateral *substantia nigra* neurons responded to both aversive (shock) and appetitive (sucrose) stimuli. Another study used optogenetics to manipulate CeA projections to the globus pallidus external segment (GPe) during stimuli presentation: activation affected positively aversive learning, while inhibition diminished aversive learning (Giovanniello et al., 2020).

CeA neurons are also involved in driving defensive behaviours. One such population is constituted by protein kinase c-δ^{+} neurons in the capsular nucleus (Kim et al., 2017). Furthermore, optogenetic activation of medial CeA cells promoted freezing, as did pharmacological inactivation of lateral CeA (but not in the medial – or entire – CeA) (Ciocchi et al., 2010). Inactivating the CeA during fear conditioning (or exclusively the lateral CeA) also abolishes expression of conditioned fear in the form of freezing (Ciocchi et al., 2010). The medial CeA, and the CeA as a whole, are also important for fear memory retrieval (Ciocchi et al., 2010). Curiously, projections from the locus coeruleus to the CeA appear essential for behaviours such as freezing in a conditioned context (Gu et al., 2020).

Furthermore, somatostatin^{+} cells of the lateral subdivision of the CeA appear to encode, not simply defensive behaviours, but particularly processing a balance between active and passive responses (Yu et al., 2016). Lower levels of activity have been linked with active defence (*i.e.* avoidance and running), while activation promotes passive defence (*i.e.* freezing and lick repression) (Li et al., 2013; Yu et al., 2016). In addition, activating a separate neuronal population (oxytocin-responsive neurons in the lateral CeA) lowers freezing levels and enhances escape behaviour in response to impending threats (Terburg et al., 2018). Considering these studies and the above-mentioned BLA results, the switch in defence mechanisms is possibly influenced by multiple cooperative mechanisms and brain regions.

1.8. Open questions and future of the field

Tools in the field of neuroscience have developed so much in the last decade that they allowed us to field-test conceptual models of neuronal function and behaviour, and to go deeper into our understanding of how certain neuronal circuits are involved in pleasure and aversion. These same tools also led us to recognize that there is enormous complexity in the way the brain encodes information with positive or negative valence, and how this translates into a certain behaviour. Many brain regions have more defined influences in valence and related roles, such as the BLA and CeA ((Giovanniello et al., 2020; Gore et al., 2015a); reviews in (Balleine & Killcross, 2006; Namburi et al., 2016; O'Neill et al., 2018; Sah et al., 2003; Šimić et al., 2021; Tovote et al., 2015)).

The nucleus accumbens is a usual suspect when we talk about reward and aversion, but the field is far from understanding how external (sensory) stimuli reach this brain region, how the valence stamp is attributed to particular stimuli, and how this is then translated into adequate behaviour – approach, avoid (or ignore). The vision that D1- and D2-MSNs were the main characters in positive/appetitive or negative/aversive stimuli processing, respectively, is no longer supported by recent evidence in the field. In numerous cases, these subpopulations do not appear to have neither antagonistic nor exclusive roles, but rather appear to work together to encode an overall outcome. Yet, the possibility of unequal levels of influence from each neuron type in the presence of specific events cannot be excluded and this is likely the future of the studies in the field. Further work would also benefit from more recently developed and specialized tools with potential in accurately identifying activated neurons, such as targeted recombination in active populations (TRAP; (Guenthner et al., 2013)) optotagging (Lima et al., 2009) and single cell RNA-sequencing (scRNA-seq) tools such as activated cell population sequencing (Act-seq; (Wu et al., 2017)).

The mechanisms underlying positive and negative valence encoding and associated behaviour are still not clear, and so, more directed and comprehensive studies, particularly works taking into account the recent technological advances, are essential, as many questions remain to be answered.

CHAPTER 2 – Objectives

2. Objectives

The aim of the present dissertation was to study the contribution of NAc neuronal populations in valence encoding, to better comprehend rewarding and aversive mechanisms. The major focus of this dissertation was the NAc, while the BLA and the CeA were used as experimental controls, considering their previously established roles in valence encoding and in reward and aversion.

This thesis was subdivided in three main objectives:

1. Identify neuronal populations encoding positive- and negative-valence stimuli by evaluating c-fos^{+} cell density in the NAc, BLA and CeA;

2. Characterize the expression and function of a neuronal activity (c-fos)-dependent viral vector after cocaine administration (positive valence) or exposure to electric footshock (negative valence);

3. Induce positive- or negative-valence associated behavioural responses in a neutral context, by optogenetic activation of positive or negative stimuli-responsive neuronal ensembles (in a real time place preference (RTPP) and a conditioned place preference (CPP) paradigm).

CHAPTER 3 – Materials and Methods

3. Materials and Methods

3.1. Animals

Wild-type C57BL/6J mice (males and females) (Charles River Laboratories, Barcelona, Spain) were kept in standard housing conditions (light/dark cycle of 12/12h with lights turned on at 08:00h; 22°C±2°C ambient temperature) with food and water *ad libitum*. Animals were housed up to a maximum of 6 mice per homecage (type 2L). All behavioural experiments were executed during the light period of the light/dark cycle.

All experiments were conducted with 2–4-month-old subjects, which were kept divided according to gender from postnatal day 21, and handled regularly for 5-10min in the week before behavioural testing to reduce handling-associated stress. Animals were also habituated to all behavioural apparatuses in at least three occasions for 10min prior to behavioural tests.

All protocols were approved by the Ethics Committee of the Life and Health Sciences Research Institute and by the national competent entity, the Direção-Geral de Alimentação e Veterinária (DGAV) (#19074). All practices and techniques were performed following established ethical and legal standards, including the European Union Directive 2010/63/EU. Health monitoring was carried out according to FELASA guidelines and all experimenters and animal facilities were accredited by DGAV.

3.2. Neural labelling and optogenetics – constructs and virus preparation

We used the pAAV-c-fos-hChR2(H134R)-eYFP-PEST-noWPRE plasmid, under the control of the c-fos minimal promoter segment (767bp), and including the 500bp intron 1 coding region comprising key regulatory elements, as well as pAAV-c-fos-eYFP-PEST-noWPRE (differing only by not including the hChR2(H134R) exon sequence). Both constructs included the ampicillin resistance (*AmpR*) gene. The plasmids were kindly provided by Dr. Karl Deisseroth, from Stanford University (vector description available at (Ye et al., 2016)).

Plasmid DNA was used to transform DH5α competent *E. coli* cells, followed by DNA isolation and simultaneous generation of glycerol socks. Briefly, 5ul of plasmid DNA received from the collaborator was added to an aliquot of DH5α competent cells, kept on ice for 30min and then rapidly exposed to heat shock (42°C; 1min) and put on ice for 3min. 500ul of SOC medium (super optimal broth with catabolite repression) was added to the mixture and sample was incubated for 1h with agitation at 37°C. This medium was then platted in LB (lysogeny broth) agar plates with ampicillin at 100μg ml^{-1}, so that only cells that had integrated the plasmid would survive, and left overnight at 37°C. Single colonies were then

inoculated into LB liquid medium with added ampicillin at 100μg ml^{-1}, and bacteria were grown at 37°C (overnight at 180rpm).

To extract and purify the replicated plasmid DNA, obtaining a high-yield product while having low levels of contaminants such as endotoxins, the NucleoBond® Xtra EF plasmid purification kit was used (Macherey-Nagel, Düren, Germany). Protocol was followed exactly as described in the kit-provided instructions for a midipreparation. DNA quantification was assessed via UV-Vis spectrophotometry (NanoDrop™ 1000; Thermo Fisher Scientific, MA, USA).

The viral constructs were packaged into an adeno-associated virus (rAAV) 5 serotype by the UNC Gene Therapy Center Vector Core (University of North Carolina, NC, USA). AAV5 vector titters were 1.2-9.9X10^{12} virus molecules/ml. Schematic representation of the sequenced vectors was performed using SnapGene®.

AAV5-c-fos-ChR2-eYFP leads to AAV c-fos-driven expression of channelrhodopsin (ChR2(H134R)) fused with enhanced yellow fluorescent protein (eYFP) for optogenetic activation of c-fos-activated neurons, while AAV5-c-fos-eYFP simply expresses eYFP in c-fos-activated neurons.

3.3. Tracing surgeries and cannula implantation

Wild-type C57BL/6J mice (>2 months old) were anaesthetized with 75mg kg^{-1} ketamine (Imalgene, Merial, Lyon, France), together with 1mg kg^{-1} medetomidine (Dorbene, Cymedica, Horovice, Czech Republic), for stereotaxic surgeries.

The animals used for initial vector characterization were injected with the ChR2 construct (AAV5-c-fos-ChR2-eYFP; 500nl per injection) bilaterally in the NAc (stereotaxic coordinates from bregma (Paxinos & Franklin, 2001): +1.3mm anteroposterior (AP), +0.9/-0.9mm mediolateral (ML), and -4.0mm dorsoventral (DV)); and in the BLA (stereotaxic coordinates from bregma: -1.6mm anteroposterior (AP), +3.3/-3.3mm mediolateral (ML), and -4.6mm dorsoventral (DV)).

The mice used for assessing the recruitment of neuronal populations were unilaterally injected with the YFP construct (AAV5-c-fos-eYFP; 1000nl total per coordinate) in three NAc stereotaxic coordinates (Paxinos & Franklin, 2001) in two-step injections (500nl in the first coordinate followed by 500nl injection in a different DV coordinate); Injection 1: +1.6mm AP, +1.2mm ML, and -4.5mm DV, then up to -3.9mm DV; Injection 2: +1.3mm AP, +1.2mm ML, and -4.7mm DV, then up to -4.0mm DV; Injection 3: +1.0mm AP, +1.2mm ML, and -4.7mm DV, then up to -3.9mm DV. Similar sequential injections (500nl each step) were performed in the BLA, with two stereotaxic coordinate sets (Paxinos & Franklin, 2001); Injection 1:

-1.4mm AP, +3.2mm ML, and -4.7mm DV, then up to -4.2mm DV; Injection 2: -1.6mm AP, +3.3mm ML, and -4.7mm DV, then up to -4.2mm DV.

For optical stimulation in the BLA, animals were unilaterally injected with the ChR2 construct (AAV5-c-fos-ChR2-eYFP; 500nl per individual injection) in the BLA (stereotaxic coordinates from bregma: -1.6mm AP, +3.3mm ML, and -4.6mm DV). To allow optogenetic stimulation, animals were also implanted with an optic fiber cannula (200µm core fiber optic; Thorlabs, Germany), with 2.5mm stainless steel ferrule (Thorlabs, Germany), in the same coordinates with the exception of DV (-4.0mm DV), being fixed to the skull using dental cement (C&B kit, Sun Medical, Shiga, Japan).

Presented values are stereotaxic coordinates from bregma according to Paxinos & Franklin (2001) and all injections were performed using a 30-gauge needle 2 Hamilton syringe (Hamilton Company, Reno, NV, USA), at a rate of 100nl min^{-1}. After injection, the syringe was left in place for 5min to allow viral diffusion.

At the end of the surgical procedures, mice were removed from the stereotaxic frame, sutured and injected with anaesthesia reversal (1mg kg^{-1} atipamezole; Antisedan). Postoperative care was carried out by administering analgesia (0.05mg kg^{-1} buprenorphine; Bupaq, Richter Pharma, Austria) 6h post-procedure, as well as once every 24h during three successive days. A multivitamin supplement and saline were also administered post-procedure when necessary.

3.4. Behavioural Assessment

3.4.1. Apparatus

Three identical operant chambers (21.59cm length x 18.08cm width x 12.7cm height - working area; Med Associates, St. Albans, VT, USA) accommodated in light- and sound-attenuating boxes, were used in the test. All chambers contained a central magazine and were equipped with a grid floor and a shocker. The source of illumination was a 100mA, 2.8W house-light installed on the top-centre of the wall opposite to the magazine wall. A computer equipped with Med-PC software (Med Associates, St. Albans, VT, USA) was used to control the equipment and record the data, and webcams (Microsoft LifeCam HD-3000) were used to acquire video footage of each individual stimulus exposure.

3.4.2. Recruitment of NAc and amygdala ensembles upon exposure to positive *vs* negative valence stimuli

Wild-type C57BL/6J mice, 2-4 months of age were randomly distributed into four groups: Shock (n=9), No Shock (n=5), Cocaine (n=8) and Saline group (n=5). In the week prior to behavioural testing,

handling was regularly performed in 5-10min sessions in all mice, aside from which they were experimentally naïve to prevent conditioning. The animals were habituated to the apparatus in three 10min habituation sessions a few days preceding the behavioural procedure. Animals were also acclimatized to the experiment room 30min prior to testing. The animals were exposed to one of two stimuli, either cocaine (appetitive stimulus) or electric footshock (aversive stimulus).

Mice were administered with cocaine via intraperitoneal (I.P.) injection (20mg/kg) (Cocaine group) or exposed to 20 1.5mA footshocks over 10min (Shock group) before being returned to the homecage. For this and the remaining of this dissertation, shock protocol was defined in agreement with Gore et al. (2015a). Controls were injected with saline via I.P. (Saline group) or left to explore in the same chambers for 10min (No Shock group). The house-light remained ON independently of the group. All animals were sacrificed 90min after stimulus exposure.

3.4.3. Temporal characterization of a c-fos-driven viral vector

Wild-type C57BL/6J mice, 2-4 months of age, were used. All mice were previously subjected to surgery to inject the AAV5-c-fos-ChR2-eYFP construct, bilaterally in the NAc and in the BLA. These animals were randomly distributed into three groups, designated hereafter as Shock 8h (n=4), Shock 16h (n=4) and the Control group (n=3). Mice were handled regularly in 5-10min sessions in the week prior to testing. All animals were accustomed to the apparatus a few days preceding the behavioural testing in three 10min habituation sessions. The subjects were moved to the experiment room 30min prior to the behavioural procedure for acclimatization. Only one stimulus session was executed with all subjects, and the house-light was kept on independent of the group.

The stimulus used was an electric footshock (aversive stimulus), in which the animals were exposed to 20 1.5mA footshocks every 30s, over 10min. Both the Shock 8h and Shock 16h groups were subjected to electric footshock, being sacrificed 8h and 16h after stimuli exposure, respectively. Controls were simply left to explore the same apparatus for 10min and sacrificed with the Shock 16h group. All animals returned to the homecage after the procedure.

3.4.4. Transient labelling and identification of neuronal populations responsive to positive *vs* negative valence stimuli

Wild-type C57BL/6J mice, 2-4 months of age, were formerly subjected to surgery to inject the AAV5-c-fos-eYFP construct in the NAc and in the BLA. Mice were randomly assigned in four groups: Shock (n=9), No Shock (n=5), Cocaine (n=8) and Saline group (n=5). In the week preceding behavioural testing,

handling was conducted frequently in 5-10min sessions in all mice. Animals were otherwise experimentally naïve to prevent conditioning. Mice were familiarized with the apparatus in three 10min habituation sessions a few days prior to the behavioural procedure. Animals were also acclimatized to the experiment room 30min before testing. The animals were exposed to one of two stimuli, either cocaine (appetitive stimulus) or electric footshock (aversive stimulus).

These animals were administered with cocaine via intraperitoneal (I.P.) injection (20mg/kg) (Cocaine group) or exposed to 20 1.5mA footshocks over 10min (Shock group) before being returned to the homecage. Controls were injected with saline via I.P. (Saline group) or left to explore in the same chambers for 10min (No Shock group). The house-light remained ON independently of the group. All animals were sacrificed 16h after stimulus exposure.

3.4.5. Optogenetic manipulation of neurons responsive to negative valence stimuli

Wild-type C57BL/6J mice, 2-4 months of age, were used in this experiment. Animals were formerly subjected to surgery to inject the ChR2 construct (AAV5-c-fos-ChR2-eYFP) in the BLA and were then implanted with an optical fiber in the same brain region.

For conditioned place preference (CPP), animals were divided into Shock (n=5) and No Shock groups (controls; n=4). For real time place preference (RTPP), all animals were exposed to shock (n=9). In the week preceding behavioural testing, handling was conducted frequently in 5-10min sessions. Three 10min habituation sessions in the apparatus were performed in the week prior to shock exposure. Animals were also acclimatized to the behavioural room 30min prior to testing.

3.4.5.1. Real Time Place Preference (RTPP)

Animals were exposed to electric footshock 15h30min prior to the behavioural test (20 1.5mA footshocks (1 shock every 30s) over the course of 10min) to induce AAV5-c-fos-ChR2-eYFP expression timed with the behavioural testing.

RTPP apparatus corresponded to a customized acrylic arena (60cm x 60cm x 40cm), comprised by two indistinguishable chambers tailored with a continual striped pattern and connected by a central opening.

RTPP was performed in agreement with the procedure used by Soares-Cunha et al. (2020), and mice could explore the apparatus freely for a period of 15min. One chamber (ON/Stimulation chamber) was paired with light stimulation, while the other was paired with no light stimulation (OFF/No Stimulation chamber). Mice started the test in the No Stimulation chamber and optical stimulation would be activated

immediately upon crossing into the Stimulation chamber. The time spent in each chamber was manually evaluated. The Stimulation chamber was counterbalanced across subjects. Data are presented as total time spent (s) in each chamber.

3.4.5.2. Conditioned Place Preference (CPP)

Mice were subjected to electric footshock 15h30min prior to the conditioning sessions (20 foot shocks at 1.5mA (1 shock every 30s) across 10min), and controls were left to explore the apparatus for 10min. This was done to time the AAV5-c-fos-ChR2-eYFP expression induction.

CPP was executed in a three compartment apparatus, with two main chambers ("white" and "black") divided by a neutral area (Med Associates Inc., St. Albans, VT, USA). The two side chambers had distinct designs on both the flooring and walls.

CPP was executed in agreement with (Coimbra et al., 2017), comprising three separate stages across three days. On day 1, a pre-test was carried out, in which the animals were free to move in the apparatus for 15min, with no stimulation. Shock exposure was performed later on the same day. On day 2, two 30min conditioning sessions were performed, in which animals were confined to a specific chamber (session 1 was in the ON/Stimulation chamber, while session 2 was in the OFF/No Stimulation). The chamber designated as stimulation-paired was randomly assigned and counterbalanced throughout animals. On day 3 (post-test), mice were again allowed to freely explore the apparatus for 15min and the time spent on each chamber was evaluated by an automated photo-beam system (Med Associates Inc., St. Albans, VT, USA). Subjects were placed in the neutral area, with the doors to the main chambers being opened at the start of the two test sessions. Results were presented in two ratios: ratio 1 – difference between the time spent in the Stimulation chamber in post-test day and the time in the same chamber on pre-test day; ratio 2 – difference between the time spent in the Stimulation chamber in post-test day and the time spent in the No Stimulation chamber in post-test day.

3.4.5.3. Optical stimulation

Optogenetic stimulation was performed using blue light, at 20Hz, with 60 5ms pulses of light being delivered every 5s for the conditioned place preference, and for all the period remaining in the Stimulation side in the RTPP.

Blue light was produced by a 473nm DPSS laser (CNI Laser, Changchun, China) and supplied to the brain via a fiber optic patch cord (0.22 NA, 200μm diameter; Thorlabs, Newton, NJ, USA), which was connected to the implanted ferrule during the test. Laser output was regulated by a Master-8 pulse generator (A.M.P.I., MN, USA).

3.5. Histological procedures

3.5.1. Sacrifice and brain sectioning

Mice were deeply anesthetized by a mixture of ketamine/medetomidine 90min/8h/16h after stimulus exposure. Animals were then transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde (PFA) solution. Brains were carefully removed and immersed for 48h in 4% PFA for fixation and then rinsed and stored in 30% of sucrose at 4°C until sectioning. Sectioning was performed coronally, in 40μm slices, on a vibrating microtome (VT1000S, Leica, Germany) and slices were stored at 4°C on 12-well plates (or long-term storage in cryoprotectant solution at -20°C) until use. Slices from the areas of interest (NAc and Amygdala) were selected using the Mouse Brain Atlas (Paxinos & Franklin, 2001).

3.5.2. Immunofluorescence (IF) for c-fos and GFP detection

Below follows the general immunofluorescence procedure, followed by the specific antibodies and incubation conditions utilized for each particular case. Specific antibodies and incubation conditions are detailed in **Table 2**.

Brain slices were washed first with phosphate buffered saline (PBS; 1X), and then with PBS/Triton-X100 (0.3%) (PBS-T). An antigen retrieval step was performed using heated citrate buffer (1X), to uncover epitopes and disrupt potential protein cross-links. Blocking, to avoid unspecific binding, was executed for 30min using 10% fetal bovine serum (FBS; Invitrogen, MA, USA) in PBS-T at room temperature (RT). Primary antibody incubation was performed overnight, followed by PBS-T washes and subsequent incubation with the appropriate secondary fluorescent antibody. All antibodies were diluted in PBS-T with 2% FBS. Slices were washed with PBS-T, incubated with DAPI (4',6-diamidino-2-phenylindole; 1:1000), washed with PBS (1X) and mounted using Permafluor (mounting media; Invitrogen, MA, USA). Slides were stored at 4°C and kept protected from light.

Table 2 – Antibody information for all immunofluorescence (IF) protocols used.

IF	Species	Binding	Concentration	Incubation Conditions	Note
c-fos	rabbit	anti-c-fos	1:1000	overnight, RT	AB_2314042 *
c-fos secondary	goat	anti-rabbit	1:1000	2h, RT	Alexa Fluor® 488 **
GFP	goat	anti-GFP	1:500	overnight, 4°C	ab6673 ***
GFP secondary	donkey	anti-goat	1:500	2h, RT	Alexa Fluor® 488

** Merck Millipore, Burlington, MA, USA ** Invitrogen, Carlsbad, CA, USA *** Abcam, Cambridge, UK*

3.6. Image acquisition and analysis

Images were collected and analysed by inverted fluorescence microscopy (Olympus Widefield Inverted Microscope IX81). About 5-10 slices for each animal were used for each analysis. Slices were classified in terms of stereotaxic coordinates using the Mouse Brain Atlas (Paxinos & Franklin, 2001) and the evaluated regions (*i.e.* the BLA, CeA and the main NAc subregions, core and shell) were drawn and measured in terms of area (mm^2) using Fiji (ImageJ) software (Schindelin et al., 2012).

Quantification of neuronal activation (c-fos^+ cell density) was performed using the Cell Counter plugin in the Fiji (ImageJ) software (Schindelin et al., 2012). Cell densities were presented as cells per mm^2.

In densitometry analysis, images (green fluorescent protein – GFP - staining) were converted to greyscale. Fluorescence levels, serving as a vector expression measure, were obtained as the mean grey value (average grey value of pixels within the selection). Fluorescence area (percentage of labelled area within a given selection, obtained by defining a pixel intensity threshold for the background) was also evaluated. This assessment was fully executed in the Fiji (ImageJ) software (Schindelin et al., 2012).

Optic fiber placement was assessed in the animals subjected to optogenetic manipulation to confirm if light stimulation was accurately conducted in the intended BLA region. For that, slices where the optic fiber was detected were classified according to Paxinos & Franklin (2001) to estimate the stereotaxic coordinates.

3.7. Statistical analysis

Normality was assessed in all data analysed by using the Shapiro–Wilk test and outliers were removed when applicable (Grubbs' test; a standard test in univariate data sets) (data not shown). If normality assumptions were met, unpaired t-tests were utilized in c-fos^{+} cell density and densitometry data sets. Otherwise, the non-parametric Mann–Whitney U test was performed. For RTPP analysis, a paired t-test was conducted, while for CPP assessments, unpaired t-tests were applied for ratio comparisons. All data analyses are presented as mean ± standard error of mean (SEM). Result differences were deemed statistically significant when $p \leq 0.05$. Statistical analysis was performed utilizing GraphpPad Prism (v8.0.2; La Jolla, CA, USA).

CHAPTER 4 – Results

4. Results

The amygdala subnuclei, the BLA and the CeA, are known to respond to various valence-specific stimuli, and while there is evidence that NAc also encodes valence, it remains unclear which neuronal populations are involved (Carlezon & Thomas, 2009; Gore et al., 2015a; Roitman et al., 2005; Steinberg et al., 2020). Thus, in order to confirm neuronal activation pattern after specific valence stimuli, and establish a temporal strategy to manipulate the activity of such neurons in the NAc (and amygdala as a positive experimental control), we used endogenous c-fos labelling (*c-fos* is an immediate early gene and marker of neuronal activity; (Krukoff, 1999)) and also tested a c-fos-based viral tracing approach (described in (Ye et al., 2016)).

4.1. Recruitment of NAc and amygdala ensembles upon exposure to positive *vs* negative valence stimuli

In order to assess if NAc and amygdala represent positive and negative valence stimuli, wild-type C57BL/6J mice were exposed to an aversive stimulus, that consisted of electric footshocks (Shock group); or to a positive stimulus, which consisted of a cocaine injection (Cocaine group) (**Figure 6a**). Control groups consisted in a No Shock group, in which animals explored the apparatus without receiving footshock, and a Vehicle group, which received an I.P. injection of saline (vehicle for cocaine). Mice were sacrificed 90min post-stimulus exposure for assessment of endogenous c-fos expression (Krukoff, 1999) by immunofluorescence.

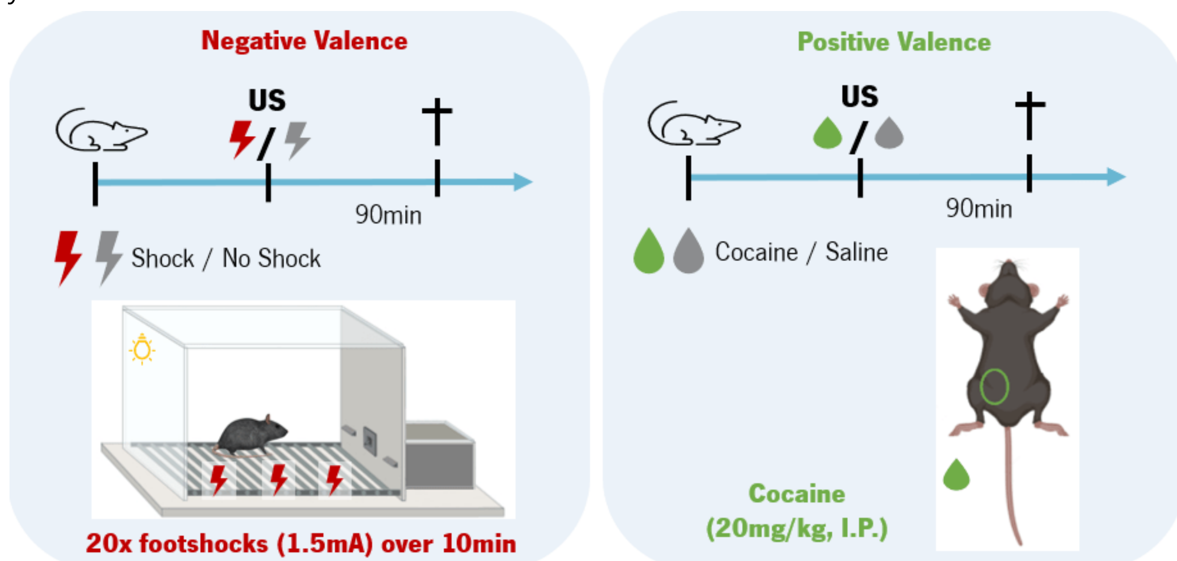


Figure 6 – Exposure to positive or negative valence stimulus for identification of responsive neuronal populations. a) Mice were exposed to 20 1.5mA footshocks over 10min (Shock group) or injected intraperitoneally (I.P.) with cocaine (20mg/kg) (Cocaine group). Controls were left to explore the chambers for 10min (No Shock group) or injected with saline via I.P. (Saline group). Sacrifice was performed 90min post-stimulus exposure. US – unconditioned stimulus.

We then calculated c-fos cell density in the NAc. The amygdala was used a positive control, as other studies have analysed this region in response to valence-related stimuli (Gore et al., 2015a; Kim et al., 2016; Namburi et al., 2015). As seen by the c-fos$^+$ cell density, footshock significantly activated the NAc core (101.9±10.95cells mm^{-2}), when compared to the No Shock group (58.20±12.33cells mm^{-2}) (**Figure 7c**; t (12) = 2.511, p = 0.0274). In the NAc shell, there were no significant differences in neuronal activation between Shock (112±9.382cells mm^{-2}) and No Shock (89.80±12.74cells mm^{-2}) groups (**Figure 7d**; t (12) = 1.408, p = 0.1844).

Regarding neuronal activation after exposure to the positive stimulus, no significant effects were found in both the NAc core (**Figure 8c**; t (11) = 1.284, p = 0.2255; Cocaine: 76.88±8.991cells mm^{-2}; Vehicle: 61.00±5.683cells mm^{-2}) or the NAc shell (**Figure 8d**; t (11) = 0.8649, p = 0.4056; Cocaine: 91.75±8.402cells mm^{-2}; Saline: 81.20±7.358cells mm^{-2}).

Similarly to the NAc core, the cell counts in the BLA showed a significant increase in c-fos$^+$ cell density in the Shock group (81.89±5.277cells mm^{-2}), comparing with the No Shock group (51.20±7.151cells mm^{-2}) (**Figure 9c**; t (12) = 3.464, p = 0.0047). In the CeA, only a tendency for increased c-fos$^+$ density was observed between the Shock (34.89±4.872cells mm^{-2}) and No Shock (20.40±4.226cells mm^{-2}) groups (**Figure 9d**; t (12) = 1.980, p = 0.0711). Concerning BLA c-fos$^+$ cell density caused by cocaine injection, no differences were observed (**Figure 10c**; t (11) = 1.339, p = 0.2076; Cocaine: 75.13±7.579cells mm^{-2}; Saline: 60.40±6.623cells mm^{-2}). Similarly, no major changes were noted in CeA neuronal activation when comparing the two groups relative to the positive stimulus (**Figure 10d**; t (11) = 0.6002, p = 0.5605; Cocaine: 28.25±4.296cells mm^{-2}; Saline: 23.80±6.430cells mm^{-2}).

These data show that endogenous c-fos$^+$ cell density was significantly higher in the NAc core and BLA in response to footshock, indicating that, in these brain regions, there are neurons that are activated by negative valence stimulus.

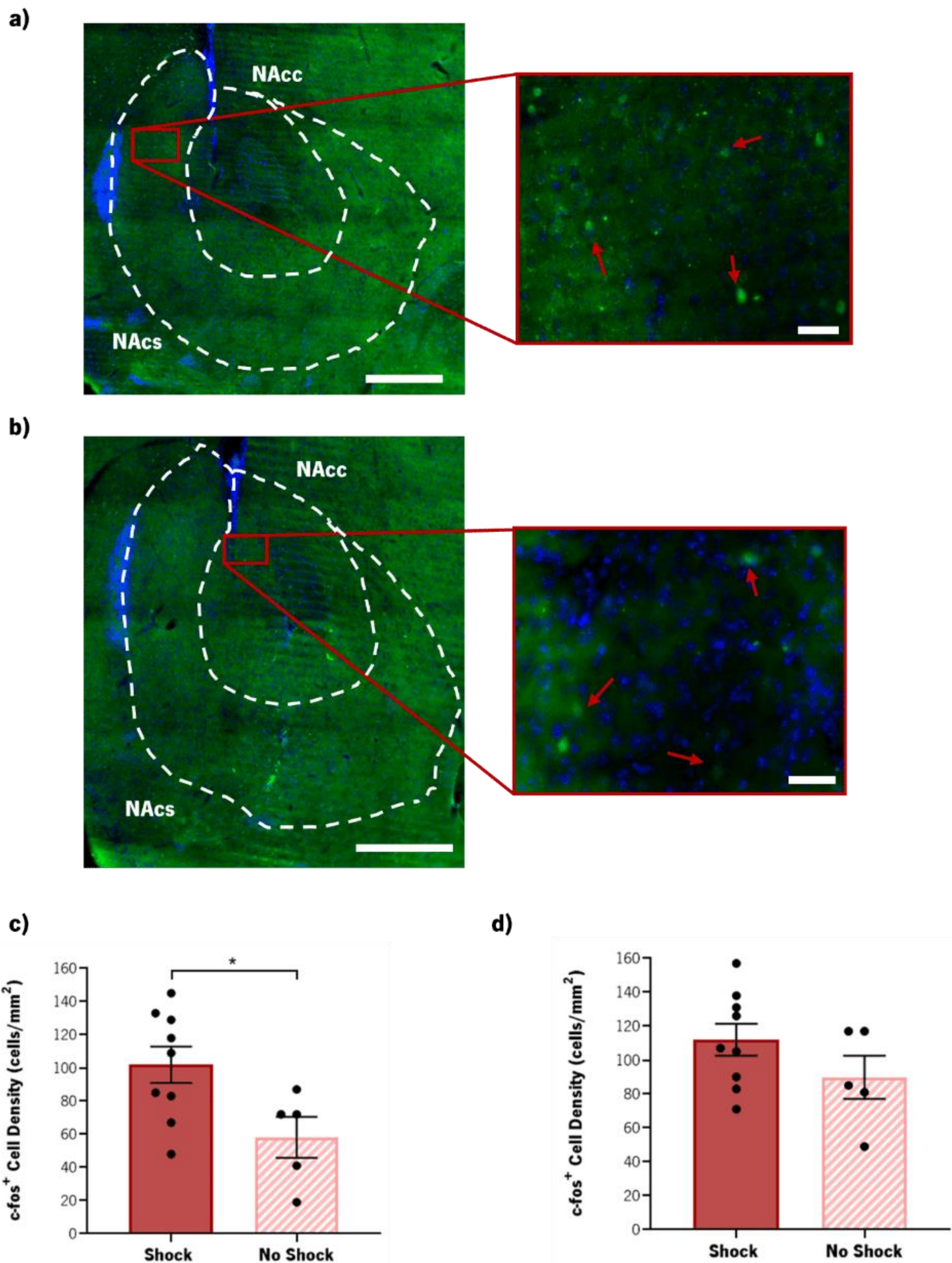


Figure 7 – Identification of neuronal populations responsive to a negative valence stimulus – endogenous c-fos+ cell density in the NAc. Representative GFP immunofluorescence images showing endogenous c-fos expression in the NAc of a **a)** Shock- and a **b)** No Shock-exposed animal (20x - left; 40x - right). Arrows point to a few examples of c-fos$^+$ cells. Scale bar 400µm (left), 40µm (right). **c)** c-fos$^+$ cell density (cells mm^{-2}) in the NAc core 90min post-footshock exposure (Shock, n=9; No Shock, n=5). **d)** c-fos$^+$ cell density (cells mm^{-2}) in the NAc shell 90min post-footshock exposure (Shock, n=9; No Shock, n=5). *$p \leq 0.05$. Data are represented as mean ± SEM.

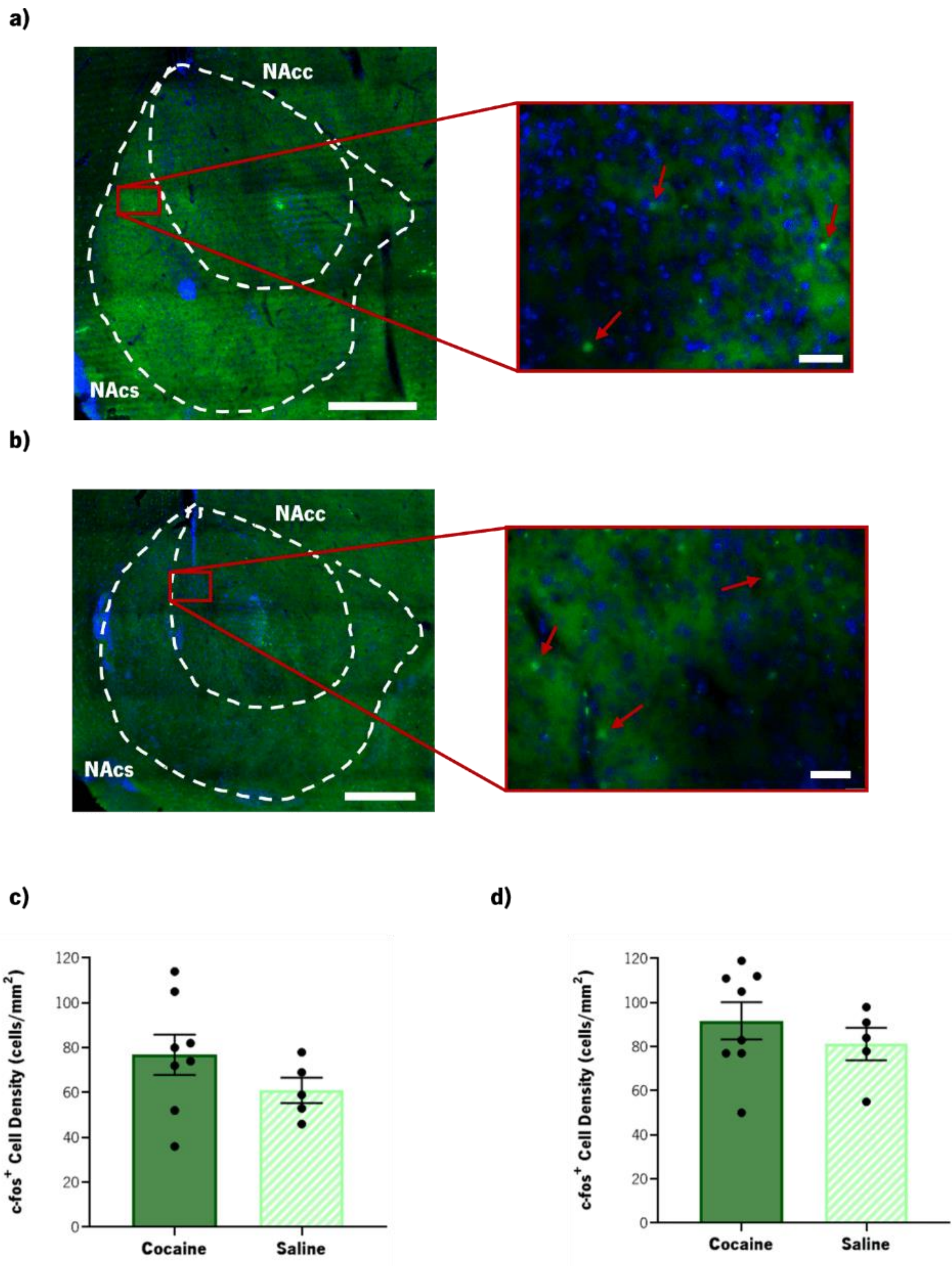


Figure 8 – Identification of neuronal populations responsive to positive valence stimuli – endogenous c-fos+ cell density in the NAc. Representative GFP immunofluorescence images showing endogenous c-fos expression in the BLA and CeA of a **a)** Cocaine- and a **b)** Saline-exposed mouse (20x - left; 40x - right). Arrows point to a few examples of c-fos+ cells. Scale bar 400µm (left), 40µm (right). **c)** c-fos+ cell density (cells mm-2) in the NAc core 90min post-cocaine injection (Cocaine, n=8; Saline, n=5). **d)** c-fos+ cell density (cells mm-2) in the NAc shell 90min post-cocaine injection (Cocaine, n=8; Saline, n=5). Data are represented as mean ± SEM.

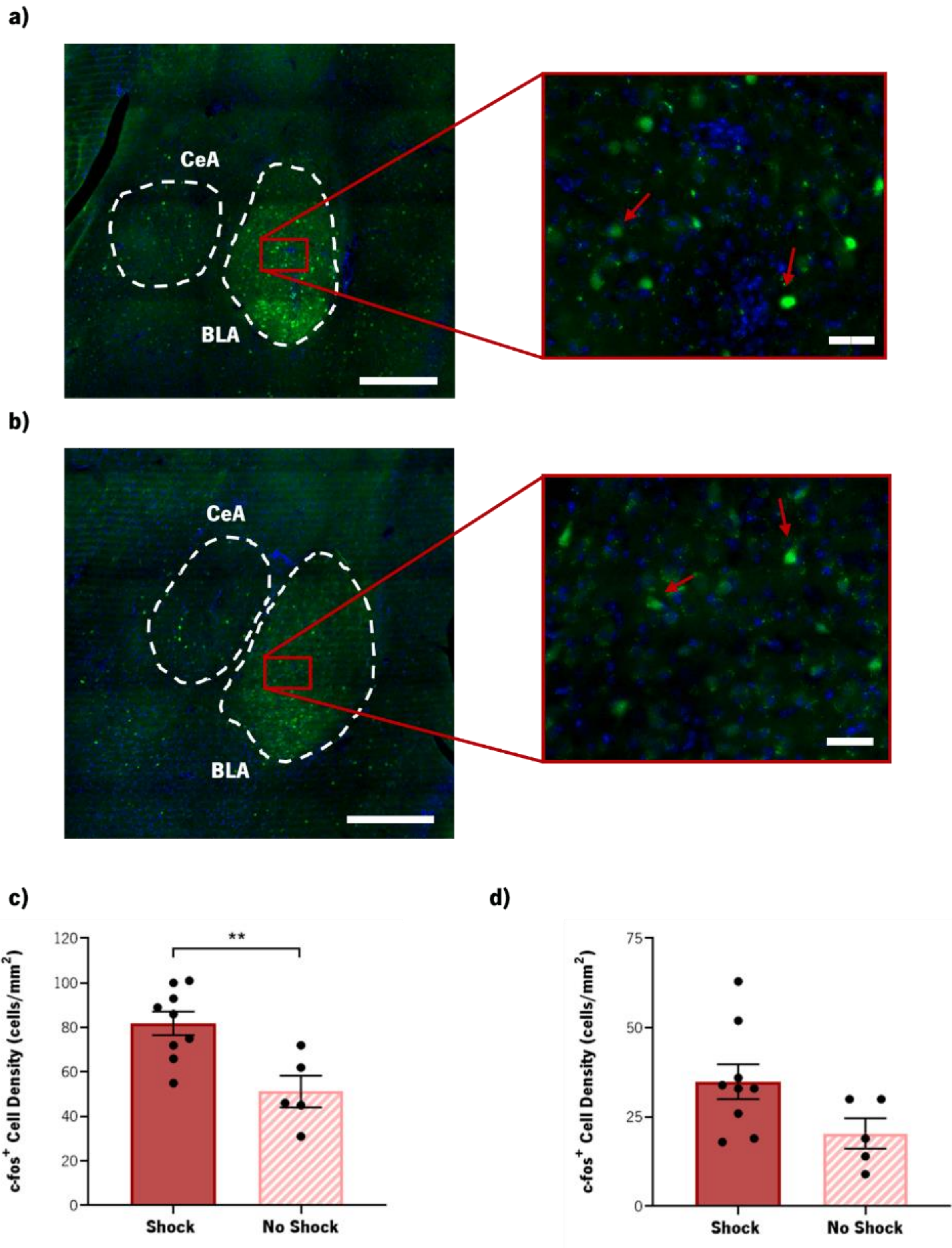


Figure 9 – Identification of neuronal populations responsive to negative valence stimuli – endogenous c-fos+ cell density in the Amygdala. Representative GFP immunofluorescence images showing endogenous c-fos expression in the BLA and CeA of a **a)** Shock- and a **b)** No Shock-exposed mouse (20x - left; 40x - right). Arrows point to a few examples of c-fos+ cells. Scale bar 400µm (left), 40µm (right). **c)** c-fos+ cell density (cells mm^{-2}) in the BLA 90min post-footshock exposure (Shock, n=9; No Shock, n=5). **d)** c-fos+ cell density (cells mm^{-2}) in the CeA 90min post-footshock exposure (Shock, n=9; No Shock, n=5). $^{**}p \leq 0.001$. Data are represented as mean ± SEM.

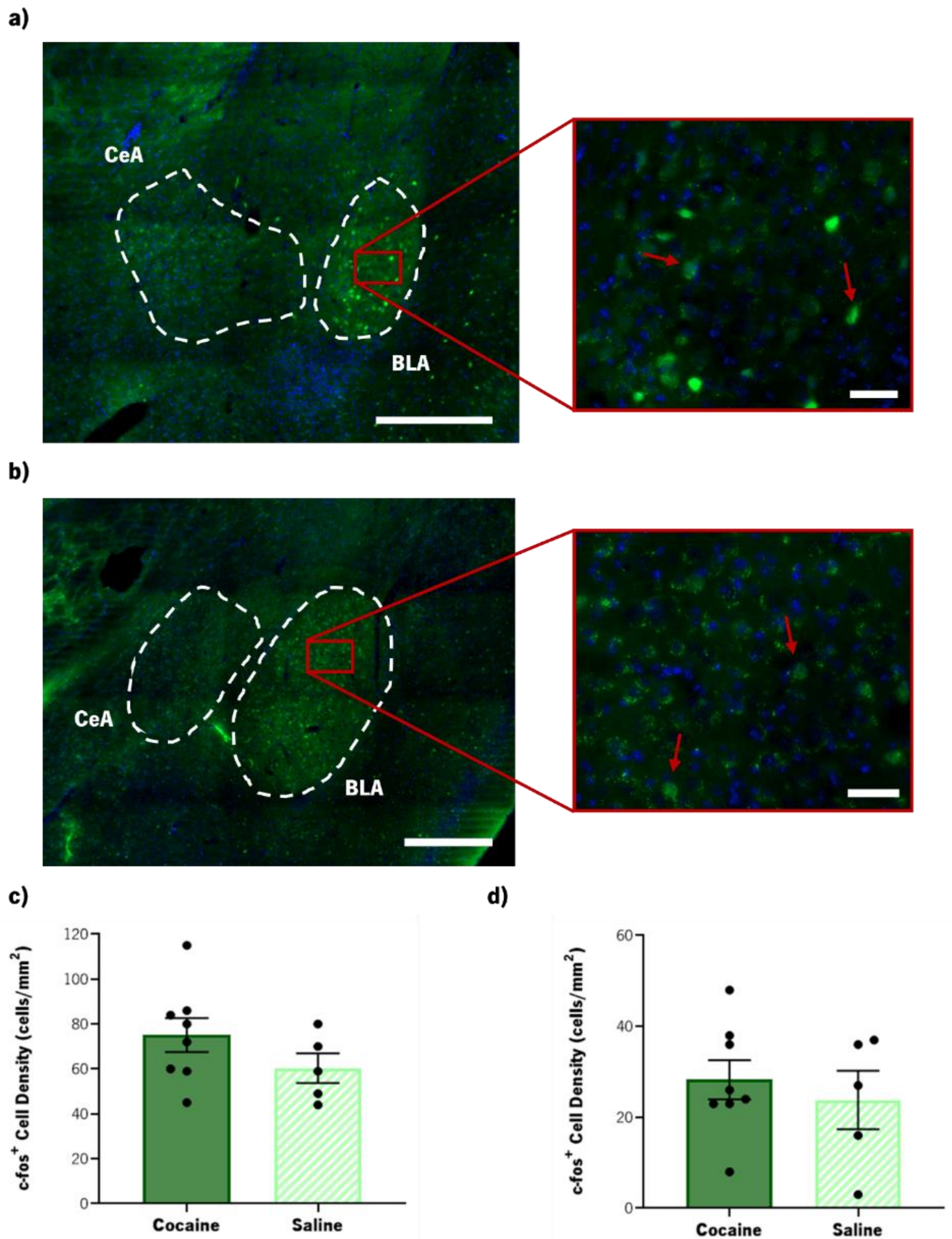


Figure 10 – Identification of neuronal populations responsive to positive valence stimuli – endogenous c-fos+ cell density in the Amygdala. Representative GFP immunofluorescence images showing endogenous c-fos expression in the BLA and CeA of a **a)** Cocaine- and a **b)** Saline-exposed mouse (20x - left; 40x - right). Arrows point to a few examples of c-fos^{+} cells. Scale bar 400µm (left), 40µm (right). **c)** c-fos^{+} cell density (cells mm^{-2}) in the BLA 90min post-cocaine injection (Cocaine, n=8; Saline, n=5). **d)** c-fos^{+} cell density (cells mm^{-2}) in the CeA 90min post-cocaine injection (Cocaine, n=8; Saline, n=5). Data are represented as mean ± SEM.

4.2. Temporal characterization of a c-fos-driven viral vector

In order to label neurons previously responsive to positive or negative valence stimuli (c-fos-expressing neurons) we used a viral vector that contains channelrhodopsin (ChR2) under the control of the c-fos promoter region (AAV5-c-fos-ChR2-eYFP vector) (**Figure 11a**), which has been previously reported in (Ye et al., 2016). This construct allows expression of ChR2 fused with a yellow fluorescent protein (eYFP), in neurons that are activated. It has a window of expression (*i.e.* labels neuronal activity) of ≈6h after c-fos activation and perdurance (*i.e.* allows observation and manipulation of labelled neurons) of up to one day (DeNardo & Luo, 2017). The virus AAV5-c-fos-eYFP (**Figure 11a**) which does not contain the ChR2 sequence was used as a control.

Wild-type C57BL/6J mice were injected with AAV5-c-fos-ChR2-eYFP bilaterally in the NAc and in the BLA (**Figure 11b**). Three weeks after surgery, animals were subjected to electric footshocks to allow c-fos-induced ChR2-eYFP expression in activated neurons (**Figure 11c**). To select an optimal timepoint for viral labelling and optical stimulation to be used in the remaining work, eYFP expression was assessed 8h and 16h after footshock exposure (**Figure 11**).

For both NAc and BLA, animals receiving footshock 8h prior to sacrifice (Shock 8h group) had very low levels of viral expression (**Figure 11d** and **11f**, respectively), while animals receiving footshock 16h prior to sacrifice (Shock 16h) had stronger viral expression (**Figure 11e** and **11g**, respectively; No Shock was used as the control groups - **Figure 11h** and **11i**). Thus, 16h timepoint was chosen for future experiments.

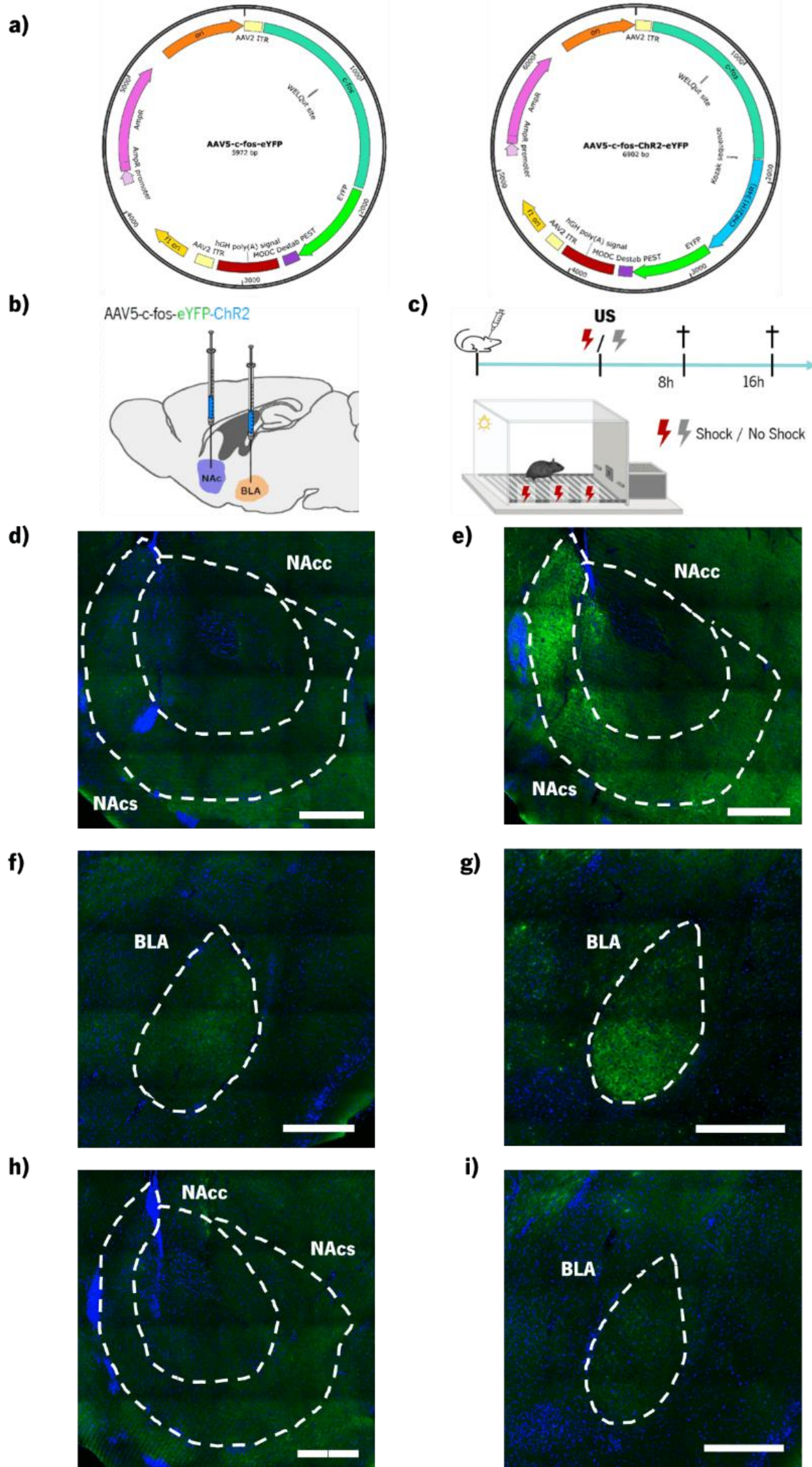

a)
AAV5-c-fos-eYFP
5972 bp
ori
AAV2 ITR
c-fos
WELQut site
EYFP
MODC Destab PEST
hGH poly(A) signal
f1 ori
AmpR
AmpR promoter
AAV5-c-fos-ChR2-eYFP
6902 bp
Kozak sequence
b)
AAV5-c-fos-eYFP-ChR2
NAc
BLA
c)
US
8h
16h
Shock / No Shock
d)
NAcc
NAcs
e)
NAcc
NAcs
f)
BLA
g)
BLA
h)
NAcc
NAcs
i)
BLA

Figure 11 – Experimental design for viral vector temporal characterization. a) Schematic representation of the plasmid vectors used in the experiment; AAV5-c-fos–eYFP (left); AAV5-c-fos-ChR2-eYFP (right). **b)** Stereotaxic surgeries for injection of the virally encapsulated construct pAAV5-c-fos-ChR2-eYFP were performed bilaterally in the NAc and in the BLA. **c)** Mice were exposed to 20 1.5mA foot shocks over 10min. Controls were left to explore the chambers for 10mim (No Shock group). Sacrifice was performed either 8h (Shock 8h group) or 16h (Shock 16h group); US – unconditioned stimulus. Representative GFP immunofluorescence images showing viral expression in the NAc in the **d)** Shock 8h and in the **e)** Shock 16h group (20x). Representative GFP immunofluorescence images showing viral expression in the BLA in the **f)** Shock 8h group and in the **g)** Shock 16h (20x). Representative GFP immunofluorescence images showing viral expression in the **h)** NAc in the No Shock group and in the **i)** BLA in the No Shock group (20x). Scale bars 400μm.

4.3. Transient labelling and identification of neuronal populations responsive to positive *vs* negative valence stimuli

Wild-type C57BL/6J mice were injected with the AAV5-c-fos-eYFP to allow eYFP expression in the NAc and BLA in response to either a positive or negative valence stimulus.

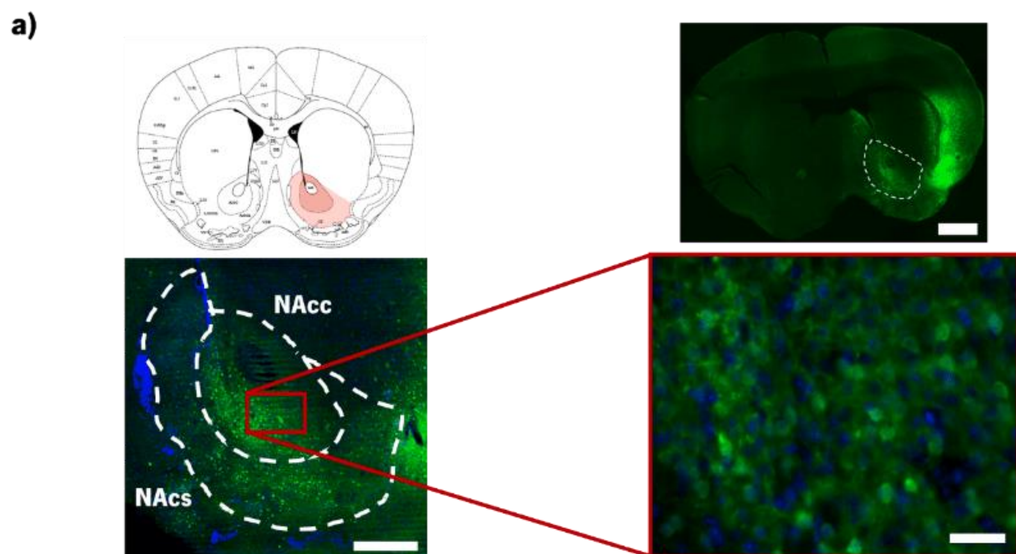
Four weeks after infection, animals were subjected to a footshock or cocaine injection as described before (16h timepoint). As an indicator of neuronal activation, we calculated fluorescence intensity of each brain region given in the form of the mean grey value (MGV), and the percentage (%) of the area presenting YFP expression (see methods for details about the calculation). Footshock exposure did not increase eYFP expression given by the MGV neither in the NAc core nor shell (NAc core – **Figure 12c**; $t(12) = 0.9221$; $p = 0.3746$; Shock: 31.31±2.296; No Shock: 27.68±3.314; NAc shell – **Figure 12d**; $t(12) = 0.8334$; $p = 0.4209$; Shock: 30.41±2.295, No Shock 27.19±3.137). In terms of area of viral expression, analysis of fluorescent area revealed no differences between Shock and No Shock animals in both NAc subregions (NAc core – **Figure 12e**; $t(11) = 1.463$, $p = 0.1715$; Shock: 65.15±3.769%; No Shock: 73.98±2.673%; NAc shell – **Figure 12f**; $t(11) = 0.6649$, $p = 0.5198$; Shock: 68.78±3.338%; No Shock: 63.74±8.134%).

Similarly, concerning cocaine administration, no differences were found in terms of fluorescence intensity for both NAc subregions (NAc core – **Figure 13c**; $t(11) = 0.3018$ $p = 0.7684$; Cocaine: 28.09±-2.976; Saline: 26.84±2.086; NAc shell – **Figure 13d**; $t(11) = 0.3744$; $p = 0.7153$; Cocaine: 25.14±2.818, Saline: 23.72±1.487. Regarding fluorescence area, no differences were observed due to the cocaine injection (NAc core – **Figure 13e**; $t(11) = 1.064$, $p = 0.3102$; Cocaine: 62.87±5.734%; Saline: 71.68±4.901%; NAc shell – **Figure 13f**; $t(11) = 0.5023$, $p = 0.6254$; Cocaine: 52.40±9.244%; Saline: 59.27±8.786%).

Regarding the BLA analysis, this brain region showed a tendency for increased activation by footshock, as reflected in the MGV data (**Figure 14c**; $t\,(11) = 1.913$, $p = 0.0821$; Shock: 46.15±1.884; No Shock: 36.31±5.901). No changes were obtained in terms of viral expression area (**Figure 14d**; $U = 18$, $p = 0.8329$; Shock: 75.45±6.465%; No Shock: 74.63±15.04%). Furthermore, no effects were found in fluorescence intensity involving cocaine exposure in this region (**Figure 15c**; $t\,(10) = 0.04114$; $p = 0.9680$; Cocaine: 35.61±4.929; Saline: 35.24±8.250). Similarly, viral expression area reflected no alterations due to cocaine administration (**Figure 15d**; $t\,(10) = 0.2899$, $p = 0.7778$; Cocaine: 63.05±11.45%; Saline: 69.00±17.97%). It should be noted that two animals were removed from BLA analysis due to the lack of viral expression in the sections, which likely indicates a problem with viral injection, as we expect to always see some degree of YFP expression.

In sum, endogenous c-fos (c-fos^{+} cell density) and densitometry fluorescence data shared similar findings, namely a similar tendency for higher neuronal activation in the BLA in response to footshock, but no significant changes in response to cocaine.

a)



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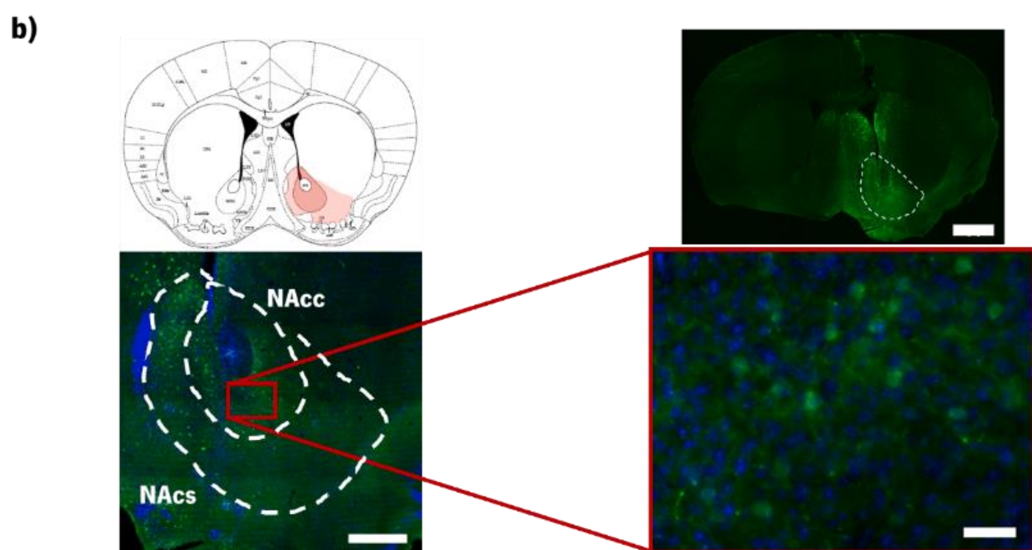
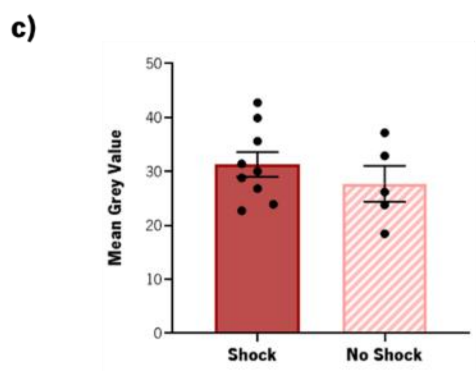
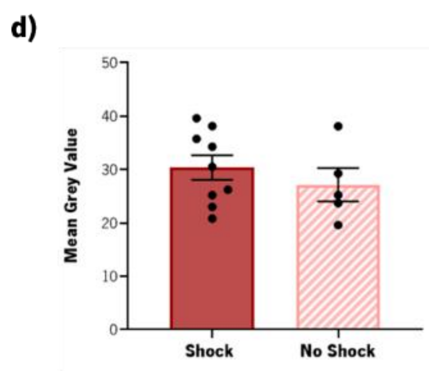


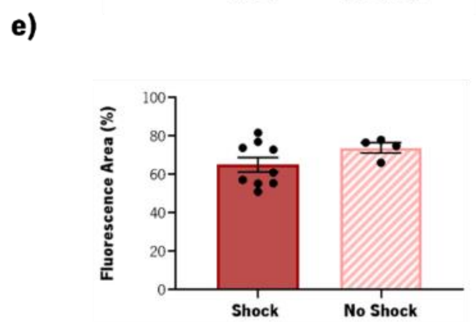
c)



d)



e)



f)

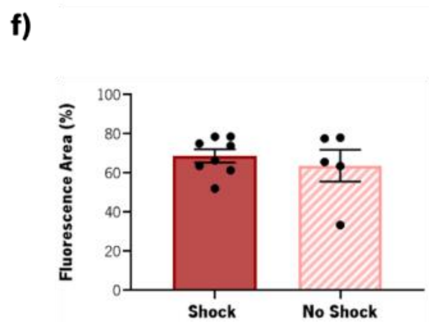
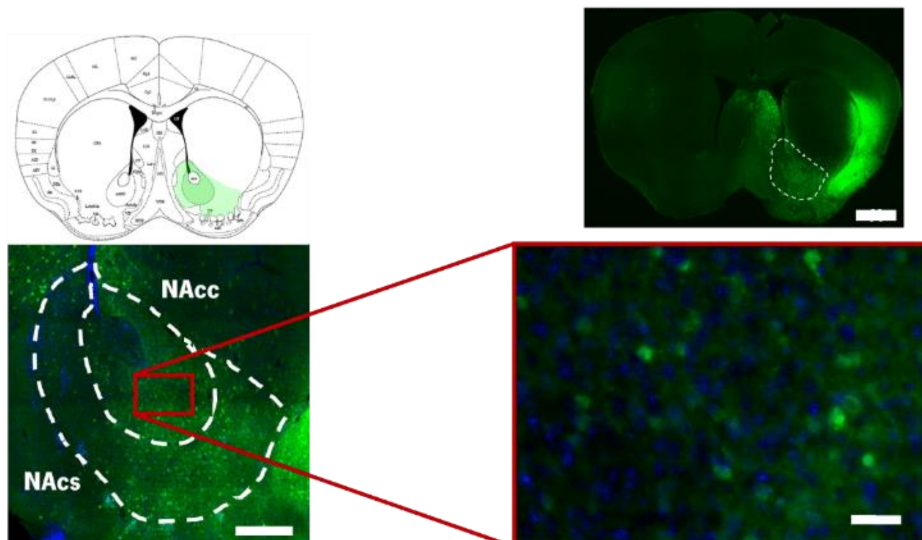


Figure 12 – Quantification of c-fos driven eYFP expression in the NAc in response to negative valence stimuli. Representative GFP immunofluorescence images showing viral expression in the NAc of a **a)** Shock- and a **b)** No Shock-exposed mouse (4x – top right; 20x – bottom left; 40x – bottom right) and respective brain atlas scheme (top left) (AP: 0.98mm; 0.86mm). Scale bar 400μm (bottom left), 40μm (bottom right) 1mm (top right). **c)** Immunofluorescence intensity (16h post-footshock exposure) measured by the mean grey value (MGV) within the defined NAc core (Shock, n=9; No Shock, n=5). **d)** Immunofluorescence intensity (16h post-footshock exposure) measured by the MGV within the defined NAc shell (Shock, n=9; No Shock, n=5). **e)** Area of immunofluorescence (16h post-footshock exposure) labelled within the defined NAc core (Shock, n=9; No Shock, n=4). **f)** Area of immunofluorescence (16h post-footshock exposure) labelled within the defined NAc shell (Shock, n=8; No Shock, n=5). Data are represented as mean ± SEM.

a)



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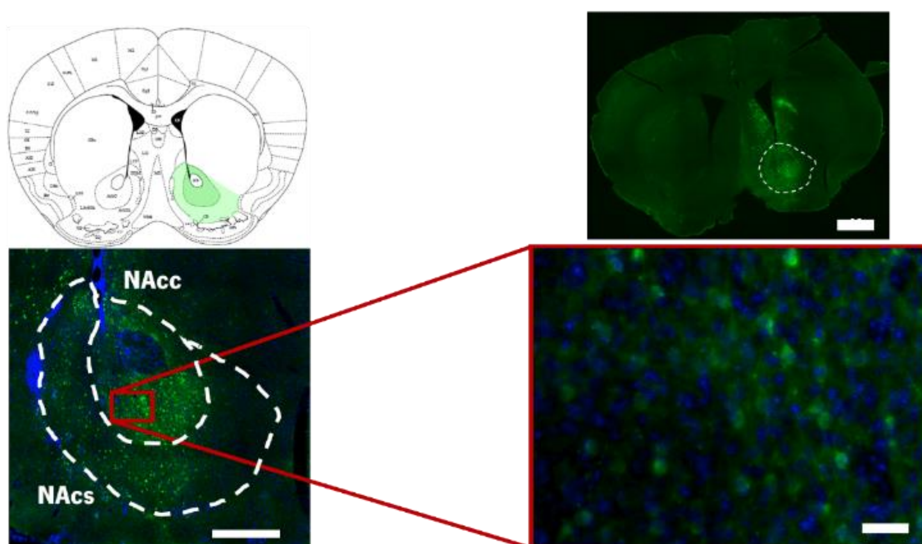

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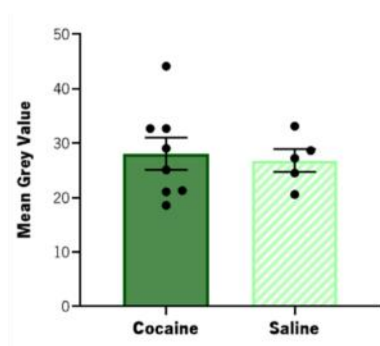

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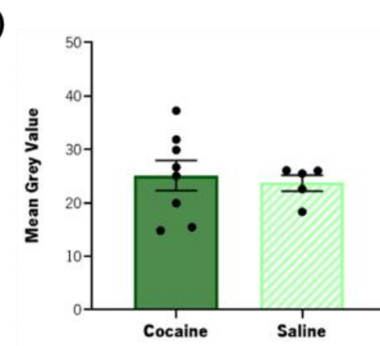

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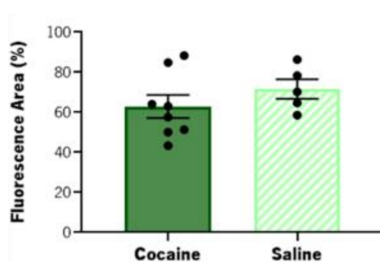


f)

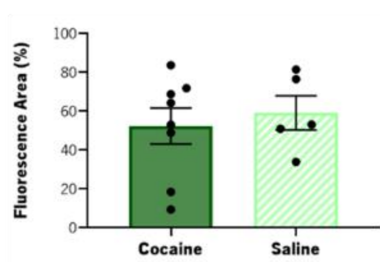


Figure 13 – Quantification of c-fos driven eYFP expression in the NAc in response to positive valence stimuli. Representative GFP immunofluorescence images showing viral expression in the NAc of a **a)** Cocaine- and a **b)** Saline-exposed mouse (4x – top right; 20x – bottom left; 40x – bottom right) and respective brain atlas scheme (top left) (AP: 0.86mm; 1.70mm). Scale bar 400μm (bottom left), 40μm (bottom right) 1mm (top right). **c)** Immunofluorescence intensity (16h post-cocaine injection) measured by the MGV within the defined NAc core (Cocaine, n=8; Saline, n=5). **d)** Immunofluorescence intensity (16h post-cocaine injection) measured by the MGV within the defined NAc shell (Cocaine, n=8; Saline, n=5). **e)** Area of immunofluorescence (16h post-cocaine injection) labelled within the defined NAc core (Cocaine, n=8; Saline, n=4). **f)** Area of immunofluorescence (16h post-cocaine injection) labelled within the defined NAc shell (Cocaine, n=8; Saline, n=5). Data are represented as mean ± SEM.

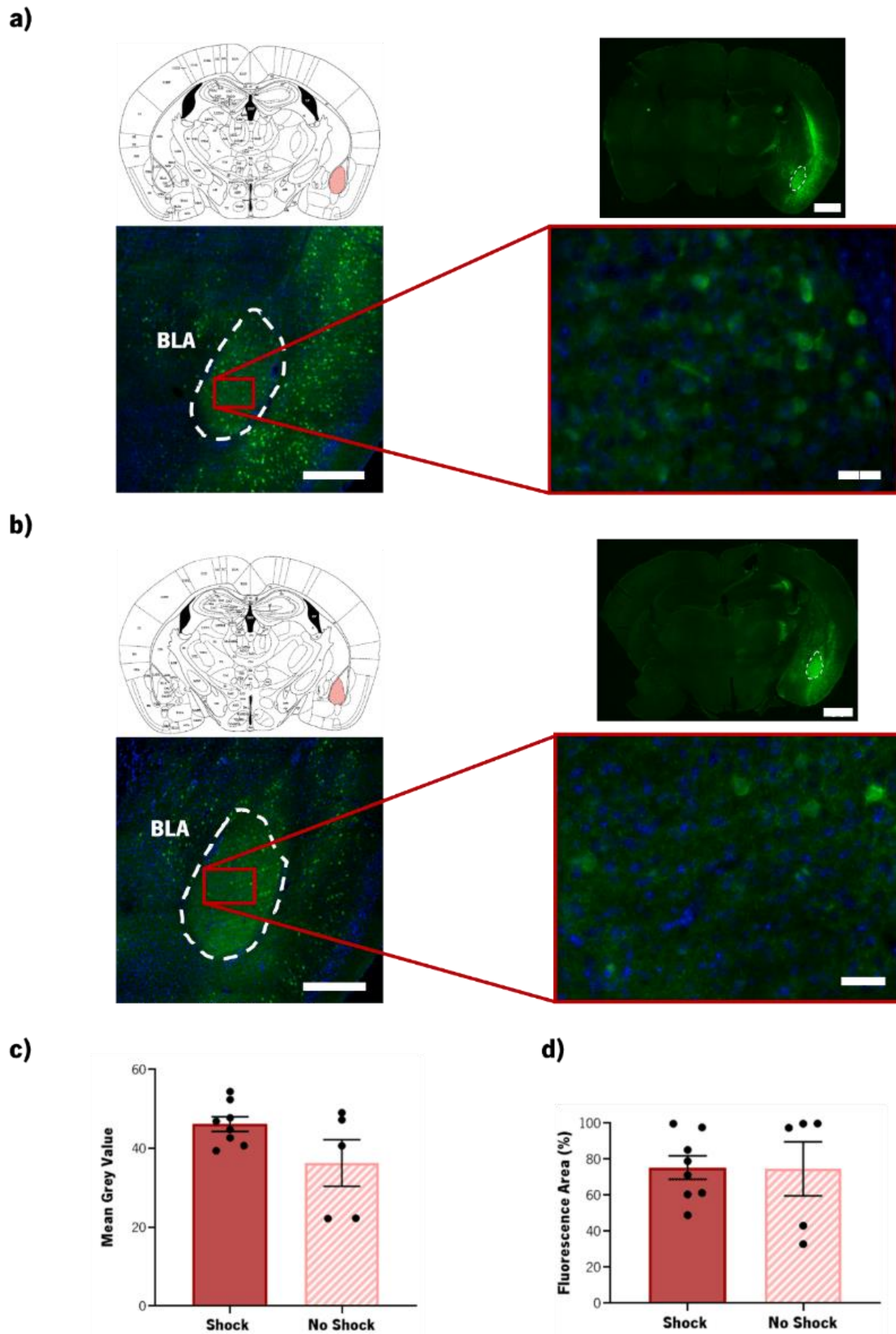


Figure 14 – Quantification of c-fos driven eYFP expression in the BLA in response to negative valence stimuli. Representative GFP immunofluorescence images showing viral expression in the BLA of a **a)** Shock- and a **b)** No Shock-exposed mouse (4x – top right; 20x – bottom left; 40x – bottom right) and respective brain atlas scheme (top left) (AP: -1.22mm; -1.34mm). Scale bar 400μm (bottom left), 40μm (bottom right) 1mm (top right). **c)** Immunofluorescence intensity (16h post-footshock exposure) measured by the MGV within the defined BLA (Shock, n=8; No Shock, n=5). **d)** Area of immunofluorescence (16h post-footshock exposure) labelled within the defined BLA (Shock, n=8; No Shock, n=5). Data are represented as mean ± SEM.

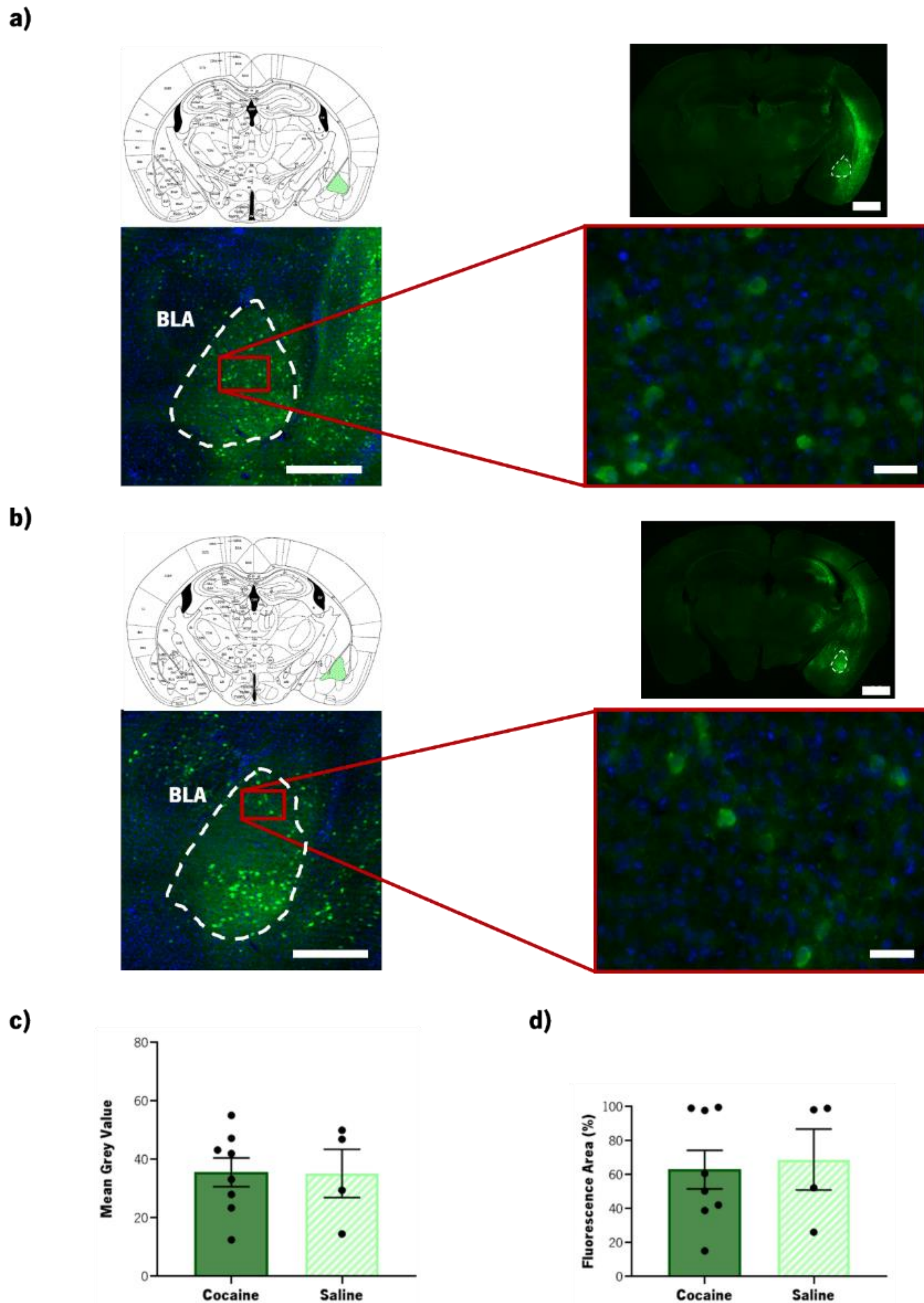


Figure 15 – Quantification of c-fos driven eYFP expression in the BLA in response to positive valence stimuli. Representative GFP immunofluorescence images showing viral expression in the BLA of a **a)** Cocaine- and a **b)** Saline-exposed mouse (4x – top right; 20x – bottom left; 40x – bottom right) and respective brain atlas scheme (top left) (AP: -1.70mm; -1.46mm). Scale bar 400µm (bottom left), 40µm (bottom right) 1mm (top right). **c)** Immunofluorescence intensity (16h post-cocaine injection) measured by the MGV within the defined BLA (Cocaine, n=8; Saline, n=4). **d)** Area of immunofluorescence (16h post-footshock exposure) labelled within the defined BLA (Cocaine, n=8; Saline, n=4). Data are represented as mean ± SEM.

4.4. Optogenetic manipulation of neurons responsive to negative valence stimuli

Studies have given various evidence for the contribution of BLA in encoding negative valence stimuli, including footshock (Gore et al., 2015a), as well as processing classical aversive behavioural responses, such as place avoidance (Gore et al., 2015a; Namburi et al., 2015; Redondo et al., 2014). Thus, in order to functionally validate the viral vector used in the previous sections, we designed an experiment that allows optical activation of negative-valence neurons in order to evaluate if it can trigger an aversive response in a neutral context. For this, wild-type C57BL/6J mice were injected with the AAV5-c-fos-ChR2-eYFP in the BLA, and implanted with an optical fiber that will allow posterior optogenetic activation (**Figure 16a**). First, mice were exposed to footshock to induce c-fos activation, and consequently ChR2-YFP expression. 15h30min after footshock exposure (to allow sufficient levels of ChR2-eYFP expression – optimized in 4.2 section) mice performed a RTPP test, in which one of the sides was paired with optical activation of negative valence neuronal ensembles (**Figure 16c**). This experimental design would allow to determine if re-activation of neurons previously responsive to footshock would result in aversive behaviour (avoidance towards the chamber paired with optical activation).

Our data show that optogenetic activation of footshock-responsive neurons induced place avoidance, as animals exhibited a tendency for spending more time in the No Stimulation chamber during the behavioural session (**Figure 16d**; $t(6) = 2.308$; $p = 0.0605$; Stimulation chamber: 368.6±35.29s, No Stimulation chamber: 531.4±35.29s). These results further validate the involvement of BLA neurons in processing negative valence stimuli, and also suggest that this construct can be used to study negative valence neurons in the amygdala. Unfortunately, and in contrast, this vector is not appropriate to study valence neurons in the NAc due to the absence of mensurable differences between control group and stimuli-exposed groups.

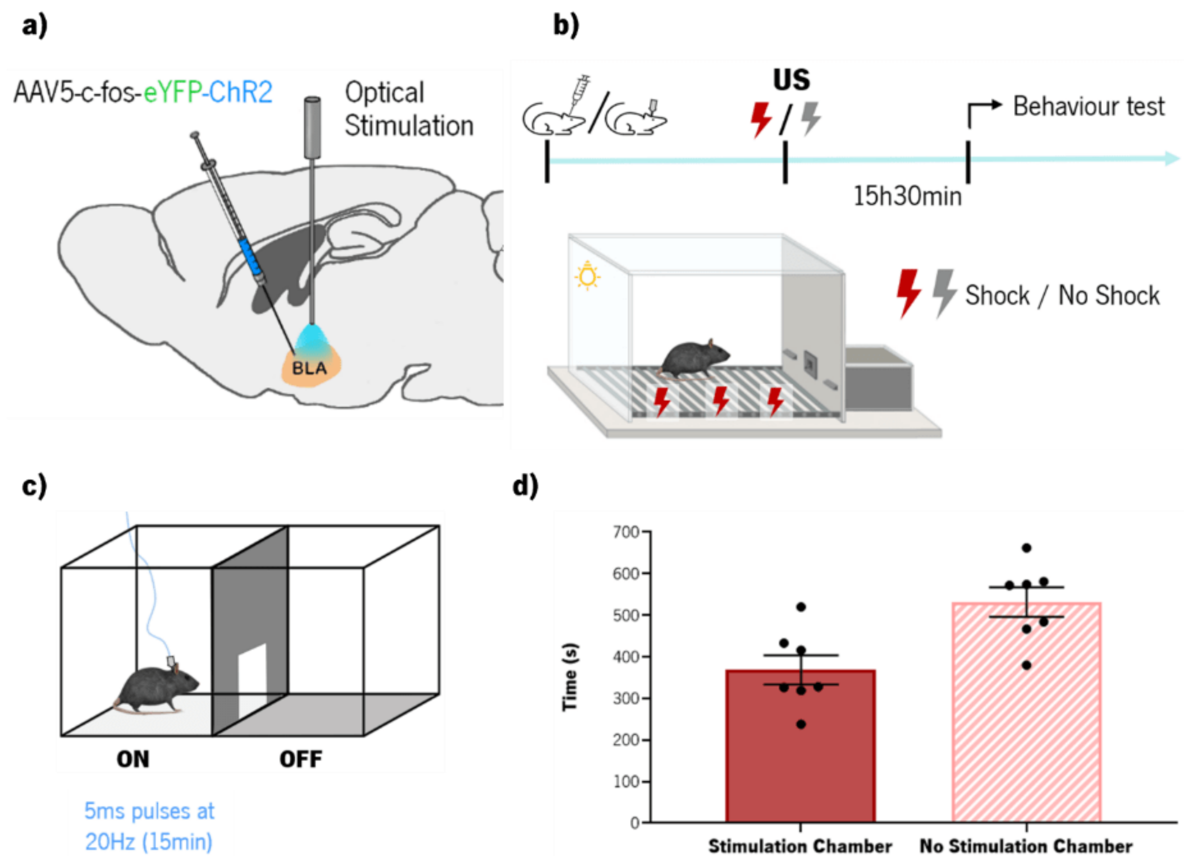


Figure 16 – Optogenetic manipulation of BLA neurons responsive to negative stimuli in the Real Time Place Preference. a) Stereotaxic surgeries for injection of the virally encapsulated construct pAAV5-c-fos-ChR2-eYFP and implantation of an optical fiber were performed in the BLA. **b)** Mice were exposed to 20 1.5mA footshocks over 10min (RTPP; CPP – Shock group) 15h30min prior to behavioural tests to induce viral expression in c-fos activated (footshock-responsive) neurons; US – unconditioned stimulus. **c)** Schematic representation of the Real Time Place Preference (RTPP) test with optogenetic stimulation protocol of 5ms pulses at 20Hz occurring in the Stimulation (ON) side of the chamber. **d)** Time spent in the Stimulation and No Stimulation sides of the chamber during the RTPP test period (n=7). Data are represented as mean ± SEM.

To further validate our construct, the CPP test (**Figure 17a**) was performed to verify if re-activation of footshock-responsive neurons would result in place avoidance. Our data show that optogenetic activation of the shock-activated population did not create any kind of conditioning effect, as there were no significant differences between Shock and Control groups regarding the difference in time spent in the Stimulation chamber in the post-test day in comparison with pre-test session (**Figure 17b**; t (6) = 1.520; p = 0.1794; Shock: 108.7±60.01; No Shock: -26.96±66.04). Furthermore, stimulated mice also showed no difference in the ratio of time spent in the Stimulation chamber in comparison with the No Stimulation chamber in the post-test session (**Figure 17c**; t (6) = 1.631; p = 0.1540; Shock: 308.0±89.17; No Shock: 96.14±94.46).

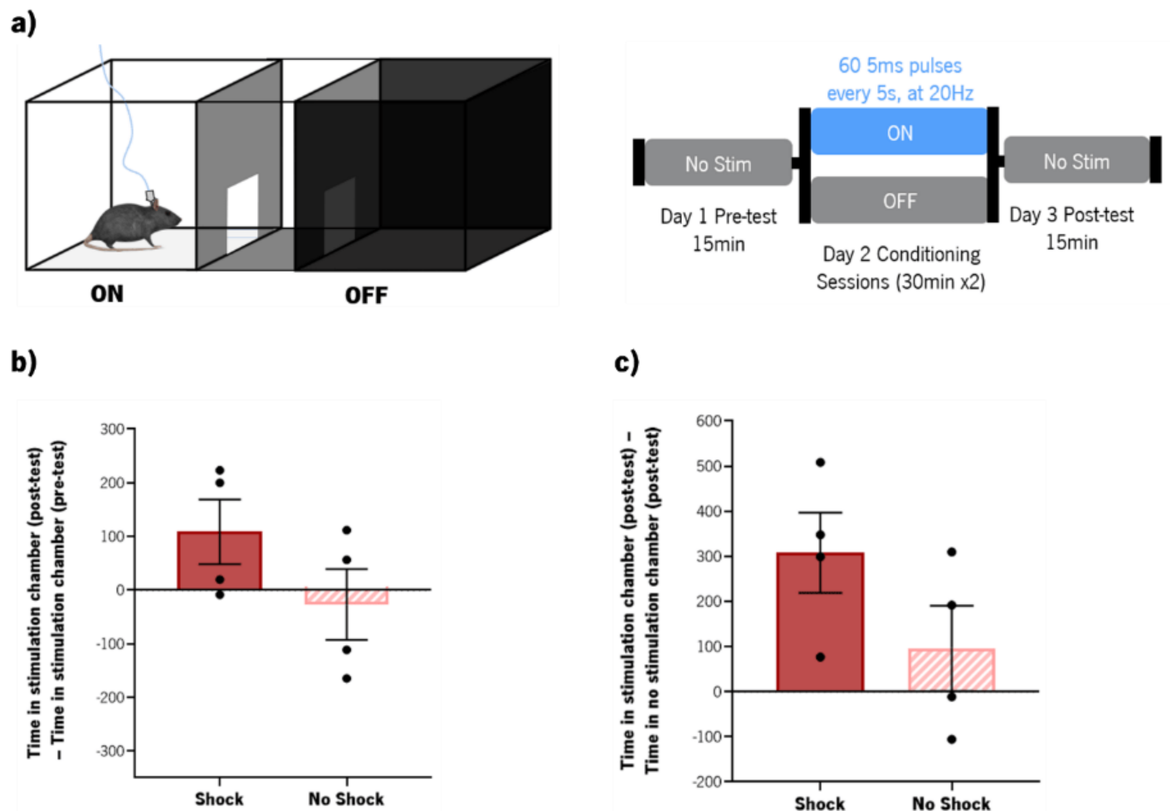
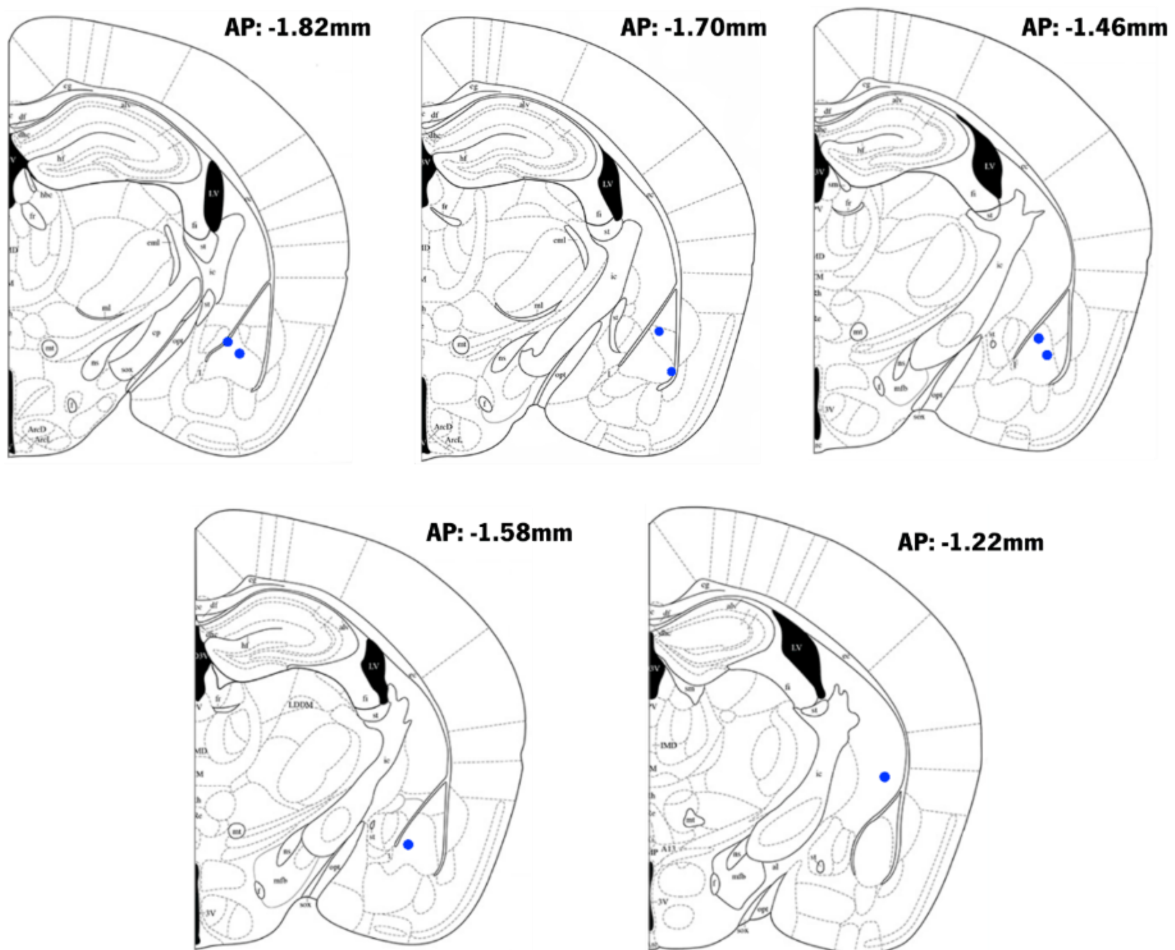


Figure 17 – Optogenetic manipulation of BLA neurons responsive to negative stimuli in the Conditioned Place Preference. a) Schematic representation of the Conditioned Place Preference (CPP) test with optogenetic stimulation protocol of 60 5ms pulses at 20Hz, every 5s, occurring in the Stimulation (ON) chamber during the respective 30min conditioning session (session 1, day 2). Pre- and post-test days lasted 15min. **b)** Ratio 1 – Difference between the time spent in the Stimulation chamber (post-test) and the time spent in the Stimulation chamber (pre-test) during the CPP test period (Shock, n=4; No Shock, n=4). **c)** Ratio 2 – Difference between the time spent in the Stimulation chamber (post-test) and the time spent in the No Stimulation chamber (post-test) during the CPP test period (Shock, n=4; No Shock, n=4).

These findings showed neither preference for nor avoidance of the chamber paired with optogenetic stimulation, since there were no differences in the calculated ratios, which accounted for change in time spent in the Stimulation chamber from the pre-test to the post-test (ratio 1) and the difference in time spent in the Stimulation and No Stimulation chamber in the post-test (ratio 2).

After behavioural assessment, to confirm if stimulation was performed in the correct region, we analysed the fiber placement for all the animals (according to (Paxinos & Franklin, 2001)). Implantation coordinates, for the most part allowing for light stimulation in the BLA, are represented in **Figure 18a**. In one animal, we could not see the fiber location in the sections (not shown), while another animal was excluded because it was shown to not be implanted near the amygdala (**Figure 18a**).



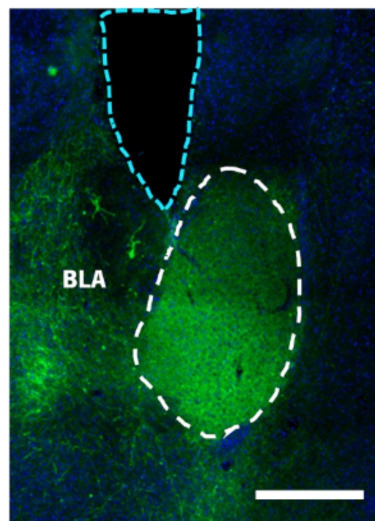


Figure 18 – Optogenetic manipulation of BLA neurons responsive to negative stimuli – histological analysis. a) Schematic representation of the optical fiber cannula implantation positioning in the BLA of the optogenetically stimulated mice (in blue). **b)** Representative GFP immunofluorescence images showing viral expression in the BLA and the optical fiber cannula implantation (in light blue) (20x). Scale bar 400μm.

CHAPTER 5 – Discussion, Conclusion and Future Perspectives

5.1. Discussion

Comprehending the neuronal mechanisms underlying reward and aversion can advance the knowledge on the pathophysiology of emotional disorders with deficits in these processes such as depression and addiction (Cooper et al., 2017; Dichter et al., 2012; Russo & Nestler, 2013). To accomplish that it is essential to define how positive and negative valence is encoded and processed in terms of neuronal populations, which may occur in a completely segregated or, to some extent, overlapped manner. How a specific valence is encoded may be related with, for example, inputs and outputs or distinct genetic profiles of the neurons involved, or even a combination of anatomical and genetic characteristics. Though several brain regions of the limbic system, including the nucleus accumbens (NAc) (Berridge & Kringelbach, 2013; Knowland & Lim, 2018; Namburi et al., 2016; Nieh et al., 2013; O'Neill et al., 2018), are known to encode valence, the type of characteristics that segregates positive from negative valence neurons remains far from being disclosed.

Recruitment of NAc and amygdala ensembles upon exposure to positive vs *negative valence stimuli*

NAc neurons are known to respond to both unconditioned and conditioned stimuli of positive and negative valence (Carlezon & Thomas, 2009). NAc neurons change their activity in response to primary aversive stimuli (*e.g.* quinine; air puffs) (Roitman et al., 2005; Yanagimoto & Maeda, 2003) and primary natural rewards, such as sucrose and saccharin (Roitman et al., 2005; Wheeler et al., 2005, 2008; Wilson & Bowman, 2004). They are also essential for processing appetitive stimuli delivered via self-administration paradigms, such as sucrose (Nicola et al., 2004a), food (Carelli, 2002; Carelli et al., 2000), water (Carelli et al., 2000), cocaine (Chang et al., 1998; Peoples et al., 1998; Peoples & West, 1996), heroin (Chang et al., 1997, 1998) and ethanol (Janak et al., 1999). Giving credit to comparative experiments with varied stimuli, neuronal processing is not equal across reward types ("natural" and "drugs of abuse") or even between specific rewards of similar nature (water *vs* cocaine, (Carelli & Deadwyler, 1994; Carelli & Wondolowski, 2003); water *vs* food *vs* cocaine, (Carelli, 2002; Carelli et al., 2000); cocaine *vs* heroin, (Chang et al., 1998); water *vs* sucrose, (Roop et al., 2002)).

Similarly, amygdala neurons encode a variety of appetitive and aversive stimuli, such as water and air-puffs, respectively (Belova et al., 2007). The BLA in particular, has populations responding to rewards like sucrose (Muramoto et al., 1993), water (Kim et al., 2016) and nicotine (Gore et al., 2015a) and negative stimuli like footshock (Gore et al., 2015a; Kim et al., 2016; Muramoto et al., 1993) and unpalatable tastes (Shabel et al., 2011). Likewise, the CeA has neuronal populations reacting to footshock

(negative) and sucrose (positive) (Steinberg et al., 2020). Due to the fact that different studies have shown that amygdala neurons respond to both positive and negative valence stimuli, we chose this region to be used as a *positive control* for the experiments presented in this thesis.

Tools to identify valence responsive neurons

Endogenous c-fos to quantify neuronal populations responsive to positive vs *negative valence stimuli*

A common basis in strategies used to understand if/how neuronal populations are involved in certain behaviours, or responsive to particular stimuli, is the evaluation of the expression of immediate early genes (IEG) (*i.e. c-fos*), which are expressed rapidly upon neuronal activity (Deubner et al., 2019). Labelling endogenous c-fos is typically used to assess neuronal activation on short-term analysis, as mRNA levels peak around 30-45min, with protein levels peaking around 1-2h (depending on specific brain region and conditions) (Bisler et al., 2002; Müller et al., 1984; Zangenehpour & Chaudhuri, 2002). Reaction to stimuli with a specific valence has thus been observed via c-fos expression levels in varied regions - either via mRNA levels, or protein, usually by c-fos$^+$ cell counts.

To identify neuronal populations encoding positive- and negative-valence stimuli we started by injecting mice with cocaine or exposing them to footshock, respectively, and then labelled endogenous c-fos to quantify neuronal activation in the NAc core and shell, as well as in the BLA and in CeA. Electric footshock was delivered at an intensity known to induce neuronal activation in the BLA, as well as generating negative-valence responses (Gore et al., 2015a). Cocaine was injected at a concentration previously shown to enhance motor activity and induce CPP, thus having a robust appetitive value (Catlow & Kirstein, 2005; Itzhak & Martin, 2002).

Our data showed that electric footshock appears to be encoded by neurons in the NAc core, since neuronal activation was higher in Shock animals in comparison to control animals, while the NAc shell did not present changes in the c-fos$^+$ positive cell density. A summary of our main results is presented in **Table 3**. This data is in agreement with previous literature, showing an overall increase in NAc c-fos-labelled activation for many aversive events, such as the case of formalin injection (Senba & Ueyama, 1997), acute forced swim stress (Cullinan et al., 1995), audiogenic stress (Campeau & Watson, 1997) and restraint stress (Cullinan et al., 1995; Senba & Ueyama, 1997). On the contrary, we saw no statistical difference in the neuronal activation in cocaine group in comparison to saline, for neither NAc subregion. This result was unexpected, considering that the NAc is known to be a central reward system region (Berridge & Kringelbach, 2015; Carlezon & Thomas, 2009; Russo & Nestler, 2013), and in particular

since it has been shown to exhibit an increase in NAc c-fos levels after exposure to both natural rewards (sucrose; (Koekkoek et al., 2021)) and drugs of abuse such as morphine (Liu et al., 1994), cocaine (Johansson et al., 1994) and amphetamine (Dalia & Wallace, 1995; Johansson et al., 1994), along with social novelty (Gómez-Gómez et al., 2019).

We performed similar analysis of the amygdala subnuclei - BLA and CeA, as a control strategy, expecting neuronal activation for both valence stimuli. c-fos$^+$ cell density was higher in the BLA following shock exposure. These results were anticipated, particularly considering the work of Gore et al. (2015a), who have reported an increase in BLA c-fos$^+$ neurons after footshock. No differences were found in the CeA after footshock exposure (although a tendency was present), which was somewhat surprising, considering this region's general involvement in aversive learning (Giovanniello et al., 2020) and previous descriptions of shock-responsive neurons projecting from the CeA to the *substantia nigra* (Steinberg et al., 2020). Regarding the cocaine group, no effect was found in neither the BLA nor the CeA, with no differences in neuronal activation between drug and vehicle (saline) injection. Both subnuclei have been observed to include neuronal populations responding to appetitive stimuli: Gore et al. (2015a), having demonstrated an increase in BLA c-fos-labelled neurons after nicotine exposure; and sucrose-responsive cells presented in the CeA (Steinberg et al., 2020).

While data in the NAc core and the BLA regions seemed in line with previous studies using aversive conditions, the data with cocaine was unanticipated. In this context, we can pinpoint some technical issues as potential explanation for the findings. First, it is important to consider that the number of animals is relatively low, which may hamper the identification of subtle neuronal activation differences between groups. Second, it is important to refer that animals were experimentally naïve prior to behavioural exposures, with handling in the week previous to testing and with only three 10min habituation sessions to the apparatus (for Shock and No Shock animals). It is plausible that increasing the time and frequency of the habituation to the apparatus and experimental rooms could reduce "unspecific" neuronal activation due to novelty (rather than to shock). Regarding positive valence stimulus experiment, we should have habituated animals to I.P. injections prior to the behavioural procedure (cocaine and saline groups) to minimize neuronal activation due to the injection (that is aversive) rather than cocaine *per se,* as this procedure is painful and can cause acute distress in the animals. We believe that these conditions would minimize unspecific neuronal activation levels, and lead to clearer results.

Apart from experimental conditions that could be improved, one should also consider some other explanations for the results. First, maybe NAc neurons do not encode cocaine positive valence as we assumed it would, though this is unlikely considering the electrophysiological data supporting changes in

neuronal firing within the NAc in response to reward consumption, and in particular cocaine (Carlezon & Thomas, 2009; Chang et al., 1997; Peoples & West, 1996; Roitman et al., 2005; Wheeler et al., 2005, 2008; Wilson & Bowman, 2004), as well calcium imaging data supporting an increase of NAc activity in response to cocaine-associated cues (Calipari et al., 2016). Second, one could hypothesize that c-fos activation is not the fittest marker to evaluate valence recruitment in the NAc, and that other IEGs could be more sensitive – such as *Arc* or *Zif268* (Choi et al., 2020; Gore et al., 2015b; Kovács, 2008).

Temporal characterization of a c-fos-driven viral vector to label neuronal populations responsive to positive vs *negative valence stimuli*

Additional conventional c-fos-dependent techniques incorporate the expression of specific genes associated with IEG gene promoters (Deubner et al., 2019). A construct can be encapsulated in a viral vector and injected in brain regions of interest to locally express (in an activity-reliant manner), for instance, fluorescent labels for neuronal tracing (*e.g.* eYFP) (Deubner et al., 2019), or extrinsic ion channels to induce neuronal activation (*e.g.* ChR2) (Ye et al., 2016). For our approach, we selected a similar strategy, using a c-fos-dependent viral vector to assess neuronal activation after exposure to electric footshock (negative valence) and cocaine administration (positive valence). The viral approach was previously used to analyse the molecular properties of neurons in the PFC that are associated with distinct behavioural experiences (Ye et al., 2016).

We first started by validating this construct, regarding optimal timeframe and the ability to transiently label NAc and amygdala neurons activated by specific-valence stimuli (similarly to endogenous c-fos). By using both endogenous c-fos and c-fos-driven viral expression of a construct, two stimuli of appetitive and aversive valence could be combined, and responsive populations could be thus compared in terms of overlap and segregation and anatomical distribution, amongst other factors. A comparable approach (with lentivirus) has been used successfully in the BLA by Gore et al. (2015a), demonstrating anatomically distinct yet marginally overlapped populations responding to negative- and positive-valence stimuli.

We first characterized the vector in terms of temporal expression levels, and selected 16h post-stimulus timepoint due to stronger expression levels in comparison to 8h timepoint. Concerning our stimuli-induced fluorescence data, the construct showed very limited success in labelling salient stimuli of negative and positive valence. The BLA did exhibit a trend regarding pAAV5-c-fos-eYFP fluorescence intensity after footshock exposure (negative valence), though not significant. No differences were found regarding cocaine group. It is, however, not definite whether the lack of significant c-fos-dependent viral

expression in our data is due to the inefficiency of the viral approach itself or, for instance, the aforementioned possibility of procedural confounding effects in the behavioural protocol.

Regarding the viral approach itself, it is relevant to consider that transduction rates fluctuate across strategies. When utilizing recombinant adeno-associated viruses (rAAVs) – frequently used due to their efficacy –, transduction rates of the many serotypes change not only across tissues, but throughout different brain regions and neuron types (Van Vliet et al., 2008). Remarkably, a comparative study using AAV viral constructs that were pseudotyped in varied AAV serotypes, either to transduce inhibitory BLA neurons or glutamatergic neurons, found that the AAV5 serotype showed much lower expression/transduction levels than other serotypes (de Solis et al., 2017). A similar serotype comparison with an AAV2 genome plasmid and CamKII-driven expression also found lesser transduction levels for the AAV5 serotype (Holehonnur et al., 2014). Concerning potential transduction issues in the NAc, although the AAV5 serotype has been shown to effectively label striatal tissue (Aschauer et al., 2013; Markakis et al., 2010; Taymans et al., 2007), the overall focus appeared to be on more dorsal sections of the striatum.

Though neither fluorescence intensity (apart from a tendency in the BLA) and area of expression indicated any difference in viral expression between animals exposed to footshock and cocaine, some animals (of varied groups) presented lower values of fluorescence area due to some slices exhibiting only minimal labelling, a possible indicator of low viral diffusion. Therefore, transduction issues may at least have contributed to the puzzling results obtained with the viral approach.

Optogenetic manipulation of neurons responsive to negative valence stimuli

Valence has, by definition, a direct impact on behaviour, given that it is comprised by both the value of a stimulus and consequent reactions induced on the individual (Berridge, 2019). One of the simplest external responses of being subjected to salient stimuli of either positive or negative valence is directed motivated movement, to either approach (rewarding value) or avoid (aversive value) (Tye, 2018). Approach and avoidance of stimuli are commonly assessed via place preference paradigms such as the conditioned place preference test (CPP) and the real time place preference (RTPP). In both, one can associate a particular stimulus to a location and infer if it is rewarding, aversive or neutral.

Optogenetics is commonly used in such paradigms, in order to evaluate how certain neuron types or populations influence physiological and/or behavioural effects (Beyeler et al., 2014). In an attempt to functionally validate our pAAV5-c-fos-ChR2-eYFP vector, we used optogenetics to activate negative-valence neurons in the BLA and assessed if one could elicit an aversive response in a neutral context. To do so, we tested animals in both a real time place preference (RTPP) and a conditioned place preference (CPP)

paradigm. Our data showed a trend for avoidance of optical activation of footshock-activated neurons in the RTPP test (spending more time in the OFF chamber), suggesting a negative valence-associated behavioural effect. This was expected due to earlier studies, such as the work of Namburi et al., (2015), in which optogenetic activation of BLA-centromedial amygdala neurons created avoidance to the stimulation-paired chamber of a real time place avoidance task. However, it is important to refer that if we excluded one animal in which implantation coordinates were impossible to determine, the effect is not so evident, however, the low sample size hampers the interpretation of the data. In the CPP, neuronal activation of footshock-responsive neurons was not enough to elicit aversion to the light-paired chamber. Thus, we were not able to induce avoidance in a previously neutral context in the CPP, as one was expecting to. This is in contrast with previous literature indicating that stimulating BLA shock-responsive neurons in an odour-learning task is sufficient to induce avoidance of the light-conditioned chamber (Gore et al., 2015a). A similar outcome was shown by Redondo et al. (2014) when using a doxycycline (dox)-based optogenetic place avoidance test with fear conditioning using footshock. These results are particularly puzzling when considering that this region is heavily involved with fear learning (Duvarci & Pare, 2014; Tovote et al., 2015), in which footshock is often employed (Bali & Jaggi, 2015), and with encoding footshock in particular (Gore et al., 2015a). This data, together with the results from fluorescence analysis, seems to suggest that this viral approach is not a suitable strategy to evaluate valence-related neuronal differences in the NAc (and amygdala) after exposure to aversive and appetitive stimuli.

However, it should also be stated that in the RTPP and CPP paradigms, factors like optical stimulation settings and the number and length of conditioning sessions vary between paradigms, and as such, may influence the conditioning results. Our optogenetic stimulation was achieved with blue light (473nm), at 20Hz in 5ms pulses (10% duty cycle), for both RTPP and CPP, with the latter being delivered in 60 light pulses every 5s. Our RTPP followed the real time place avoidance approach of Namburi et al., (2015) in terms of stimulation, while our CPP followed the intracranial optical self-stimulation (ICSS) light stimulation protocol. In Gore et al. (2015a), the Pavlovian task presented an odour co-terminating with 2s stimulation (20Hz; 20% duty cycle), while Redondo et al. (2014) used 15ms pulses (20Hz, 30% duty cycle). Applying an alternative optogenetic protocol might clarify if the absence of effect is related with the neuronal activation pattern or with the viral strategy used.

Table 3 – Summary of the main results obtained by evaluating neuronal activation after exposure to negative- (footshock) and positive-valence (cocaine) stimuli via c-fos+ cell density (NAc, BLA, CeA), c-fos-dependent eYFP expression (NAc, BLA) and of optogenetically-induced avoidance behaviour in RTPP and CPP paradigms due to activation of BLA shock-responsive neurons.

– no changes; ↑ higher values/avoidance behaviour noted; ≈ (↑) tendency for higher values/avoidance behaviour noted

		c-fos+ cell density	eYFP MGV	eYFP fluorescence area	RTPP	CPP
NAc	**-**	↑	–	–		
	+	–	–	–		
BLA	**-**	↑	≈ (↑)	–	≈ (↑)	–
	+	–	–	–		
CeA	**-**	≈ (↑)				
	+	–				

5.2. Conclusion

Studies have provided evidence involving the NAc in responding to stimuli of positive- and negative-valence. However, which specific neurons process each valence (and how that arises) still requires further exploration.

Here, we have shown that electric footshock significantly activates neurons of NAc core and of the BLA regions of the brain, as evaluated by endogenous c-fos cell counting. Surprisingly, cocaine did not elicit significant changes in neuronal activation in these brain regions. Several hypotheses could explain the absence of differences in the cocaine group, such as the fact that experimental conditions were not optimal – a very high level of "unspecific" c-fos recruitment was observed. One could also consider that other IEG could better reflect NAc (and amygdala) activation patterns.

We also tested a viral labelling approach to apply in more complex experiments to assess which neuronal populations encode positive *versus* negative valence, as well as their potential segregation or overlap. The viral strategy showed a tendency for higher neuronal activation in the BLA with footshock, with no differences in the NAc. No differences with cocaine were found. This suggests that this methodology is not suitable for our goals.

Optogenetic activation of footshock-responsive neurons in the BLA led to a tendency for place avoidance behaviour in a RTPP paradigm, a typical aversion-related reaction, but this effect was absent in the CPP test.

Overall, our results indicate that the viral strategy tested in this dissertation is not the best one to evaluate valence encoding in the NAc. Therefore, other tools should be used to label valence-responsive neurons in the NAc, as briefly introduced in the section below.

5.3. Future perspectives

Additional studies are still necessary to identify and characterize neuronal populations encoding negative- and positive-valence stimuli in the NAc and study neuronal influence in valence-specific behaviours. Many relevant methodological advancements have been developed in recent years, allowing for more accurate and clear identification of neurons.

One of the possible strategies that can be used to label valence neurons is the TRAP system (Targeted Recombination in Active Populations; (Guenthner et al., 2013)) (with the TRAP2 mouse line). This transgenic mouse line allows permanent genetic access to neurons activated by a given stimulus (Allen et al., 2017; DeNardo et al., 2019). This system is also c-fos-driven and leads to expression of a tamoxifen-inducible Cre recombinase (CreER) in activated cells. By introducing a Cre-dependent effector gene (either transgenic or virally-delivered), one can temporally restrict the expression of the effector gene to a particular moment in time. This ensures that only in neurons that are activated in the presence of tamoxifen, Cre-based recombination takes place, leading to permanent expression of the effector gene. If the effector gene encodes a constitutively fluorescent protein such as tdTomato, we can have a time-locked, c-fos-specific labelling of neuronal populations activated by specific valence stimuli. This strategy has been used with success, for example, to label and characterize water deprivation-responsive neurons in the hypothalamic median preoptic nucleus (Allen et al., 2017), study the influence of prelimbic neurons in remote memory retrieval (DeNardo et al., 2019), study the involvement of NAc core D1- and D2-MSNs in sucrose- and cocaine-seeking (Bobadilla et al., 2020) and to label quinine-responsive disgust-associated neurons in the CeA (Tanaka et al., 2021).

Optotagging (Photostimulation-assisted Identification of Neuronal Populations (PINP); (Lima et al., 2009)) enables the classification of neurons in *in vivo* electrophysiological recordings, via optical targeting of specific genetically-identified neuronal populations (*e.g.* using Cre-driven opsins) and analysis of the optical-evoked response. This has been used, for instance, to study valence in BLA projections by Beyeler et al. (2018).

Additionally, single-cell RNA sequencing (scRNA-seq) tools allow transcriptome-wide genetic characterization of single cells (Ziegenhain et al., 2017). This can be applied to identify activated neuronal populations (by focusing on IEG expression and potential genetic markers) in an unbiased and high-

throughput manner. Wu et al. (2017), for example, developed Act-seq (Activated Cell Population Sequencing) - an scRNA-seq approach, and used it to study neuronal activation by acute stress in the medial amygdala.

Overall, this toolbox (and others) opens new avenues for a better understanding of neuronal activation in specific NAc subpopulations due to exposure to positive and negative valence stimuli. This knowledge on valence encoding might, in a long term perspective, identify target neuronal subpopulations or mechanisms that would contribute for developing new molecular approaches for treatment of emotional disorders with NAc disfunctions.

CHAPTER 6 – References

6.1. References

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