The effects of reward devaluation on cue-evoked modulation of sucrose seeking and neuronal ensemble plasticity in Nucleus accumbens

Meike Claudia Sieburg

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I hereby declare that this thesis has not been and will not be, submitted in whole or in part to another University for the award of any other degree. The work in this thesis is entirely my own except where due acknowledgement and reference was made.

Signature: .....

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Abstract

Animals must learn the relationship between food and the environmental cues that predict their availability for the successful procurement of nutrient sources. These cues can gain powerful control over food seeking, but these cue-evoked behaviours must remain flexible and updated upon changes in internal states such as the perceived desirability of food. Recalling these cue-food associations activate subsets of neurons termed 'neuronal ensembles' in motivationally relevant brain areas such as the striatum. However, how neuronal ensembles are recruited and physiologically modified following the update of these learned associations has not fully elucidated. To investigate this, we examined the effects of reward devaluation on ensemble plasticity at the levels of recruitment, excitability, and synaptic physiology in sucrose conditioned *Fos-GFP* mice that express green fluorescent protein (GFP) in recently activated neurons. Neuronal ensemble activation patterns and their physiology were examined using immunohistochemistry and *ex vivo* electrophysiology, respectively. First, devaluation via four days of *ad libitum* sucrose consumption, but not caloric satiation, attenuated the ability of the cue to evoke sucrose seeking. Thus, changes in the hedonic, incentive value of sucrose, and not caloric need drove cue-induced sucrose seeking. Also, devaluation attenuated the cue's ability to recruit a neuronal ensemble in nucleus accumbens (NAc), but not dorsal striatum. Next, devaluation prevented the cue from recruiting a hyper-excitable, GFP+ ensemble in the NAc, but did not alter the physiology of excitatory synapses on these GFP+ neurons. Our findings provide new insights into how updates in the hedonic value of sucrose critically modulates the flexibility of sucrose seeking and recruitment of ensembles with an altered excitability phenotype in the NAc shell.

Abbreviations

aCSF: artificial cerebrospinal fluid
AHP: afterhyperpolarisation
AMPA: α-amino-3-hydroxy-5-methyl -4-isoxazolepropionic acid
AP: action potential
BK: big conductance
BLA: basolateral amygdala
CeA: central nucleus of the amygdala
CR: conditioned response
CRE: cAMP response element
CREB: cAMP response element-binding protein
CS: conditioned stimulus
D1R: dopamine-1 receptor
D2R: dopamine-2 receptor
DA: dopamine
DLS: dorsolateral striatum
DMS: dorsomedial striatum
DS: dorsal striatum
eEPSC: evoked excitatory postsynaptic current
EGFP: enhanced green fluorescent protein
EPSC: excitatory postsynaptic current
ERK: extracellular signal–regulated kinase
GABA: gamma-aminobutyric acid
GFP: green fluorescent protein
HP: hippocampus
IEG: immediate early gene
ICS: intracellular solution
IL: infralimbic
IPSC: inhibitory postsynaptic current
ITI: intertrial interval
K: potassium
KIR: inwardly rectifying potassium channel
LH: lateral hypothalamus
LiCl: Lithium Chloride
LTD: long-term depression
LTP: long-term potentiation
MAPK: mitogen-activated protein Kinase
mPFC: medial prefrontal cortex
MSN: medium spiny neuron
Na: sodium
NAc: nucleus accumbens
NMDA: N-methyl-D-aspartate
OFC: orbitofrontal cortex
PBS: phosphate buffered saline
PFA: paraformaldehyd
PFC: prefrontal cortex
PL: prelimbic
PPR: paired pulse ratio
Ri: input resistance
RI: random interval
SD: standard deviation
SEM: standard error of the mean
sEPSC: spontaneous excitatory postsynaptic current
sIPSC: spontaneous inhibitory postsynaptic current
SK: small conductace
TRE: tetracycline response element
tTA: tetracycline transactivator
UR: unconditioned response
US: unconditioned stimulus
VP: ventral pallidum
VTA: ventral tegmental area

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1 General introduction

1.1 Associative learning: food-cue associations

1.1.1 The significance of food-cue associations for humans and non-human animals

Humans and non-human animals alike form associations between environmental cues and the food whose availability they predict to guide food seeking behaviour (Arenas & Farina, 2012; Croney, Adams, Washington, & Stricklin, 2003; Nederkoorn, Smulders, & Jansen, 2000; Petrovich, 2013; Whitfield, Köhler, & Nicolson, 2014). Such cues (e.g. smells) help optimize the localization of food sources, obtain motivational significance and exert substantial control over foraging behaviour (Nederkoorn et al., 2000; Petrovich, 2011; Petrovich & Gallagher, 2007). Importantly, animals need to adapt their cue-responsivity according to the current attractiveness of the food. One factor that determines the current attractiveness of a food is the satiety level of an animal. Therefore, reactivity to food-associated cues is decreased following satiation (P. Holland & Rescorla, 1975; Parkes & Balleine, 2013; E. A. West & Carelli, 2016). However, in today's society of readily available highly palatable, energy dense food, this behavioural flexibility can malfunction and environmental cues associated with palatable food may promote food seeking behaviour even in the absence of homeostatic hunger (Davidson, Giesbrecht, Thomas, & Kirkham, 2018; Jones, Sample, Hargrave, & Davidson, 2018). This maladaptation can lead to dysregulation of food consumption patterns, leading to overeating, one of the leading causes for the development of obesity or overweight (defined as a BMI ≥ 30 and BMI = 25 - 30, respectively) (Boswell & Kober, 2016; Kosheleff et al., 2018). In humans this process is illustrated by a wealth of studies reporting a link in children or adolescents between the amount of television watched, specifically unhealthy food advertisements (acting as cues)

and unhealthy food choices, as well as current and adult body weight (Halford et al., 2008; Kelly et al., 2016; Lobstein & Dibb, 2005; Viner & Cole, 2005). Eating related-disorders, the development of obesity and overweight, and comorbidities such as cardiovascular diseases, diabetes, muscoskeletal diseases, and cancer are on the rise (S. C. Smith, 2007). In 2016, 39% of adults worldwide were overweight and 13% obese, a number that nearly tripled since 1975. The costs of obesity and overweight affect not only medical and prevention costs, but also economic costs due to loss of productivity, hence the estimated economic cost of obesity in 2014 in the US was 2.8% of the gross domestic product (Dee et al., 2014; Finkelstein, Trogdon, Cohen, & Dietz, 2009; McKinsey Global Institute, 2014; Specchia et al., 2015; Tremmel, Gerdtham, Nilsson, & Saha, 2017). Therefore, elucidating the behavioural and neurobiological processes underlying the flexibility of food-cue associations is crucial in order to contribute to the prevention of overeating and subsequent disorders.

1.1.2 Experimental modelling and behavioural analysis of learning of food-cue associations

Early on in the scientific exploration of associative learning two main types of environmental relationships emerged (Fanselow & Wassum, 2016). The first is the association of two stimuli, which is today called Pavlovian or classical conditioning (I. P. Pavlov, 1927) and the second is the association of an action and a stimulus, which is today called instrumental or operant conditioning (Edward L. Thorndike, 1898). Thorndike first discovered that actions with a positive outcome are more likely to be repeated and actions with no or a negative outcome tend to not be repeated when he put cats in “puzzle boxes” they could only escape from through specific actions (E L Thorndike, 1905). He termed this learning rule “law of effect”. It was Skinner however who coined the term operant conditioning, and optimised experimental procedures by inventing experimental operant conditioning chambers (“Skinner box”) with a variety of schedules of reinforcement (Skinner, 1969). Whereas

operant conditioning relies on action-outcome associations, classical conditioning relies on stimulus-outcome or stimulus-stimulus associations (Bolles, 1972; Mackintosh, 1974; Rozeboom, 1958). In his famous initial experiments, Pavlov measured salivation in dogs in response to different stimuli (I. Pavlov, 1929; I. P. Pavlov, 1927). First, he rang a bell, an initially neutral stimulus termed conditioned stimulus (CS) to the dogs, which did not elicit any salivation. Presentation of food, the unconditioned stimulus (US), however did elicit salivation, the unconditioned response (UR). After the two stimuli had repeatedly been presented together, even presentation of just the CS elicited salivation, now called the conditioned response (CR): Interestingly, a more accurate translation from the original Russian would be ”conditional” instead of “conditioned”, however as “conditioned” is widely used today we will use this term throughout this study (Fanselow & Wassum, 2016; Robert A. Rescorla, 1968). Since then there has been much work to establish learning theories and to define rules for the CS-US association that goes beyond the scope of the current study (for a review see Fanselow & Wassum, 2016).

Under specific circumstances, such as overtraining or after second order conditioning, in which the CS itself acquires reinforcing properties, associative learning can form stimulus-response associations, which are non goal-directed (Mackintosh, 1974; Robert A Rescorla, 1974; Edward L Thorndike, 1911). These associations are also called habits and thought to lack the sensory specifics of the US reinforcement, as the CS itself acquired generalized reinforcing properties (Dickinson & Balleine, 2002; Fanselow & Wassum, 2016). Due to this lack of sensory details, a common way of testing if an association is between a goal-directed, stimulus/action and an outcome in Pavlovian conditioning/operant conditioning or if it is a habit, so a stimulus-response association, are devaluation procedures (Pickens & Holland, 2004). This entails reducing or altering the value of the US and measuring the CR, which will be adapted in the case of stimulus/action-outcome but not stimulus-outcome

associations (Pickens & Holland, 2004). Common ways to reduce the value of a positive US is pairing it with an aversive stimulus, such as illness-inducing rotation, Lithium Chloride (LiCl) injections, or foot shock (P. Holland & Rescorla, 1975; Erin C Kerfoot, Agarwal, Lee, & Holland, 2007; Kraemer, Hoffmann, Randall, & Spear, 1992; Singh, McDannald, Haney, Cerri, & Schoenbaum, 2010). In the case of appetitive conditioning it is also possible to reduce the US value without making it aversive through sensory specific satiety or selective satiation (Glueck, Dennis, Perrotti, Torres, & Papini, 2015; P. Holland & Rescorla, 1975; Parkes & Balleine, 2013; E. A. West & Carelli, 2016). Such modifications of the magnitude of appetitive value can additionally be used to draw conclusions about aforementioned behavioural flexibility, the ability of an animal to adapt cue-elicited responding according to the current value of the food reward, which in turn depends on the internal satiety level.

1.2 Neurocircuitry of motivation and reward

1.2.1 Overview

The striatum as part of the basal ganglia is considered to be an integrative hub using information from cortical, thalamic, and limbic inputs to select appropriate actions and send according signals to downstream output areas (Redgrave, Prescott, & Gurney, 1999). Therefore, this region has been shown to play a role in a variety of functions including motivation, reward guided and motor learning, as well as cognitive processes (Burke, Rotstein, & Alvarez, 2017). The basal ganglia and specifically the striatum is evolutionarily conserved and most developed in mammals, making it suitable for the use of animal models (Reiner, Medina, & Veenman, 1998). In the following paragraphs we will outline information about function, anatomy, biochemistry, circuitry and physiology of the striatal subareas most relevant for the current study, a broad overview of the mentioned pathways can be seen in Figure 1.

1.2.2 General striatal anatomy, biochemistry, and physiology

The principal projection neurons in the striatum are GABA (gamma-aminobutyric acid) ergic medium spiny neurons (MSNs), constituting 95% of all neurons in rodents and less in humans (Freund, Powell, & Smith, 1984; Graveland & Difiglia, 1985; Kita & Kitai, 1988). Their spontaneous activity consists of no or low frequency bursts (C J Wilson & Kawaguchi, 1996; Charles J. Wilson, 1993). Moreover, MSNs are known to transition between a more excitable up-state and a less excitable down-state in membrane potential *in vivo*, but not in slices as glutamatergic inputs are missing (C J Wilson & Kawaguchi, 1996; Charles J. Wilson, 1993). This transition is dependent on excitatory synaptic input from upstream areas, whereas the membrane fluctuations and potential firing depend on voltage-dependent intrinsic membrane properties (C J Wilson & Kawaguchi, 1996). The remaining 5% of striatal neurons are interneurons, with 1% constituting cholinergic, tonically active interneurons with long-lasting afterhyperpolarisation and 4% being aspiny GABAergic interneurons (F. M. Zhou, Wilson, & Dani, 2002). The latter have be divided into three traditional categories: 1) Parvalbumin expressing, fast-spiking interneurons, 2) somatostatin, neuropeptide Y, oxide synthase expressing, low-threshold firing and persistent depolarizing plateau interneurons, and 3) calretinin expressing interneurons (Burke et al., 2017; Y Kawaguchi, 1993; James M. Tepper & Bolam, 2004). However this categorization of GABAergic interneurons is constantly challenged with addition of new classes due to technological advances (Faust, Assous, Shah, Tepper, & Koós, 2015; Ibanez-Sandoval et al., 2010, 2011; J.M. Tepper & Koós, 2016). Neurochemically, the striatal subregions are not clearly delineated, pointing towards similar densities of the different cell types, with the greatest chemoarchitechtonic complexity and heterogeneity in the ventral striatum (Gangarossa et al., 2013; Prensa, Richard, & Parent, 2003; Voorn,

Vanderschuren, Groenewegen, Robbins, & Pennartz, 2004). Striatal MSNs can further be divided according to their dopamine (DA) receptor expression and projection areas. Traditionally, two main projection pathways of approximately equal size have been described, both ultimately projecting to the thalamus (Y. Smith, Bevan, Shink, & Bolam, 1998). The direct pathway consists of dopamine 1 receptor (D1R) containing MSNs expressing substance P and dynorphin and projecting from the striatum directly to the ventral tegmental area (VTA) or substantia nigra and internal globus pallidus (humans) / entopeduncular nucleus (rodents). The indirect pathway contains dopamine 2 receptor (D2R) and encephalin expressing MSNs projecting to the same target areas but indirectly via external globus pallidus (humans) / ventral pallidum (VP) (rodents) and the subthalamic nucleus (Albin, Young, & Penney, 1989; R. M. Beckstead & Cruz, 1986; Charles R. Gerfen et al., 1990; Yasuo Kawaguchi, Wilson, & Emson, 1990; Pan, Penney, & Young, 1985). As all projections in the direct and indirect pathways are GABAergic, except for the glutamatergic subthalamic nucleus neurons, overall an activation of the direct pathway increases thalamic output, whereas an activation of the indirect pathway decreases thalamic output (Y. Smith et al., 1998).

1.2.3 Nucleus accumbens

Together with the olfactory tubercle, the Nucleus accumbens (NAc) forms the ventral striatum and it can further be divided in core and shell subregions, according mostly to functional and afferent differences (Heimer, Zahm, Churchill, Kalivas, & Wohltmann, 1991; Zahm & Brog, 1992). Major glutamatergic input to the NAc originates from the medial prefrontal cortex (mPFC), medial thalamus, basolateral amygdala (BLA), and hippocampus (HP), the VTA provides dopaminergic input, and the VP provides GABAergic input (Brog, Salyapongse, Deutch, & Zahm, 1993; R C Malenka, Nestler, & Hyman, 2009; Voorn et al., 2004). The main output area of NAc MSNs besides the VP and VTA as part of the direct

and indirect pathways, respectively, is the lateral hypothalamus (LH) (Mogenson, Swanson, & Wu, 1983). Both NAc inputs and outputs have been shown to be organised in a topographic manner along a dorsolateral to ventromedial gradient (Berendse, Graaf, & Groenewegen, 1992; Voorn et al., 2004). The aforementioned striatal direct/indirect projection pathway division for the NAc has recently been challenged, however with these findings in mind, it is still used for the interpretation of many new findings (Gagnon et al., 2017; Gallo et al., 2018; Ji et al., 2017; Kupchik et al., 2015; Macpherson & Hikida, 2018; R. J. Smith, Lobo, Spencer, & Kalivas, 2013). This issue arises partially from the highly heterogeneous organisation of the NAc, especially the shell (Gangarossa et al., 2013; Prensa et al., 2003; Zahm & Brog, 1992).

The NAc has long been known to be implicated in goal directed, motivated behaviour and stimulus reward associations. This is reflected in the pattern of inputs to the NAc, mostly conveying limbic information. Early studies have showed that rats reliably perform VTA, DA dependent, as well as NAc intracranial self-stimulation paradigms, establishing a clear connection between the NAc and direct reward-associated behaviours (A. G. Phillips & Fibiger, 1978). Lesion studies from the late 80s then pointed towards an additional role of the NAc in reward-associated cue reactivity but not habitual behaviours (Cole & Robbins, 1987; Robbins, Cador, Taylor, & Everitt, 1989; Robbins, Giardini, Jones, Reading, & Sahakian, 1990). This role has been shown to be DA dependent, as DA depletion in NAc impaired acquisition and expression of Pavlovian conditioning (J. A. Parkinson et al., 2002). DA is now known to be released in the NAc in response to primary reinforcers as well as reward-associated cues (Cacciapaglia, Saddoris, Wightman, & Carelli, 2012; Hajnal, Smith, & Norgren, 2004; Sackett, Saddoris, & Carelli, 2017; Taha, 2005). The specific involvement of the core and shell subregions in this appear to differ however. Whereas the core mediates the acquisition and also required for the expression of cue-reward associations, the shell is

required for the expression but not the acquisition of Pavlovian approach behaviours, demonstrated by lesion and glutamate or DA inactivation studies (Dalley et al., 2005; J. A. Parkinson et al., 2002; John A. Parkinson, Willoughby, Robbins, & Everitt, 2000). Instead, the shell is involved in conveying the reinforcing properties of a reward or a reward-associated cue itself, as DA levels, NAc activation, and the number of phasically activated neurons here correlated with reward magnitude (Ahn & Phillips, 1999; Genn, Ahn, & Phillips, 2004; Erin C Kerfoot et al., 2007; Taha, 2005; E. A. West & Carelli, 2016). In contrast, Singh and colleagues reported rats with either core or shell lesions to be unable to flexibly adapt responding after reward devaluation, indicating that even though based on aforementioned studies the shell may mediate reward magnitude, the core is also necessary to flexibly adapt cue-responding (Singh et al., 2010).

1.2.4 Dorsal striatum

Similarly to the NAc, the glutamatergic inputs and outputs of the dorsal striatum (DS) are organized in a topographic manner, further dividing the DS into dorsomedial (DMS) and dorsolateral (DLS) striatum (Voorn et al., 2004). Specifically, the majority of glutamatergic afferents originates in sensorimotor cortex or thalamus (Kemp & Powell, 1971; Voorn et al., 2004). Dopaminergic input to the DS is provided by the substantia nigra (Robert M. Beckstead, Domesick, & Nauta, 1979). The main efferents of the DS are direct and indirect pathways ultimately projecting to substantia nigra (Maurin, Banrezes, Menetrey, Mailly, & Deniau, 1999; Voorn et al., 2004).

The DS is known to be involved in both goal-directed and habitual, non-goal directed behaviours and stimulus-response associations. This is reflected in the topographic organization of inputs to the DS, mostly conveying information related to motor learning or sensorimotor information (Voorn et al., 2004). Lesioning of the DS impair acquisition and performance of habitual, non-conditioned discriminative tasks but not amphetamine-induced

amplification of reward associated cue reactivity (Cole & Robbins, 1987; Robbins et al., 1989, 1990). More targeted studies using excitotoxic lesions, pharmacological inactivation, or NMDA (N-methyl-D-aspartate) receptor block revealed that the DLS is mainly involved in habit formation and stimulus-response behaviours, whereas the DMS mediates response-outcome associations and goal-directed behaviours (Corbit & Janak, 2010; Yin, Knowlton, & Balleine, 2004, 2005; Yin, Ostlund, Knowlton, & Balleine, 2005).

1.3 Neurocircuitry of hunger and food intake

Feeding behaviour, food related reward, and motivation are similarly to other rewards mediated by the aforementioned mesocorticolimbic network, however they are also influenced by signalling pathways unique to food rewards (see Figure 1 for a broad overview). Information from the periphery about mechanical and chemical stimulation by nutrients but also reward related signals are sent via neuronal subpopulations in the sensory branch of the vagus nerve from the gut to the brain where they influence mesolimbic DA (W. Han et al., 2018; Hellström et al., 2004). Additional information from the periphery about physiological hunger and satiety are sent to the hypothalamus via circulating hormones such as ghrelin, leptin, and insulin, which are produced in the stomach, adipocytes, and pancreas, respectively. They act primarily on the arcuate nucleus of the hypothalamus where they are integrated into signals sent to orexin-producing neurons in the LH. The LH has long been known as the “feeding center” of the brain, with early studies using LH lesions or stimulation inhibiting or increasing feeding behaviour, respectively (Anand & Brobeck, 1951; Delgado & Anand, 1952). Orexin, also known as hypocretin, is a neuropeptide involved in the stimulation of appetite and food seeking behaviour, via its actions in areas including the VP and VTA and hence on downstream DA signals in the NAc, as well as the NAc shell itself (Daniel C Castro, Cole, & Berridge, 2015; Cone, McCutcheon, & Roitman, 2014; Nakamura et al., 2000; Peyron et al., 1998; Thorpe & Kotz, 2005). In order to further determine the

exact role of these projections, pilot studies performed by Castro and colleagues using optogenetics support the view that orexin LH neurons are generally involved in mediating motivation for food, whereas only the specific orexin projections from LH to a so called hedonic hotspot in the posterior VP appear to be involved in hedonic impact of food (Daniel C Castro et al., 2015; Ho & Berridge, 2013). Similarly, whereas orexin in the NAc shell generally mediates motivation for food, in the anterior medial shell, the hedonic hotspot, orexin mediates hedonic liking of food (Daniel C. Castro, Terry, & Berridge, 2016). In addition to the orexinergic projections, the LH sends excitatory glutamatergic projections to the VTA, constituting another way to impact DA release in the NAc (Watabe-Uchida, Zhu, Ogawa, Vamanrao, & Uchida, 2012).

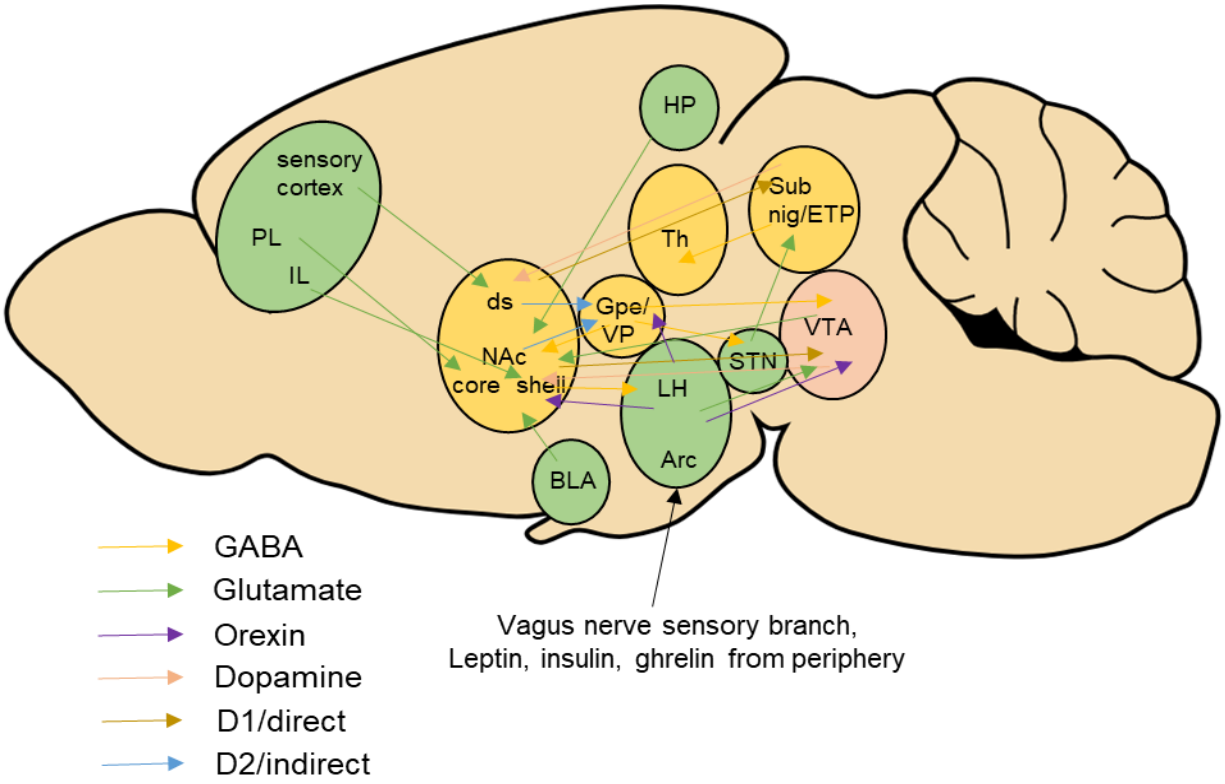

Figure 1: Selected neurocircuitry of appetitive motivation and reward. Note that direct and indirect pathways from NAc overlap and are not entirely separate as in ds (not shown, see Smith et. al 2013). PL = prelimbic cortex, IL = infralimbic cortex, ds = dorsal striatum, Nac = Nucleus accumbens, BLA = basolateral amygdala, LH = lateral hypothalamus, Arc = arcuate nucleus, STN = subthalamic nucleus, VTA = ventral tegmental srea, VP = ventral pallidum, Gpe = external globus pallidus, Th = thalamus, HP = hippocampus, Sub nig = substantia nigra, ETP = entopeduncular nucleus. For references see text.

It has long been know that opioids stimulate feeding behaviour, however the details of the extent to which this is due to modulation of hedonic liking or motivation to consume food is still being discussed (Sanger & McCarthy, 1981). Early evidence for the involvement of opioids in feeding behaviour comes from a study increasing food intake using morphine microinjections in NAc and VTA (Mucha & Iversen, 1986). Conversely, opioid antagonists have been shown to reduce food seeking as well as binge-like eating of palatable food, but not normal chow (Giuliano, Robbins, Nathan, Bullmore, & Everitt, 2012; Ward, Nicklous, Aloyo, & Simansky, 2006). More specifically, together with aforementioned orexin, opioids play an important role in NAc shell and VP "hedonic hotspots", where they mediate hedonic liking of food (D. C. Castro & Berridge, 2014; Pecina, 2005; K. S. Smith, 2005). However, in the remaining NAc shell as well as other brain areas including amygdala, prefrontal cortex and striatum opioid signalling mediates motivation for food rather than hedonic impact (D. C. Castro & Berridge, 2014; Difeliceantonio, Mabrouk, Kennedy, & Berridge, 2012; S. V. Mahler & Berridge, 2009; Pecina, 2005; M. Zhang & Kelley, 2000). A recent study has investigated the projection from NAc shell to LH in particular and revealed that μ-receptor agonism in NAc shell mediates intake of palatable food and LH firing in response to palatable food associated cues (Tandon, Keefe, & Taha, 2017).

Similarly to orexin and opioids, endocannabinoids are mediators of hedonic eating and palatability (Stephen V. Mahler, Smith, & Berridge, 2007). Endocannabinoid receptors are expressed in the striatum including NAc (Fusco et al., 2004; J. P. Gong et al., 2006; Moldrich & Wenger, 2000). In line with this, microinjection of endocannabinoids into a hedonic hotspot in dorsal medial shell has been shown to increase orofacial hedonic reactions to intraoral sucrose, ingestion of a sweet solution, as well as eating behaviour in rats (Kirkham, Williams, Fezza, & Di Marzo, 2002; Stephen V. Mahler et al., 2007; Shinohara, Inui, Yamamoto, & Shimura, 2009). Further studies revealed that this also increases Fos expression in the LH,

indicating an increase in hypothalamic activity after NAc shell endocannabinoid agonism (Soria-Gómez et al., 2007).

Taken together, the mesocorticolimbic reward network interacts with peripeheral signals mostly via the LH using neuropeptides besides traditional neurotransmitters to integrate information about physiological state, nutrients, and palatability to drive food seeking behaviour.

1.4 Neuronal plasticity: definitions, types, and adaptations induced by associative learning

1.4.1 Overview

Neuronal plasticity is a broad term for adaptations in function or structure of neuronal circuits based on intrinsic or extrinsic factors and can be divided in numerous categories (Ganguly & Poo, 2013). The earliest type of neuronal plasticity occurring in an organism is during neurodevelopment, when during embryonic neurogenesis new neurons are created and migrate depending on biochemical gradients. Similarly to this prenatal neurogenesis, in adulthood certain areas, namely the olfactory bulb/lateral ventricle and the HP are capable of neurogenesis (Altman & Das, 1965; Eriksson et al., 1998; Lledo, Alonso, & Grubb, 2006; Ming & Song, 2011). Additionally, during early postnatal development a so called critical period of increased neuronal plasticity drives development of circuits in areas such as the visual and motor system based on sensory experience, spontaneous neuronal activity, and genetics (V. Anderson, Spencer-Smith, & Wood, 2011; Espinosa & Stryker, 2012; David H. Hubel & Wiesel, 1998; Martin, 2005). Turrigiano and colleagues were the first ones to describe homeostatic plasticity, a process regulating the balance of excitation and inhibition in neuronal circuits so that neuronal firing is stabilized in a range to optimally transmit signals despite ever changing outside factors (G. G. Turrigiano, Leslie, Desai, Rutherford, & Nelson,

1998). Besides acting on synapses via synaptic scaling, it is now known that homeostatic mechanisms also include adaptations of intrinsic factors regulating overall neuronal excitability (G. Turrigiano, 2011). Metaplasticity is considered to be the “plasticity of plasticity”, meaning the impact of neuronal activity on the ability of synapses to undergo long-term alterations and constitutes another form of neuronal plasticity (Abraham & Bear, 1996). But the most relevant form of neuronal plasticity for the current study is activity-dependent plasticity, which can act on a short or long-term scale and at the site of synapses or on in intrinsic factors at extrasynaptic sites along the axonal, dendritic, and somatic membrane (Citri & Malenka, 2008; W. Zhang & Linden, 2003). This form of neuronal plasticity will be discussed in the following sections in the context of its cellular mechanisms, associative learning, and neuronal ensemble specific adaptations.

1.4.2 Cellular mechanisms underlying neuronal plasticity

Adaptive changes can be implemented at two main sites of a neuron: the synapse or the axonal, dendritic, and somatic plasma membrane. Alterations at the synapse can be short-term and last milliseconds to minutes or long-term, lasting hours or longer (Abbott & Regehr, 2004). Short-term synaptic plasticity can help synapses to filter incoming signals of a specific frequency range depending on its neurotransmitter release probability (Abbott & Regehr, 2004). A way to measure this is to apply a paired-pulse protocol, where two pulses separated by an increasing interval are given and the responses to the two stimuli are recorded (Citri & Malenka, 2008). Whereas short-term plasticity mostly serves computing functions, long-term synaptic plasticity encodes past experiences as differences in synaptic weights to alter behavioural output. The most widely researched forms of experience-dependent long term synaptic plasticity are LTP (long-term potentiation) and LTD (long-term depression) (Bliss & Lømo, 1973; M. Ito & Kano, 1982). They are thought to be cellular mechanisms underlying learning and memory in many brain different brain areas, increasing

(LTP) or decreasing (LTD) synaptic efficiency in response to a stimulus (Bear & Malenka, 1994; Whitlock, Heynen, Shuler, & Bear, 2006). To date there have been many accounts of different forms of LTP/D depending on brain area, cell type, developmental stage, and signalling cascades involved (Robert C. Malenka & Bear, 2004). A major form is NMDA receptor dependent LTP/D at Schaffer collaterals in the hippocampal CA1 region. This requires sustained and coordinated glutamatergic activation from the presynaptic neuron to depolarize the postsynaptic neuron via sodium influx through α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, subsequent removal of the magnesium block of NMDA receptors, and hence an elevation of postsynaptic calcium levels (Robert C. Malenka, 1991; Robert C. Malenka & Nicoll, 1993; Mulkey & Malenka, 1992). This then activates signalling cascades in the postsynaptic terminal, altering synaptic transmission via the incorporation or dissociation of AMPA receptors as well as structural changes of the postsynaptic neuron (Luscher, Nicoll, Malenka, & Muller, 2000; Malinow & Malenka, 2002; Masanori Matsuzaki, Honkura, Ellis-Davies, & Kasai, 2004). The size and timing of the postsynaptic increase in calcium levels appears to determine if LTP or LTD is induced and if the synapse is strengthened or weakened, respectively (Citri & Malenka, 2008; Robert C. Malenka & Nicoll, 1993). Additional forms of long-term synaptic plasticity include presynaptic LTP, as observed in the hippocampal CA3 region, metabotropic glutamate receptor-dependent LTD, which amongst other areas takes place in the VTA, and endocannabinoid-mediated LTD, which occurs in the striatum (Citri & Malenka, 2008). In the NAc synapses can undergo several types of synaptic plasticity, such as short-term plasticity, manifesting as synaptic depression as a consequence of depletion of the readily available release pool of synaptic vesicles (Hjelmstad, 2004; Zucker & Regehr, 2002). Additionally, “classical” NMDA receptor-dependent postsynaptic LTP has been reported in hippocampal and cortical projections, as well as presynaptic LTD depending on retrograde endocannabinoid signalling and postsynaptic mGluR5 activation (Anwyl, 2006; LeGates et al., 2018; Robbe,

Kopf, Remaury, Bockaert, & Manzoni, 2002; Schotanus & Chergui, 2008b; Thomas & Malenka, 2003). Schotanus and colleagues demonstrated a mGluR5-dependent LTP triggered by high-frequency stimulation to be D1 receptor modulated (Schotanus & Chergui, 2008a). Some of these processes are thought to underlie reward-related behaviours, such as conditioned place preference for natural rewards and the acute effects of ethanol (Ji, Saha, & Martin, 2015; LeGates et al., 2018).

Whole-cell recordings from brain slices are performed to assess synaptic plasticity in acute slices. Typically, during recordings, the neuron is held at a constant membrane potential using the voltage clamp technique in order to assess postsynaptic currents, namely spontaneous and evoked excitatory postsynaptic currents (EPSCs). These can provide information about synaptic strength, and with the help of channel-specific blockers AMPA receptor and NMDA receptor mediated components can be distinguished to calculate the AMPA receptor/NMDA receptor current ratio (M A Ungless, Whistler, Malenka, & Bonci, 2001). A change in ratio is usually interpreted as a change in number of functional AMPA receptors, presuming a stable level of NMDA receptor expression, however NMDA receptor expression has been shown to be regulated as a means of synaptic plasticity (Rebola, Srikumar, & Mulle, 2010). Furthermore, the spatial alignment of presynaptic release sites and postsynaptic receptors appears to be crucial, due to quick diffusion of released glutamate (Biederer, Kaeser, & Blanpied, 2017). Additional (non-electrophysiological) ways of investigating synaptic strength are the quantification of receptor proteins via Western Blot or the measurement of the postsynaptic calcium signal using calcium indicators. A paired-pulse ratio (PPR) protocol of repeated paired stimulation with increasing interval serves the investigation of short-term plasticity often based on neurotransmitter vesicle release probability (Suter, Smith, & Dudek, 1999). For weaker instances of paired pulse-induced short-term plasticity postsynaptic mechanisms such as postsynaptic receptor

desensitization, saturation, surface diffusion or relief of polyamine block may play a role as well (Blitz, Foster, & Regehr, 2004; Heine et al., 2008; Zucker & Regehr, 2002). Taken together, these parameters provide information about the location of pre- and/or post-synaptic alterations.

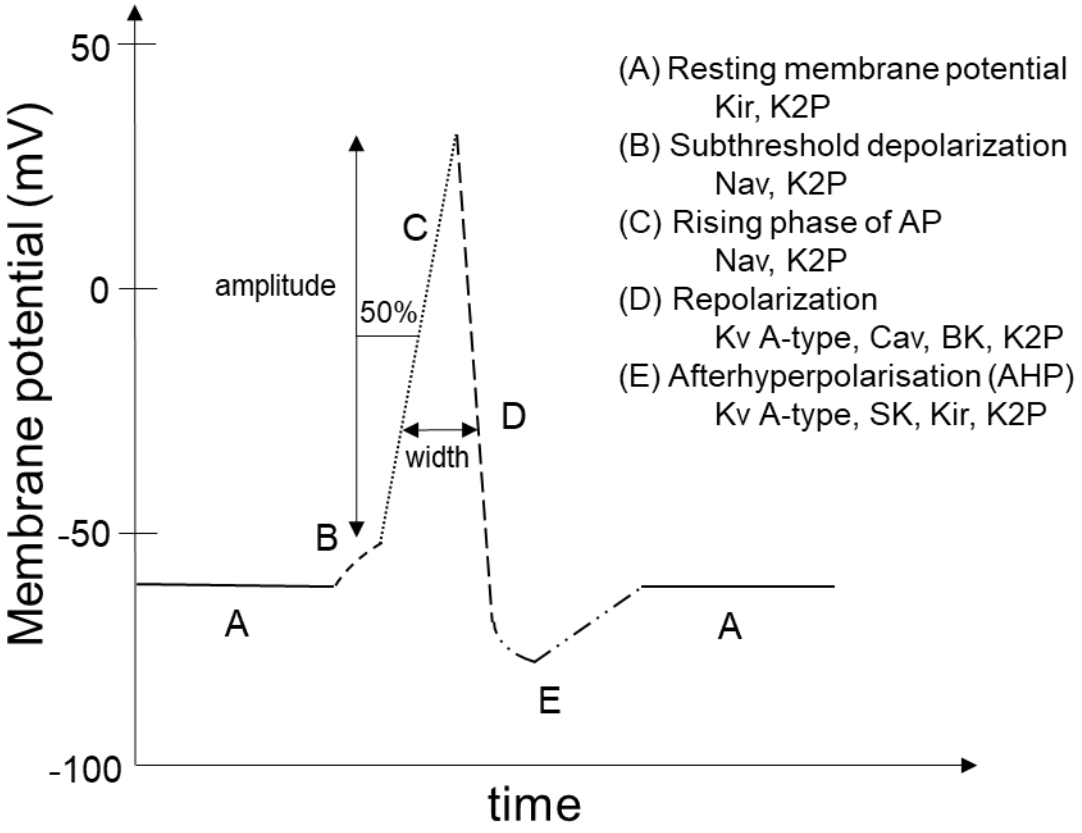

Figure 2: Schematic outline of an action potential (AP) and involvement of major classes of ion channels in its different phases. AP parameters measured in the current study are AHP, AP half-width (the width of the AP at 50% amplitude), and AP amplitude.

Besides these synaptic alterations, changes in intrinsic factors at the dendritic, axonal, and somatic membrane can induce neuronal plasticity. Intrinsic factors constitute mostly voltage gated ion channels for potassium, sodium, and calcium, as well as calcium-dependent

potassium channels. Voltage gated sodium channels open rapidly at depolarising potentials, causing a rapid influx of sodium ions, ultimately leading to firing of an AP (Hodgkin & Huxley, 1952b). These channels close and deactivate rapidly, contributing to the short duration of spikes and ensuring unidirectional transmission of the signal. The physiological role of voltage-gated calcium channels is to elicit neurotransmitter release at the presynaptic site, but calcium influx through these depolarization-activated channels is also involved in mediating neuronal plasticity (Sjostrom & Nelson, 2002). Calcium itself can also activate certain potassium channels (BK and SK channels), which are known to mediate the re- and hyperpolarization after an AP peak (Sah, 1996). The third class of voltage-gated ion channels, potassium channels, are the biggest class and one important process they are involved in is repolarisation after firing of an AP, as well as maintenance of the resting membrane potential (RMP) (Hodgkin & Huxley, 1952b; Nisenbaum & Wilson, 1995). For an overview of the main classes of ion channels and their involvement in AP firing, see Figure 2. Neuronal plasticity in striatal MSNs and other neurons can be induced by changes in the expression or function of these intrinsic factors via modulation on neuronal excitability (D'Ascenzo et al., 2009; Dong et al., 2006; Kourrich, Calu, & Bonci, 2015; Ma et al., 2013; Perez, White, & Hu, 2006; Podda, Riccardi, D'Ascenzo, Azzena, & Grassi, 2010b; Schiffmann, Lledo, & Vincent, 1995; Whitaker et al., 2017; X.-F. Zhang, 2002; X. F. Zhang, Hu, & White, 1998).

Similar to the intracellular recordings investigating synaptic plasticity, experiments measuring alterations in intrinsic excitability are performed using the current clamp technique in acute brain slices. However, unlike the voltage clamp technique, the neuron is exposed to a constant current injection regulating the membrane potential. Increasing amounts of current are now injected into the neuron, starting at negative and ending at positive values, eventually eliciting AP firing. The parameters that can later provide

information about intrinsic excitability and underlying changes in intrinsic factors are AP parameters, for instance half width, afterhyperpolarisation (AHP), amplitude, as well as number of spikes at each current step. Passive membrane parameters include the RMP, input resistance (Ri), and rheobase, the minimal current needed to elicit spiking (Suter et al., 1999).

However, the APs produced at the axon hillock are also propagated backwards into the dendrites, which is mediated and modulated by dendritic voltage-gated sodium, calcium, and potassium channels (Cai, Li, & Sesti, 2007; M. Day, Wokosin, Plotkin, Tian, & Surmeier, 2008; Stuart & Hausser, 2001). This happens more slowly than the propagation along the axon due to a lower density of voltage-gated sodium ion channels in the soma and dendrites compared to the axon, making them less excitable (Rapp, Yarom, & Segev, 1996).

1.4.3 Activity-dependent neuronal plasticity induced by associative learning

Experiences about past events shape the behavioural reaction to future events by modifying the underlying neuronal circuits in different ways. This ability for neuronal adaptation forms an integral part of healthy brain function and is known to be malfunctioning in psychiatric and eating-related disorders (Kasanetz et al., 2010; S. Liu et al., 2016; Morin et al., 2017; Oginsky, Maust, Corthell, & Ferrario, 2016; K. L. Smith et al., 2015). Hebb was amongst the first to suggest that past activity can induce lasting structural and functional neuronal plasticity and hence, as known today, form the basis for associative learning (Donald O Hebb, 1949). He stated in his neuronal assembly theory that “synaptic knobs develop with neuronal activity“, or as it was put later “neurons wire together if they fire together”, describing coordinated firing of a pre- and postsynaptic cell and the resulting synaptic plasticity (Donald O Hebb, 1949; Löwel & Singer, 1992). Early evidence for activity-dependent synaptic plasticity as mechanism driving classical associative learning comes from studies researching the gill withdrawal reflex in the sea slug *Aplysia* (Hawkins, Abrams,

Carew, & Kandel, 1983). Since then, a variety of associative learning paradigms has been used to demonstrate synaptic plasticity in motivationally relevant brain areas. Fear conditioning, a widely studied form of associative learning has long been associated with synaptic plasticity in hippocampus and amygdala depending on the experimental parameters. Whereas cued fear conditioning is mediated by the amygdala, contextual fear requires an intact hippocampus (R. G. Phillips & LeDoux, 1992). Cued fear conditioning has been shown to strengthen amygdalar synapses and photostimulation of relevant ensembles in the amygdala elicits avoidance behaviour (Namburi et al., 2015; Rogan, Staubli, & LeDoux, 1997). Similarly, optogenetic activation of a amygdala ensemble active during contextual fear conditioning induced freezing behaviour (X. Liu et al., 2012). Interfering with LTP formation in CA1 and CA3 via genetic mutations or AMPA receptor immobilization reduces contextual fear memory, confirming the importance of hippocampal synaptic plasticity in this type of associative learning (Otto et al., 2001; Penn et al., 2017; Rampon et al., 2000). The VTA, which sends DA projections to NAc, undergoes synaptic alterations after cocaine self-administration and food-cue association (Chen et al., 2008; Stuber et al., 2008). Glutamatergic synapses in NAc itself have been shown to increase their strength after Pavlovian sucrose-cue conditioning (Namburi et al., 2015). This plasticity has been shown to be NMDA receptor dependent, as Pavlovian, but not instrumental memory consolidation using an appetitive reward was shown to depend on NMDA receptor and D1 receptor signalling in NAc (Dalley et al., 2005; Hernandez, Andrzejewski, Sadeghian, Panksepp, & Kelley, 2005; Parker, Beutler, & Palmiter, 2011). However, D1 receptor signalling may also induce synaptic strengthening via mobilization of AMPA receptors in the NAc (Mangiavacchi & Wolf, 2004). Additionally, extending the *in vitro* findings of Schotanus and colleagues, aforementioned mGluR5 receptors in the Nac shell have been shown to be involved in cue-evoked reward seeking *in vivo* (Kumaresan et al., 2009; Schotanus & Chergui, 2008a).

Similar to synaptic plasticity, early evidence for changes in intrinsic factors as mechanism behind the formation of an associative memory also comes from invertebrates due to the simple experimental conditions (Alkon, 1984; Gainutdinov, Chekmarev, & Gainutdinova, 1998). Alkon used classical conditioning in the mollusc *Hermissenda* to alter the inherent phototaxic response of the animal and showed that this was based on long-lasting changes in intrinsic excitability (Alkon, 1984). The probably first study demonstrating permanently altered intrinsic excitability elicited by Pavolvian conditioning however was performed in cats using eyeblink conditioning and intracellular recordings in motor cortex (Brons & Woody, 1980). Later studies revealed adaptations in intrinsic excitability induced by various forms of associative learning in motivationally relevant brain areas. A rewarded operant olfactory discrimination task has been shown to increase excitability in HP, BLA and piriform cortex (Motanis, Maroun, & Barkai, 2014; D Saar, Grossman, & Barkai, 1999; Zelcer et al., 2006) and fear conditioning had similar effects in the HP (McKay, Matthews, Oliveira, & Disterhoft, 2009), lateral amygdala (Sehgal, Ehlers, & Moyer, 2014), and infralimbic (IL) (Song, Ehlers, & Moyer, 2015). Surprisingly however, in some instances fear conditioning had the opposite effect, where it decreased excitability in IL cortex (Santini, Quirk, & Porter, 2008), prelimbic (PL) cortex (Song et al., 2015) and BLA (Motanis et al., 2014).

Taken together, associative learning induces widespread activity-dependent alterations at the levels of synapses and/or neuronal excitability in many motivationally relevant brain areas. However, these two mechanisms are known to occasionally occur concurrently and hence are not always separate or independent of each other (Antonov, Antonova, Kandel, & Hawkins, 2001; Campanac & Debanne, 2008; A. G. Carter & Sabatini, 2004; N. Kuczewski et al., 2008; Nicola Kuczewski, Porcher, Lessmann, Medina, & Gaiarsa, 2008; Li, Lu, Wu, Duan, & Poo, 2004; Rosenkranz & Grace, 2002; Stuart & Hausser, 2001; Titley, Brunel, & Hansel, 2017; Xu, 2005).

1.4.4 Neuronal ensembles mediate associative learning and exhibit unique physiological properties

Donald Hebb's aforementioned neuronal assembly theory (D. O. Hebb, 1949) has set the foundation for what we know today as 'neuronal ensembles' (Cruz et al., 2013b). These ensembles are sparsely distributed sets of neurons that are activated by specific stimuli and/or during learned behaviours. These ensembles can also serve as memory storage substrates or 'engrams' (Tonegawa, Liu, Ramirez, & Redondo, 2015). In response to certain stimuli not an entire brain area is activated but a subset of behaviourally relevant neurons, as Hubel and Wiesel demonstrated early on by recording firing patterns in the cat visual cortex elicited by light stimuli of different size and shape (D. H. Hubel & Wiesel, 1959; Josselyn, Köhler, & Frankland, 2015; Nicolelis, Fanselow, & Ghazanfar, 1997). However, mostly due to technical limitations the majority of studies in the past investigated a random subset of neurons in a given brain area, independently of the neuronal activity during behaviour. This may be the reason for conflicting results in the aforementioned literature on intrinsic plasticity, as they might reflect a sum of up- and downregulation of neuronal excitability in a random selection of neurons containing variable numbers of activated and non-activated neurons. In recent years it has become apparent that behaviours controlled by cue-reward associations are mediated by these sparsely distributed neurons in motivationally relevant brain areas that express the protein product of the immediate early gene (IEG) *Fos* (Cruz, Javier Rubio, & Hope, 2015; C. M. A. Pennartz, Groenewegen, & Lopes da Silva, 1994; Suto et al., 2016; Warren et al., 2016; E. A. West & Carelli, 2016; Whitaker et al., 2016, 2017; Ziminski et al., 2017a; Ziminski, Sieburg, Margetts-Smith, Crombag, & Koya, 2018).

Fos mRNA peaks approximately 20 minutes after induction and its protein product Fos peaks at 90 minutes, with degradation of the signal setting in after 4 - 5 hours (Rubio et al.,

2015; Sheng & Greenberg, 1990; Xiu et al., 2014). *Fos* induction requires extended glutamatergic activation eliciting calcium influx via NMDA receptors and voltage-gated calcium channels, which then activates extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK)-dependent phosphorylation of cyclic AMP (cAMP)-responsive element-binding protein (CREB) via ribosomal S6 kinase and phosphorylation of ELK1-serum response factor, both part transcription factors of the *Fos* promoter (Cruz et al., 2013a). Increased levels of CREB have been linked to increased neuronal excitability and behavioural performance and artificial modulation of CREB in turn is used to modify neuronal excitability (Dong et al., 2006; Sano et al., 2014; Y. Zhou et al., 2009). Hence, Fos is a marker that reflects prolonged and enhanced synaptic and cellular activity levels.

The learning and memory research field is making great advances now due to the advent of new transgenic techniques that allow Fos-expressing neurons to be characterised and manipulated. By doing so, behaviourally-relevant neuronal ensembles and their underlying adaptations are identified (Cruz et al., 2013a; Tonegawa et al., 2015). These new techniques take advantage of transgenes (e.g. *GFP,* green fluorescent protein) that are coupled to the promoter of the immediate early gene (IEG) *Fos*.

In the last 6 - 7 years, the *Fos-GFP* transgenic rodent lines have been used to investigate the physiological properties of neuronal ensembles activated during learned behaviours or by specific stimuli (Barth, 2004; Cifani et al., 2012; Koya et al., 2012; Whitaker et al., 2017; Ziminski et al., 2017b, 2018). These lines allow for the visual identification of recently activated neuronal ensembles in acute brain slices and therefore permit researchers to conduct electrophysiological recordings selectively from those neurons. Hence, aforementioned studies have been able to identify differences in AMPAR/NMDAR ratio between behaviourally activated ensembles and surrounding neurons in mPFC and NAc after stress-induced reinstatement of palatable food seeking and context-specific

sensitisation of repeated cocaine-induced locomotion, respectively (Cifani et al., 2012; Koya et al., 2012). Additionally, ensemble-specific differences in intrinsic excitability have been revealed in the PL after food self-administration, and in the NAc after cue-induced sucrose seeking and conditioned cocaine locomotion (Whitaker et al., 2017; Ziminski et al., 2017b, 2018). After the identification of such differences in physiological properties between behaviourally active neuronal ensembles and surrounding neurons functional manipulations of the parameters in question allow to investigate the functional role of these ensembles in learned behaviours (Hsiang et al., 2014).

By using Fos-based techniques to manipulate behaviourally activated neuronal ensembles researchers have been able to demonstrate the functional role of these neurons in learned behaviours. To this end, the Daun 02 method, optogenetics, as well as DREADD (designer receptor exclusively activated by designer drug) have been used. In the Daun 02 method, on a transgene the *Fos* promoter is driving the expression of an enzyme, β-galactosidase in strongly activated neurons (Cruz et al., 2013b; McReynolds, Christianson, Blacktop, & Mantsch, 2018). The inactive prodrug Daun 02 is then injected and converted by β-galactosidase to the active daunorubicin which permanently inactivates neurons (Cruz et al., 2013b; McReynolds et al., 2018). As the enzyme is only present in recently activated neurons only those are inactivated. By exposing animals to a certain stimulus, eliciting a behavioural response such as cue-evoked food seeking, then injecting Daun 02 and exposing the animals to the same stimulus, the absence or attenuation of the behavioural response indicates that the Fos-expressing ensemble is functionally relevant for the behavioural response (Cruz et al., 2013b; McReynolds et al., 2018). This method has been used to implicate NAc neuronal ensembles in context specific cocaine-induced psycho- and locomotor sensitisation (Koya et al., 2009b; Mattson et al., 2008). Additionally, different neuronal ensembles in the vmPFC have been shown to drive context-induced reinstatement

of heroin seeking as well as recall and extinction of food self-administration (Bossert et al., 2011; Warren et al., 2016).

Optogenetics, which utilises light to artificially excite or inhibit neurons expressing opsins, light-sensitive membrane proteins, has been combined with the Fos-tTA mouse line to demonstrate the functional role of hippocampal neuronal ensembles in context-induced fear conditioning (X. Liu et al., 2012). In the Fos-tTA transgene the *Fos* promoter drives the expression of a tetracycline (tet)- off transcriptional activator (tTA) protein in strongly activated neurons only. This protein binds to a TRE-promoter driving expression of another transgene, allowing its transcription, in the case of the study by Liu and colleagues channelrhodopsin-2 coupled to a fluorescent protein (Cruz et al., 2013b; X. Liu et al., 2012). Additionally, doxycycline binds to tTA and inhibits its ability to induce gene expression, hence if it is removed from the animals diet a time window for transgene expression only in strongly activated neurons opens, for instance during a behavioural task or stimulus presentation (Cruz et al., 2013b). Liu and colleagues showed that light-induced activation of the ensemble active during contextual fear conditioning elicited freezing behaviour in a different context (X. Liu et al., 2012). Similarly, Garner and colleagues used the Fos-tTA mouse line but combined it with DREADD in an attempt to demonstrate this involvement of hippocampal neuronal ensembles in contextual fear conditioning (Garner et al., 2012). In this study, the inducible transgene consisted of an artificial receptor which can be activated by an injectable drug, clozapine-N-oxide or clozapine, and in the absence of doxycycline excites the strongly activated neurons it is expressed in (Cruz et al., 2013b; Garner et al., 2012; McReynolds et al., 2018). Possibly due to technical and methodological issues however the chemical reactivation of the neuronal ensembles active during fear conditioning did not produce freezing behaviour in a different context (Cruz et al., 2013b; McReynolds et al., 2018).

1.5 Aims and hypotheses

Associative memories about appetitive cue-reward associations are encoded in neuronal ensembles in motivationally relevant brain areas. The NAc specifically plays an important role in encoding and updating the reward value and it is known that the NAc activation patterns are altered by aversive reward devaluation. Specifically, it has been demonstrated that the number of phasically firing neurons in NAc shell is reduced after reward devaluation, but the underlying mechanisms of plasticity are unclear (E. A. West & Carelli, 2016). However, this type of devaluation might not only modulate how the positive value is processed but also engages neuronal systems involved in aversion, which are distinct from those related to rewarding behaviours (Namburi et al., 2015). Therefore, here we are using a non-aversive, satiety-based reward devaluation procedure to investigate the impact of a decrease in outcome value on cue-elicited sucrose seeking and activation patterns in the striatum. Associative learning induces intrinsic and synaptic plasticity in motivationally relevant brain areas including NAc and some of these adaptations are ameliorated by disruption of the associative cue-reward contingency, such as extinction learning (Ziminski et al., 2017a). During devaluation however the cue-reward contingency is maintained while the decreased reward value is transferred to the cue. The aim of this study is to elucidate how devaluation modulates neuronal ensemble recruitment and underlying intrinsic and synaptic neuroplasticity in the NAc shell.

We hypothesise that non-aversive devaluation of a sucrose reward decreases cue-evoked sucrose seeking and that this is reflected in alterations in activity patterns as well as neuronal plasticity in NAc shell.

2 The effect of reward devaluation on cue-evoked sucrose seeking and striatal neuronal ensemble activation patterns

2.1 Introduction

Animals use environmental stimuli predicting food availability, often called food-associated cues (e.g smells), to guide foraging behaviour and optimize the location of nutrient sources (Arenas & Farina, 2012; Croney et al., 2003; Whitfield et al., 2014). Following appetitive Pavlovian learning when these cue-food associations are established, cues linked to food rewards obtain motivational significance and predictive properties regarding food availability. In turn these cues become capable of exerting powerful control over food seeking and consumption (Petrovich, 2011; Petrovich & Gallagher, 2007). However, animals also need to flexibly adapt their reactivity to food-predictive cues according to the current attractiveness of food rewards. For example, a food reward can become less attractive after excessive consumption, due to increased satiety. Consequently, the updated decreased value of the food reward is integrated with the existing cue-food association in order to inhibit responses to food-associated cues (P. C. Holland & Rescorla, 1975; Parkes & Balleine, 2013; E. A. West & Carelli, 2016). Many eating-related disorders and resulting conditions such as binge-eating and obesity are characterized by malfunctioning of this behavioural flexibility, making it an important pursuit to elucidate underlying behavioural and neural mechanisms (de Zwaan, 2001; Dingemans, Visser, Paul, & Van Furth, 2015; M. E. Roberts, Tchanturia, Stahl, Southgate, & Treasure, 2007; Yanovski, Nelson, Dubbert, & Spitzer, 1993).

An extensive array of studies have highlighted the role of the striatum in food-seeking behaviours. The dorsal striatum (DS) is known to be implicated in habitual, non goal-directed behaviours. Early lesion studies demonstrated the importance of this area for the performance of these so-called stimulus response habits, which are insensitive to outcome devaluation and hence non-goal dependent (B. J. Everitt, Cador, & Robbins, 1989; Mishkin, Malamut, & Bachevalier, 1984; Robbins et al., 1990). The Nucleus accumbens (NAc), part of the ventral striatum, on the other hand is known to play an important role in goal-directed, non-habitual behaviours, such as cue-evoked food seeking, which are sensitive to modifications of the reward value. In support of this, *in vivo* electrophysiological recordings have shown that neurons in the NAc are activated in response to sucrose-associated cues (J. J. Day, Wheeler, Roitman, & Carelli, 2006; Roitman, Wheeler, & Carelli, 2005; Wan, 2006). Additionally, inactivation of the NAc by infusion of GABA agonists or lesion of dopaminergic (DA) neurons inhibit the expression of appetitive Pavlovian approach behaviour (Blaiss & Janak, 2009; J. A. Parkinson et al., 2002). Also, sucrose-associated cues increase DA release in the NAc, particularly in the shell subregion, which is positively correlated with reward value, thus further highlighting NAc's role in encoding reward value (Cacciapaglia et al., 2012; J. J. Day, Roitman, Wightman, & Carelli, 2007; Sackett et al., 2017). Finally, NAc lesioned rats fail to modify their cue responding following devaluation of food reward, indicating their inability to utilise information regarding updated reward value (Singh et al., 2010).

Although NAc's role in updating changes in reward value has been extensively studied, the neuronal activity patterns that underlie these changes in striatal subregions have not been fully characterised yet. Recent studies have indicated that a minority of sparsely distributed, activated neurons called 'neuronal ensembles' play a pivotal role in a variety of cue-evoked behaviours including food-seeking (Cruz et al., 2015; Warren et al., 2016; Whitaker et al., 2017). Using the protein product of the immediate early gene *Fos* as an activity marker, it

has been shown that neuronal ensembles in the NAc are selectively activated in response to sucrose-associated cues and that this activation pattern is modulated by reward devaluation (E C Kerfoot, Agarwal, Lee, & Holland, 2007; Ziminski et al., 2017a). However, many studies to date have investigated NAc ensemble activity following reward devaluation by pairing a food reward with aversive stimuli (e.g. lithium chloride) (E C Kerfoot et al., 2007). Aversive and rewarding stimuli are known to engage different neurobiological pathways modulating NAc activity via inputs from upstream areas such as the ventral tegmental area (VTA). Therefore, here we aimed to investigate neuronal underpinnings of updating reward value using a 'satiation' procedure (i.e. overconsumption of a food reward) without the interference of aversion-specific processes (Brischoux, Chakraborty, Brierley, & Ungless, 2009; Mark A Ungless, Magill, & Bolam, 2004; Yamaguchi, Sheen, & Morales, 2007).

Therefore, in this chapter we examined the effects of sucrose reward devaluation via four days of *ad libitum* sucrose access on cue-evoked sucrose seeking behaviour and striatal activity using Fos immunohistochemistry in sucrose conditioned mice. Based on the role of the NAc, but not DS, in updating the value of a food reward, we hypothesized that reward devaluation attenuates cue-evoked sucrose seeking and modulates neuronal activation patterns in the NAc, but not DS.

2.2 Materials and Methods

2.2.1 Animals

Male wild-type C57BL/6 mice were purchased from Charles River UK. Male heterozygous *Fos-GFP* mice (https://www.jax.org/strain/014135, RRID:IMSR_JAX:014135) on a C57BL/6 background that originated from the laboratory of Allison Barth (Carnegie Mellon University) were obtained from the in-house breeding programme at the University of Sussex (UK). All mice were housed 2 - 3 per cage and maintained on a 12 hour light/dark cycle (lights on at 7:00) at a temperature of 21 ± 1 °C and 50 ± 5 % humidity, and had access to standard chow (BK001 E Rodent Breeder and Grower diet, SDS) and *ad libitum* water. One week prior to and during the entire duration of the behavioural experiments, mice were food restricted to 90 % of their baseline body weight. Mice were 9 - 10 weeks old at the beginning of behavioural testing. *Fos-GFP* mice were used for experiments examining the effects of devaluation on Pavlovian approach, a way of measuring cue-evoked sucrose seeking and Fos expression (Figs.2 A, B, 3), and wild-type mice were used for the experiments examining the effects of caloric satiation on Pavlovian approach (Figs.2 C, D). *Fos*-GFP mice express a GFP-Fos fusion protein in recently activated neurons. These mice perform similarly to wild type mice in conditioning experiments and have been used in multiple published studies (Barth, 2004; Cifani et al., 2012; Cruz et al., 2013a; Koya et al., 2012; Ziminski et al., 2017b, 2018; unpublished observations). We used both strains due to availability in order to adhere to the 3 R's. All experiments were conducted during the light phase and in accordance with the UK Animals (Scientific Procedures) Act of 1986 after ethical approval by the University of Sussex Animal Welfare and Ethical Review Body.

2.2.2 Behavioural experiments

2.2.2.1 Apparatus

A similar procedure as described in Ziminski et al., 2017 was used (Ziminski et al., 2017b). Briefly, all behavioural procedures were carried out in conditioning chambers (15.9 x 14 x 12.7 cm, Med Associates) enclosed within a sound attenuating and light-resistant cubicle. Each chamber was fitted with a recessed magazine situated in the center of one side-wall which dispensed 10 % sucrose solution serving as the unconditioned stimulus (US). An infra-red beam detected head entries into the magazine. The house light was situated in the side panel and was on for the duration of the behavioural experiments. A mechanical click generator served as an auditory conditioned stimulus (CS) (Med Associates). Initiation and running of behavioural protocols, including the recording of head entries into the food magazine, was performed using Med-PC IV (MedAssociates Inc., RRID:SCR_014721).

2.2.2.2 Behavioural procedures

Prior to conditioning, mice underwent a single session of magazine training, which began following the initial head entry into the food magazine. During this session they received forty presentations of 10 % sucrose solution (13 µl) in the food magazine on a random interval 30 (RI30) schedule in order to get accustomed to the sucrose delivery magazine. The next day, mice underwent 11 - 12 Pavlovian conditioning sessions (on average 24 minutes per session; 1 -2 times daily in the morning (8 - 12 noon) and/or afternoon (12 noon – 5 pm) over 7 consecutive days. The illumination of the house light indicated the start of

each session, which consisted of six 120 s CS presentations (presented simultaneously in all conditioning chambers), separated by 120 s RI inter-trial interval (ITI) periods. During each 120 s CS period, 13.3 µL of 10 % sucrose solution was delivered into the magazine on a RI-30 s schedule. Following conditioning, mice remained in the colony room for 7 - 9 days until test day. Three days following the final conditioning session, mice were randomly allocated to one of two groups for the remaining 4 - 6 days for: 1) devaluation experiments in which all mice continued to be food restricted, and one group of mice (Devalued group) received *ad libitum* sucrose solution in their home cage whereas the control group (Non-devalued group) did not; 2) caloric satiation experiments in which one group of mice (*ad libitum* chow group) received *ad libitum* chow whereas the Control group continued to be food restricted until test day. For the caloric satiation experiments we also ran an additional Unpaired group which received the same amount of sucrose as the paired mice but in the home cage, independent from the conditioning chambers and the cue. This was done to make sure that none of the observed behavioural changes were due to general sucrose consumption. On test day, mice underwent Pavlovian approach testing, a way to measure cue-evoked sucrose seeking consisting of a single session that was similar to the conditioning session, but under extinction conditions (i.e. without sucrose delivery). This was done to prevent the mice from experiencing the decreased value of the sucrose in the presence of the cues and to exclude neuronal processes to be driven by sucrose consumption. For a timeline of the behavioural procedure see Figure 1 A.

2.2.3 Fos immunohistochemistry

Following testing for Pavlovian approach, mice from the devaluation experiments remained in the conditioning chambers for an additional ~1 h to allow for optimal Fos expression (Ziminski et al., 2017b). Subsequently, they were anaesthetized using sodium pentobarbital in saline (1:10, 10 ml/kg, 200 mg/kg, i.p.). Mice were transcardially perfused with ice cold

PBS (potassium buffered saline, 137 mM NaCl, 10 mM P_4O_3-, 2.7 mM KCl, pH 7.4) for 5 minutes (5 ml/min) and with ice cold 4 % paraformaldehyde (PFA, Sigma-Aldrich catalog no 158127) for 20 minutes (5 ml/min) using a peristaltic pump (Masterflex L/S, Cole Parmer). Thirty minutes after the end of the perfusion brains were removed, post-fixated in 4 % PFA at 4 ºC for approximately 22 h, and then cryoprotected in 30 % sucrose solution in PBS for 3 - 5 days. Brains were frozen on dry ice and stored at -80 ºC until further use. Brains were sliced into 30 µm coronal sections containing NAc and DS (AP 1.5 - 1.0; Paxinos, G and Franklin, 2012) using a cryostat (Leica CM 1900, Leica Microsystems) and stored in PBS and azide (0.2 %) or cryopreservant.

Free-floating slices were washed 3 times for 10 minutes in PBS, incubated in 0.3 % hydrogen peroxide in PBS for 15 - 20 minutes to block endogenous peroxidase activity and subsequently washed 3 times in PBS. To block unspecific binding sites and permeabilize cell membranes, slices were incubated in 3 % NGST (normal goat serum triton, RRID:AB_2336615; Vector Laboratories) for 1 h. Slices were incubated in primary antibody (rabbit anti c-Fos, sc-52, LOT: A2914, Santa Cruz Biotechnology, 1:8000, RRID:AB_2106783) in 3 % NGST over night at 4 ºC. Next, slices were washed 3 times in PBS and incubated in the secondary antibody (biotinylated anti-rabbit lgG H+L, Vector labs, 1:600, RRID:AB_2313606) in 1 % NGST for 2 h. After 3 subsequent washes in PBS slices were incubated in ABC solution (RRID:AB_2336818, Vectorlabs) for 1 h and then washed twice in PBS. Slices were incubated in 0.04 % DAB, 0.05 % nickel ammonium sulfate, 0.04 % hydrogen peroxide in PBS for approximately 30 minutes and washed 3 times in PBS. Slices were mounted in water onto Superfrost slides (Fisher) and dried overnight. For dehydration, slides went through the following steps: 2 x distilled water on ice 3 minutes, 30 % ethanol 2 minutes, 60 % ethanol 2 minutes, 90 % ethanol 2 minutes, 95 % ethanol 2 minutes, 100 % ethanol 2 minutes, 100 % ethanol 2 minutes, 2 x HistoClear (National

Diagnostics) 10 minutes. Finally, slides were coversliped using Histomount (National Diagnostics), dried overnight and stored at room temperature.

Brightfield images of the NAc shell, core, and DS were taken using a QI click camera (Qimaging) attached to an Olympus BX53 brightfield microscope and iVision-Mac software (Biovision Technologies, version 4.0.15, RRID: SCR_014786). Fos-positive cells were counted manually bilaterally in a blind manner at a magnification of 100x using iVision software. The analysis was restricted to medial proportions due to low Fos expression in lateral NAc shell, core, and DS.

2.2.4 Experimental Design and Statistical Analysis

Data were analysed and visualized using GraphPad Prism 6 (Graphpad software, RRID:SCR_002798), SPSS (IBM SPSS statistics, RRID:SCR_002865), and Excel (Microsoft). All data is presented as mean ± SEM. In text and figures values of mean ± 2xSD were considered outliers. In chapter 2 for the Fos analysis three outliers were detected in the NAc shell and four outliers were detected in the DS. Normal distribution was assessed using visual inspection of the data and Kolmogorov-Smirnoff tests. For repeated measures data, normality testing was performed on the standardized residuals. If independent sample data sets or the majority of a repeated measures data set deviated from normal distribution, non-parametric tests were used to confirm the results of the parametric tests. In data sets following a Gaussian normal distribution, an alternative method of outlier detection was used (Grubb's test). See Appendix 1 for normality testing and non-parametric tests, as well as reanalysis of data with altered number of outliers according to Grubb's. ANOVAs with significant interactions or main effects were followed up by *Fisher's* LSD post-hoc test. This test is used regularly to compare between a low number of groups (for Fos analysis with more groups a Sidak test was used) which show a significant main effect in an ANOVA,

and/or significant interaction (Howell, 2014; Rubio et al., 2015; Whitaker et al., 2016; Ziminski et al., 2017b).

2.2.4.1 Behavioural data

Total number of head entries into the sucrose-delivery magazine during acquisition were analysed using a two-way repeated measures ANOVA including cue presentation (ITI, CS) and session (1 - 12) as within-subjects factors. The test data was analysed using two-way mixed ANOVAs using cue presentation (ITI, CS) as within-subjects factor and devaluation (Non-devalued, Devalued) or caloric satiation (Control, *ad libitum* chow) as between-subjects factor. Body weights were analysed using an unpaired two-tailed t-test. Four mice from the *ad libitum* chow and Devalued groups had to be excluded from the test analyses due to equipment malfunction.

2.2.4.2 Fos expression

Fos quantification data was analysed using multiple unpaired two-tailed t-tests comparing the number of Fos+ cells per square millimetre between Non-devalued and Devalued conditions independently for each brain area (NAc shell rostral, NAc shell caudal, NAc core rostral, NAc core caudal, dorsal striatum rostral, dorsal striatum caudal). A Sidak test was performed to correct for multiple comparisons. Brain sections from one mouse were damaged and could not be used for cell quantification.

2.3 Results

2.3.1 Acquisition of Pavlovian conditioning

We assessed the establishment of a cue-sucrose association following 12 sessions of Pavlovian conditioning, during which an auditory cue (clicker) was repeatedly paired with sucrose delivery (Figure 1 A). Mice made significantly higher levels of head entries into the sucrose delivery magazine during CS presentations (cue and sucrose presentation) due to decreased responding during the ITI (no cue, no sucrose) (Figure 1 B). A two-way repeated measures ANOVA including cue presentation (ITI, CS) and session (1 - 12) as within-subjects factors revealed a significant effect of cue presentation ($F_{1,31} = 321$, $p \leq 0.001$), session ($F_{11,341} = 9.957$, $p \leq 0.001$), and interaction of the two factors ($F_{11,341} = 18.12$, $p \leq 0.001$), indicating that they had learned the association between the cue and sucrose delivery.

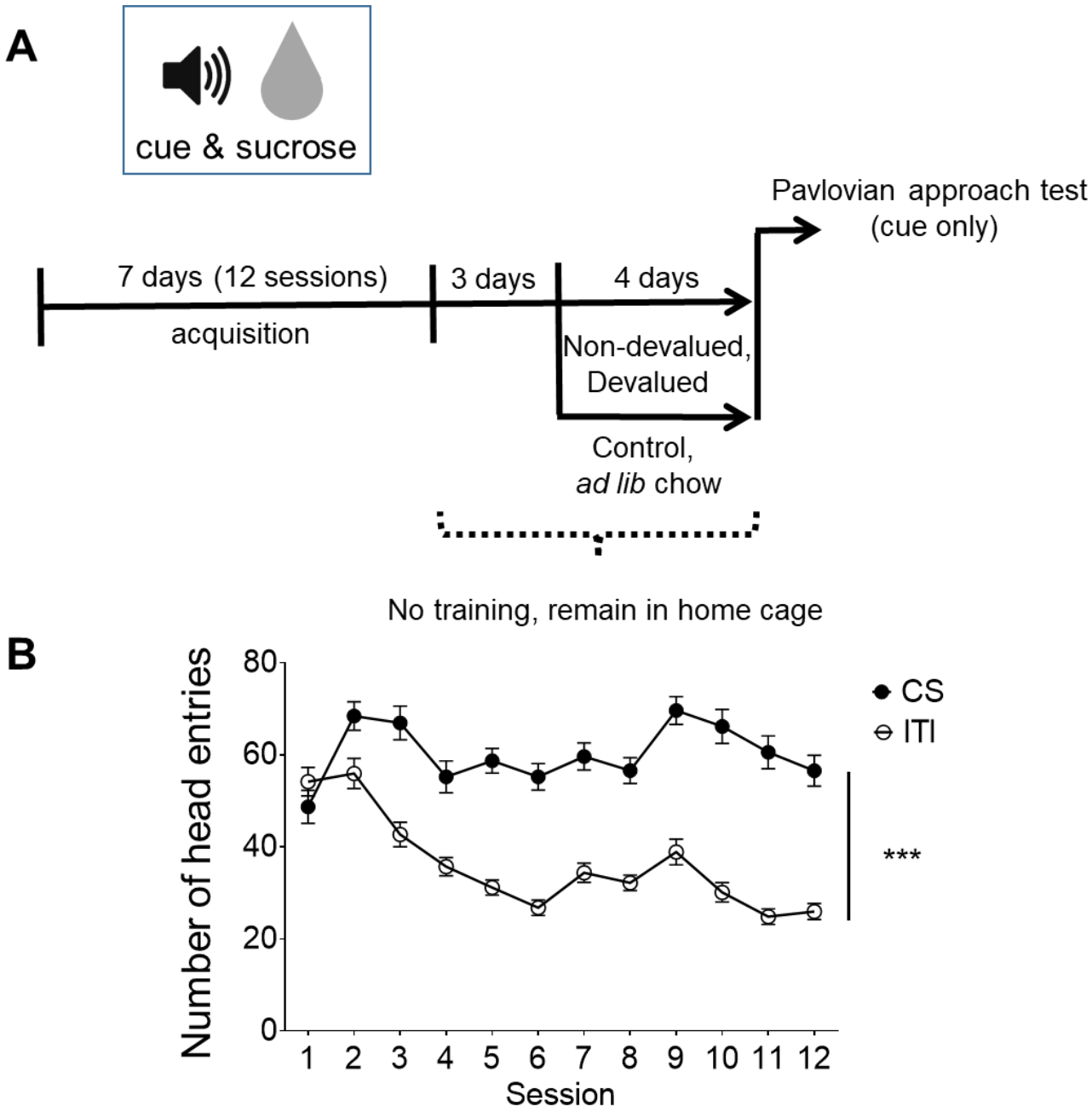

Figure 1: After 12 sessions of acquisition mice learned to associate a cue with sucrose delivery. (A) Timeline of behavioural experiments. (B) Number of head entries in sucrose delivery magazine during acquisition in response to sucrose-associated cue (CS) are significantly higher than during intertrial interval (ITI), n = 32, asterisk indicate main effect of trial, ***$p < 0.001$. Symbols indicate mean ± SEM.

2.3.2 Pavlovian approach is attenuated by sucrose reward devaluation but not caloric satiation

Seven days after the last acquisition session and after 4 - 6 days of either *ad libitum* chow or 10 % sucrose solution in the home cage mice underwent Pavlovian approach testing under extinction conditions (no sucrose delivery, only cue, see Figure 1A).

2.3.2.1 The effects of reward devaluation on Pavlovian approach

We first assessed the effect of sucrose reward devaluation on Pavlovian approach. A two-way mixed ANOVA using cue presentation (ITI, CS) as within-subjects factor and devaluation (Non-devalued, Devalued) as between-subjects factor showed a significant effect of cue presentation ($F_{1,28}$ = 27.84, $p < 0.001$) and a significant interaction of cue presentation X devaluation ($F_{1,28}$ = 5.275, $p = 0.03$). Post-hoc tests revealed a significant difference in head entries in the Non-devalued group between CS and ITI ($p < 0.001$). Also, it revealed significantly lower CS-induced head entries in the Devalued compared to Non-devalued group ($p = 0.008$). Thus, as in the Devalued group the cue-evoked sucrose seeking behavior that was displayed in the Non-devalued group was not present anymore, sucrose devaluation attenuated cue-elicited Pavlovian approach (Figure 2 A).

Frequent sucrose consumption results in weight gain (Te Morenga, Mallard, & Mann, 2013). Thus, as a measure for sucrose consumption, we measured the body weights of Devalued and Non-devalued mice. An unpaired two-tailed t-test (t_{30} = 8.629, $p < 0.001$) revealed that mice in the Devalued group exhibited significantly higher body weights than their Non-devalued counterparts (Figure 2 B), indicating that mice in the Devalued group consumed a significant amount of sucrose.

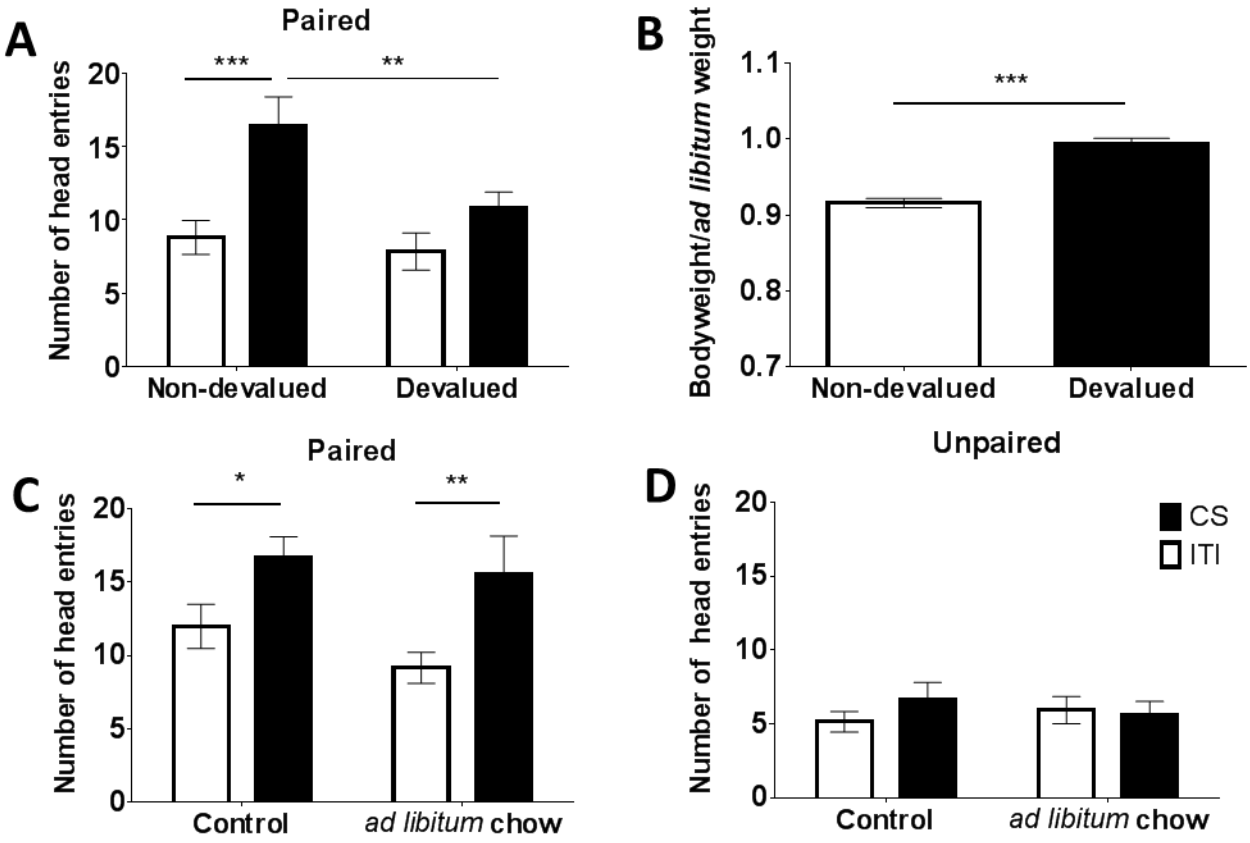

Figure 2: Sucrose reward devaluation, but not caloric satiation, attenuates Pavlovian approach behaviour. (A) Number of head entries during Pavlovian approach test in Paired group in Non- devalued and Devalued animals. Head entries during the cue are significantly higher only in the Non-devalued condition. ** $p < 0.01$, *** $p < 0.001$. n = 14-16 per group. (B) Bodyweight in Non- devalued animals is significantly lower than in sucrose group. ***$p < 0.001$. n = 16 per group (C) Number of head entries during Pavlovian approach test in Paired group in *ad libitum* chow and Control animals. Head entries during the cue are significantly higher. *$p \leq 0.05$, ** $p \leq 0.01$. n = 12 - 14 per group. (D) No difference in number of head entries during Pavlovian approach test during sucrose associated cue (CS) and intertrial interval (ITI) in Unpaired group in *ad libitum* chow and Control animals. n = 14 - 16 per group. All values are mean ± SEM.

2.3.2.2 The effects of caloric satiation on Pavlovian approach

Next, to rule out the effects of increased caloric consumption during the devaluation procedure, we trained an additional group of animals using the same behavioural procedure as above, but instead we provided them with *ad libitum* chow in their home cages in place of sucrose (Figure 1 A). In the Paired group (Figure 2 C), caloric satiation did not modulate cue-evoked sucrose seeking compared to the Control group, but cue presentations increased the number of head entries during the CS, as shown by a two-way ANOVA using cue presentation (ITI, CS) as within-subjects factor and caloric satiation (Control, *ad libitum* chow) as between-subjects factor (interaction cue presentation x caloric satiation $F_{1,24} = 0.334$, $p = 0.569$; cue presentation $F_{1,24} = 14.26$, $p < 0.001$; caloric satiation $F_{1,24} = 1.081$, $p = 0.31$). Targeted post-hoc tests revealed a significant difference between the number of head entries made during CS and ITI in Control mice ($p = 0.03$) and *ad* libitum chow animals ($p = 0.007$). As in contrast to the devaluation experiment (Figure 2 A) we did not find an interaction here, this suggests that cue-evoked sucrose seeking was not attenuated by caloric need alone. In contrast, in Unpaired mice (Figure 2 D) neither group of mice exhibited increased cue-evoked sucrose seeking, indicated by the absence of an effect of cue presentation ($F_{1,28} = 0.53$, $p = 0.27$; within-subjects factor; ITI, CS). Moreover, there was no effect of caloric satiation ($F_{1,28} = 0.01$, $p = 0.92$) or interaction ($F_{1, 28} = 1.242$, $p = 0.275$). Hence, the mice did not display cue-evoked sucrose seeking as they never established an association between the two. This result confirms that the effects observed in the paired sucrose and chow groups were not due to external factors such as handling-induced stress or exposure to the conditioning chambers.

2.3.3 Sucrose reward devaluation attenuates Fos expression in Nucleus accubens but not dorsal striatum

Next, we assessed the effects of devaluation on neuronal ensemble activity in the NAc and the DS, which differ in their behavioural roles, by examining the number of Fos-expressing neurons in these areas (Figure 3). Unpaired two-tailed t-tests revealed a significant reduction in Fos positive cells in rostral NAc shell ($t = 2.761$, $p = 0.041$) and rostral NAc core ($t = 2.735$, $p = 0.041$) in the Devalued group compared to Non-devalued group, but not in caudal NAc core ($t = 2.163$, $p = 0.122$) or shell ($t = 1.12$, $p = 0.597$) indicating that a smaller ensemble was recruited in these brain areas following sucrose devaluation (Figure 3 A - C). We also assessed whether these expression patterns would generalise to the DS, a brain area that has been shown to play a role in habitual responses, a behaviour that is distinct from those mediated by the NAc. We found no significant differences between Control and Devalued groups (caudal $t = 0.467$, $p = 0.641$, rostral $t = 1.046$, $p = 0.597$) indicating that sucrose reward devaluation decreases ensemble size in the NAc, but not DS (Figure 3 D, E).

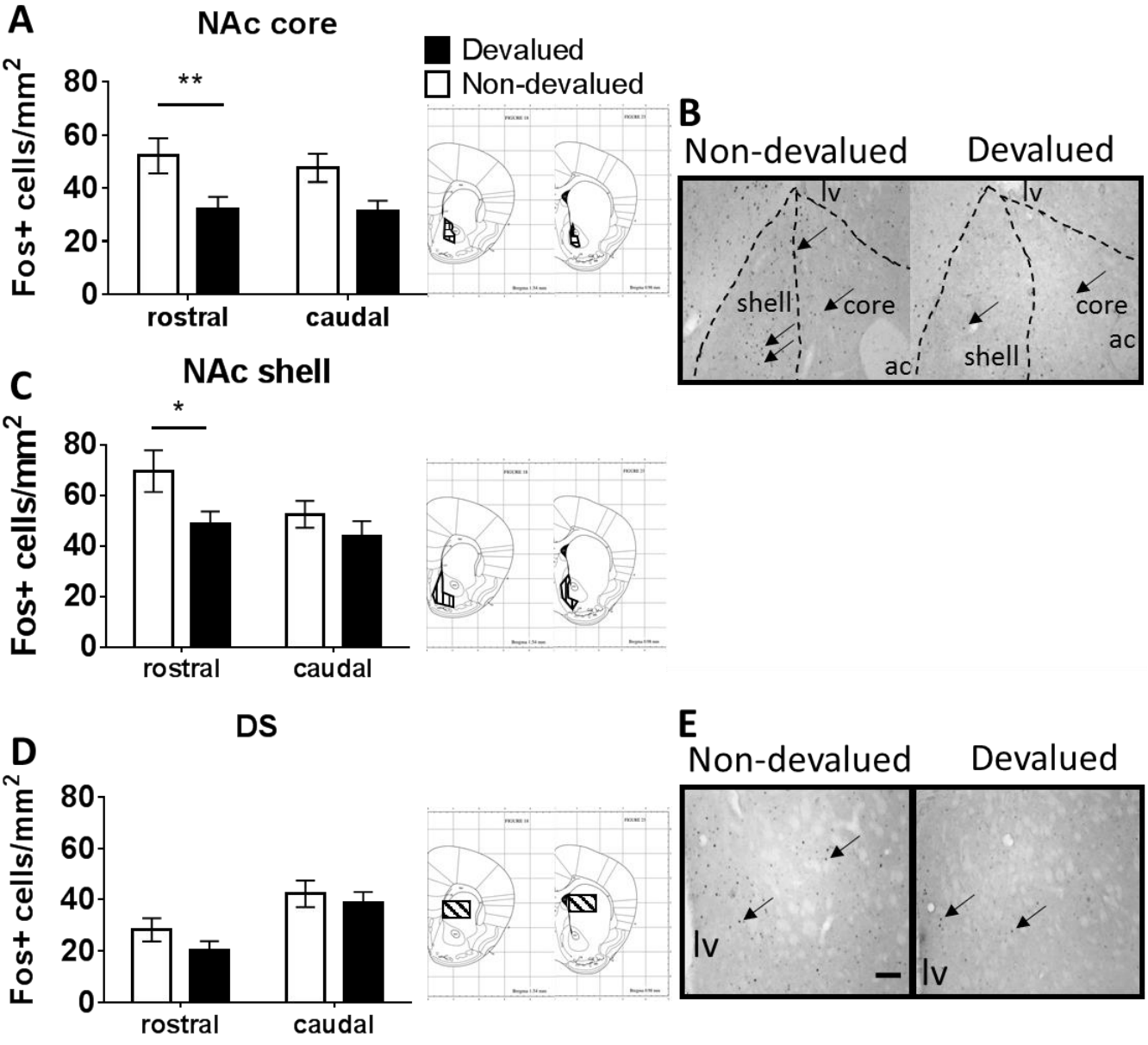

Figure 3: Fos expression in the striatum. (A) In rostral, but not caudal nucleus accumbens (NAc) core sucrose devaluation decreased the number of Fos positive cells. N = 13 - 15 per group, * $p \leq 0.05$. (B) Representative image of Fos staining in NAc in Non-devalued and Devalued groups. (C) In caudal and rostral NAc shell sucrose devaluation decreased Fos expression. N = 13 - 14 per group, * $p \leq 0.05$. (D) In caudal and rostral dorsal striatum (DS) sucrose devaluation did not modulate Fos expression. N = 13 - 14 per group. (E) Representative image of Fos staining in DS in Non-devalued and Devalued groups. All values are mean ± SEM. Arrows indicate exemplary Fos positive cells. ac = anterior commissure, lv = lateral ventricle, scale bar 100 µm, schematic overviews modified after Paxinos and Franklin, 2001.

2.4 Discussion

Here we examined the influence of sucrose reward devaluation on cue-evoked sucrose seeking and neuronal ensemble size using a Pavlovian conditioning paradigm. We found that devaluation, but not caloric satiation alone, attenuated cue-evoked sucrose seeking. Moreover, devaluation decreased the size of the recently activated, cue-responsive neuronal ensemble in the NAc shell and core, but not DS. Hence, behavioural responding to sucrose cue presentations depends on utilising information on the current attractiveness or value of sucrose reward.

2.4.1 Reward devaluation by *ad libitum* reward exposure

Our results are in line with previous studies showing attenuation of cue-evoked sucrose seeking after food reward devaluation. Commonly used methods of reward devaluation decrease the value of the US, by decreasing the reward's attractiveness through sensory specific satiety (Glueck et al., 2015; P. C. Holland & Rescorla, 1975; Parkes & Balleine, 2013; E. A. West & Carelli, 2016) via excessive consumption of a particular food reward. Here we utilised a similar approach to devalue the US via *ad libitum* exposure to sucrose. This approach does not involve any aversive stimuli (e.g. LiCl, rotation or footshock, see Holland & Rescorla, 1975; Erin C Kerfoot, Agarwal, Lee, & Holland, 2007; Kraemer, Hoffmann, Randall, & Spear, 1992; Singh, McDannald, Haney, Cerri, & Schoenbaum, 2010) and thus our neurobiological findings may not be confounded by neuronal activation induced by aversive memory recall.

2.4.2 Hedonic, but not homeostatic needs control cue-evoked sucrose seeking

The attenuation of cue-evoked sucrose seeking after sucrose devaluation is likely due to a reduction in hedonic properties of sucrose. Sweetness generates positive affective responses across many mammalian species, as measured by quantification of

consummatory licks (Davis, 1973), operant responding (Madsen & Ahmed, 2014; Reilly, 1999), taste-induced facial expressions (Pfaffmann & Norgren, 1977; J E Steiner & Glaser, 1995), and self-reported pleasantness (D. a Thompson, Moskowitz, & Campbell, 1976). Our results are likely to reflect a decrease in pleasurable properties of sucrose due to oversaturation in the oral cavity, not an effect of decrease in homeostatic need. This is supported by our finding that even though *ad libitum* sucrose exposure increases bodyweight back to 100 %, caloric satiation alone by *ad libitum* chow exposure did not attenuate cue-evoked sucrose seeking. Additionally, it has recently been shown that overconsumption of sucrose in mice leads to a decrease in palatability and consequently a decrease in consumption (Strickland, Austen, & Sanderson, 2018). Similarly, increasing the concentration of sucrose in a solution is known to lead to a U-shaped curve of palatability (Sclafani & Clyne, 1987; D. a Thompson et al., 1976; Wundt, 1905). Due to this decreased palatability of the sucrose reward, mice in the current experiment are likely to have updated the outcome representation according to its new less attractive and palatable value and accordingly adapted their cue-evoked sucrose seeking.

Caloric satiety induces mechanisms including stimulation of the vagus nerve via gastric distension (R. J. Phillips & Powley, 1998) and release of adipocytic, intestinal and pancreatic peptides such as leptin, cholecystokinin and insulin, leading to activation of the brain stem and subsequently lateral hypothalamus (LH) (Cano, Caicoya, & Ruiz-Gayo, 2003; Hellström et al., 2004; Porte, Baskin, & Schwartz, 2002; Ritter, Covasa, & Matson, 1999). However, these mechanisms potentially induced by caloric satiation in previously food restricted mice were not sufficient to attenuate cue-evoked sucrose seeking, and therefore *ad libitum* sucrose induced satiety is likely to occur via different mechanisms. One factor could be that the sucrose was presented in liquid form whereas the chow was in solid form. Carbohydrate calories have been shown to be consumed more readily in liquid form than in solid form

(Dimeglio & Mattes, 2000), possibly leading the mice to consuming more calories of the sucrose solution than the solid chow while eliciting less satiety, due to faster gastrointestinal passage and less gastric distension. Another factor to consider is the difference in hormonal response to chow and sucrose. Indicators for metabolic syndrome such as increased plasma insulin and glucose levels have been found in rats after a period of sucrose-rich diet as short as 3 - 4 weeks, but not after only 5 days, making it unlikely that the subjects in the current study suffered from chronic metabolic changes due to their sucrose devaluation (Beilharz, Maniam, & Morris, 2014; Pranprawit, Wolber, Heyes, Molan, & Kruger, 2013; Soria, D'Alessandro, & Lombardo, 2001). The acute effects of a sucrose solution however differ from those of standard chow as this contains a smaller amount of sucrose, triggering a more pronounced increase in plasma insulin and glucose levels and presumably subsequently a higher activation of brainstem, hypothalamus and other downstream targets (Bowtell et al., 2000).

Artificial non-caloric sweeteners, such as saccharin, have been previously used in order to tear apart hedonic and homeostatic aspects driving sucrose-seeking behaviour. Grimm and colleagues demonstrated that sucrose and saccharin elicit similar levels of cue-elicited seeking behaviour and concluded that this behaviour does not depend on caloric need (Aoyama, Barnes, & Grimm, 2014). In line with this, consumption of a saccharin solution has been shown to elicit similar effects on AMPA receptor recruitment in NAc as sucrose consumption, indicating that Grimm's behavioural findings may extend to the level of synaptic plasticity (Tukey et al., 2013). Phasic dopamine release in NAc however was larger in response to sucrose- compared to saccharin-associated cues, indicating that palatability alone may not be sufficient to drive cue-evoked food seeking behaviour (Mccutcheon, Beeler, & Roitman, 2012). These seemingly conflicting results may be due to different experimental setups, including the concentration of saccharin, its taste, as well as the

animals' habituation to the inherently bitter solution (or pellet) and the water deprivation status. Before future studies using saccharin can be used to further elucidate the exact roles of the hedonic and homeostatic components of sucrose in behavioural and physiological changes, these parameters need to be refined to avoid any differences in taste and hence palatability between sucrose and saccharin.

2.4.3 CS-US Contingency degradation as a potential mechanism?

An alternative possibility to explain our behavioural results would be a weakening of the unconditioned stimulus (US)-predictive properties of the CS. This can be achieved by contingency degradation and entails presenting the US independently of the CS, attenuating cue-elicited approach (R A Rescorla, 1966; Robert A. Rescorla, 1968). This may indicate that in the current study, the presentation of the sucrose *ad libitum* in the home cage may have produced Pavlovian contingency degradation, resulting in a reduction of the cue's excitatory properties. However, sucrose exposure occurred outside of the training context in the home cage which may not effectively degrade the CS-US contingency since devaluation has been shown to be context-sensitive and is potentiated when performed in the original training context (Parkes, Marchand, Ferreira, & Coutureau, 2016). Hence, we favour the interpretation that our devaluation effects were not due to CS-US contingency degradation.

2.4.4 The reduction in Fos likely represents a decrease in the incentive properties of sucrose

After reward devaluation we found significant decreases in Fos expression in the NAc core and shell, but not DS. Correspondingly, performance of goal-directed tasks involving rewarding outcomes is known to be mediated by the NAc, but not DS, which is known to be more implicated in habitual, non-goal directed behaviour. This has been shown by early studies demonstrating that lesions to the NAc but not DS impair amphetamine-induced

amplification of reward-associated cue responsivity (Robbins et al., 1989). Conversely, acquisition and performance of a habitual, non-conditioned discrimination task is impaired by DS but not NAc lesions (Cole & Robbins, 1987; Robbins et al., 1989, 1990). Additionally, microinjections of amphetamine in the NAc but not DS produce conditioned place preference to the context in which the drug was given (Carr & White, 1983) . Later studies using more targeted lesions or inactivation revealed that it is the dorsolateral subportion (DLS) which mediates habitual behaviours, whereas the dorsomedial portion (DMS) is involved in goal directed behaviours (Yin et al., 2004; Yin, Knowlton, et al., 2005; Yin, Ostlund, et al., 2005). This seems at odds with our Fos data as we mostly investigated the DMS but did not see any changes in Fos expression after a goal-directed task. A study by Corbit and colleagues however investigated the involvement of the DS subregions in stimulus outcome association specifically by using Pavlovian conditioning similar to the current study, whereas previous studies had used instrumental conditioning, hence investigated response outcome associations (Corbit & Janak, 2010). Inactivation of the DLS only affected responding in Pavlovian, not instrumental conditioning and in the DMS it affected instrumental conditioning but only in the posterior DMS inactivation attenuated responding in a Pavlovian paradigm (Corbit & Janak, 2010).

Taken together, these studies demonstrate that the NAc is involved in motivational processes and goal-dependent stimulus-reward associations, whereas the DLS is more important for habitual, non-goal directed stimulus-response behaviours and instrumental, stimulus outcome associations (Cole & Robbins, 1987; Corbit & Janak, 2010; Mishkin et al., 1984; Robbins et al., 1989, 1990). Additionally, the anterior DMS is critical for instrumental response outcome associations but not Pavlovian stimulus outcome associations, and the posterior DMS is required for both (Corbit & Janak, 2010). Since in the present study cue-evoked sucrose seeking was sensitive to devaluation, this behaviour was a goal-directed

behaviour, and not an inflexible, habitual response. In light of this, it is interesting that we observed decreased recruitment of NAc, but not DS, specifically anterior DMS and parts of DLS, ensembles following devaluation, further supporting the different functional roles of these areas stated in the literature.

In the current study, it is likely that the decrease in incentive properties of sucrose after devaluation is reflected in the observed decreases in NAc Fos expression. Both, NAc core and shell, have been shown to mediate the hedonic properties of sucrose, as shown by increased levels of dopamine after sucrose consumption and a correlation between NAc firing and DA levels and sucrose palatability (Genn, Ahn, & Phillips, 2004, Hajnal, Smith, & Norgren, 2004; Taha, 2005, but also see Bassareo, De Luca, Di Chiara, 2002). Similarly, sucrose associated cues are known to increase dopamine levels in NAc core and shell (Cacciapaglia et al., 2012; Papageorgiou, Baudonnat, Cucca, & Walton, 2016; Sackett et al., 2017).

However, in addition to these complementary functions, the NAc core and shell are likely to play distinct roles related to reward-associated cues and motivated behaviours which cannot be derived from our present data but from the literature (Valentina Bassareo, De Luca, & Di Chiara, 2002; Floresco, McLaughlin, & Haluk, 2008; Saddoris, Cacciapaglia, Wightman, & Carelli, 2015; E. A. West & Carelli, 2016). For example, the cue-elicited increase in NAc dopamine shown by Saddoris and colleagues is most pronounced and long lasting in the NAc shell, and correlates with the magnitude of the reward, contributing to evidence for a special role of the shell in tracking the value of an outcome (Ahn & Phillips, 1999; Beyene, Carelli, & Wightman, 2010; Cacciapaglia et al., 2012; Genn et al., 2004; Sackett et al., 2017). Further evidence for this role is provided by studies that show an increased number of Fos protein expressing cells and also increased *Fos* mRNA levels in the NAc shell in response to reward-associated cues in sign-trackers who assign incentive value to cues, but not goal-

trackers (Flagel et al., 2011; Cogan et al. 2018). Additionally, Blaiss and Janak have shown that muscimol-induced inactivation of the core and shell to attenuate Pavlovian approach behaviour, whereas only inactivation of the shell decreased behavioural efficiency and hence might be involved in inhibiting unwanted responses (Blaiss & Janak, 2009). In line with this, a study measuring cue-induced reinstatement of food seeking in which animals lever pressed for a cue followed by a food reward, pre-test muscimol-induced core inactivation inhibited cue-induced responses, indicating a disrupted cue-outcome association, whereas shell inactivation persistently increased responding, leaving the animals unable to adapt to the extinction conditions of the cue-only reinstatement test (Floresco et al., 2008). Similarly, decreasing the value of a sucrose reward or a sucrose-predictive cue, through devaluation or extinction, respectively, has been shown to decrease NAc shell activation and the number of phasically cue-responsive neurons (E C Kerfoot et al., 2007; E. A. West & Carelli, 2016; Ziminski et al., 2017a). Additionally, West and Carelli have shown that in the shell the number of neurons whose activity is cue-modulated is decreased after outcome devaluation, whereas cue-responsivity in the core predicted behavioural flexibility after devaluation (E. A. West & Carelli, 2016). Interestingly, the current literature supports our finding that it is specifically the rostromedial proportion of the NAc shell playing a role here (E C Kerfoot et al., 2007; Reynolds & Berridge, 2002; Stratford & Kelley, 1997). Finally, underlining the role of the core in the formation of CS-US associations are findings of impaired acquisition of Pavlovian conditioning after glutamate antagonist infusion or lesion of the core but not shell (Dalley et al., 2005; John A. Parkinson et al., 2000).

To summarise, it seems likely that whereas the NAc shell encodes the current value of a reward, its reinforcing properties, and appropriate adaptation of behaviour, the core is more

important for the formation and expression of the CS-US association, explaining why in the current study we observed decreases in both, NAc shell and core Fos expression.

2.4.5 The role of local inhibitory mechanisms in decreased Nucleus accumbens ensemble activation

At the local circuit level, there are several possible mechanisms behind the observed decreases in NAc activation. One possibility is that the neuronal activation after devaluation is reduced internally via lateral inhibition, directly between medium spiny neurons (MSNs), the GABAergic principal neurons of the NAc (Chang & Kitai, 1985; Chang, Wilson, & Kitai, 1982; Taverna, Canciani, & Pennartz, 2005). Alternatively, lateral inhibition between local interneurons and MSNs may control the size of the cue-responsive ensemble (Burke et al., 2017). Finally, feedforward inhibition of specific inputs in the NAc may play a role in ensemble selection and devaluation.

Taverna and colleagues have suggested that lateral inhibition between principal cells within the NAc and its modulation by dopamine may play a role in ensemble selection and response selection, meaning it could be involved in the behavioural flexibility required for the task in the current study (Burke et al., 2017; Taverna et al., 2005). However, synapses between MSNs are relatively weak and predominantly located on distal dendrites, hence firing of individual MSNs is insufficient to affect firing of other cells (M. J. Tunstall, Oorschot, Kean, & Wickens, 2002; Charles J. Wilson, 2007). With a sufficiently high number of MSNs firing together and inhibiting a different group of cells, while not being interconnected amongst themselves, an effect of MSN ensemble firing on nearby ensembles may appear plausible. However, practical evidence for this is lacking and the probability of a strong connectivity between MSN ensembles becomes less likely with an increasing number of ensembles (Charles J. Wilson, 2007).

Recently, Burke and colleagues have raised the point that local interneurons as well as MSNs are part of functional ensembles, with a shift in neuronal activation patterns possibly based in changes in connectivity or intrinsic cellular properties taking place during learning (Albin et al., 1989; Burke et al., 2017). Cholinergic interneurons for instance have recently been shown to play a specific role in relation to food reinforcers in the NAc, as they seem to specifically mediate motivation underlying satiety signals, potentially by inhibiting NAc outputs from MSNs (Aitta-aho et al., 2017). This however only is in line with the reduced NAc activity we observed in the Devalued group if inputs to NAc project directly onto interneurons, which then inhibit local MSNs, as a local MSN-interneuron-MSN circuit would cause disinhibition and increased NAc activation.

Rather than lateral inhibition taking place within the NAc, feedforward inhibition may contribute to ensemble selection, modifying NAc output in response to an unaltered input directly (Charles J. Wilson, 2007). Cortical inputs specifically have been demonstrated to be filtered by feedforward inhibition by striatal interneurons (Mallet, 2005). In our Devalued group, feedforward inhibition may be upregulated and hence NAc activity as seen by Fos expression being downregulated.

Taken together, this raises the intriguing possibilities that the ensemble consisting of MSNs and interneurons recruited after devaluation is attenuating the activity of the previously active ensemble and that inputs may be filtered differentially in Devalued animals via feedforward inhibition.

2.4.6 The role of decreased cortical and midbrain input in decreased Nucleus accumbens ensemble activation

The NAc is a region receiving glutamatergic inputs from several brain areas including the basolateral amygdala (BLA), hippocampus (HP), and medial prefrontal cortex (mPFC), and receives dopaminergic (DA) input from the ventral tegmental area (VTA). These afferent areas are implicated in encoding reward-associated cues and goal-directed behaviours, thus the observed decrease in activation following reward devaluation may occur from the modulated input from these upstream areas (for a review see Burke, Rotstein, & Alvarez, 2017; Voorn, Vanderschuren, Groenewegen, Robbins, & Pennartz, 2004).

2.4.6.1 Cortical areas

The BLA plays an important role in the association of cues and food (for review see Barry J Everitt, Cardinal, Parkinson, & Robbins, 2003; Holland & Gallagher, 1999). A wealth of studies have shown the involvement of the BLA in behavioural flexibility, the ability to adapt cue-responding according to the current value of the outcome. Johnson and colleagues compared several different paradigms of outcome devaluation to measure the role of the BLA in behavioural flexibility and concluded that the complexity of the task plays a decisive role (Johnson, Gallagher, & Holland, 2009). Specifically, they and others found that in multiple-reinforcer paradigms, but not single-reinforcer paradigms the BLA is involved in the updating of the cue value to align with the updated reward value after devaluation, but not the expression of the behaviour once the update happened. This discrepancy between paradigms may be due to processing of detailed sensory information about the different available rewards being mediated by the BLA, whereas more generic reward properties do not require BLA activation (Hatfield, Han, Conley, Gallagher, & Holland, 1996; Johnson et al., 2009; Ostlund & Balleine, 2008; Pickens et al., 2003; Wellman, 2005; Elizabeth A. West et al., 2012). Importantly, as discussed by West and colleagues, the BLA is specifically

involved in updating the value of the cue to the new value of the reward, but not the updating of the reward value itself, as BLA lesions or inactivation only affected cue responding but not reward consumption (Johnson et al., 2009; Simmons & Neill, 2009; Wellman, 2005; Elizabeth A. West et al., 2012). Therefore, in the context of the present study, which used a single reinforcer paradigm, the BLA is unlikely to drive the decreased activation pattern observed in the NAc after outcome devaluation. Interestingly however, a recent study by Calhoon et al. showed that in a state of homeostatic hunger the BLA to NAc positive valence mediating projection is more active compared to during satiety (Beyeler et al., 2018; Calhoon et al., 2018). For the current study this means that in sucrose devalued mice, which reached 100% body weight, the BLA to NAc projection may have become less active. This offers an alternative explanation for the current results based on reduced input from the BLA to the NAc during reward devaluation.

The mPFC projects to the NAc, with the ventral proportion, the infralimbic (IL) cortex mainly projecting to the shell and the dorsal proportion, the prelimbic (PL) cortex manly projecting to the core (Berendse et al., 1992; McGeorge & Faull, 1989; Vertes, 2004; Voorn et al., 2004).. Besides this anatomical distinction, a functional distinction has been suggested and found support by many studies (Moorman, James, McGlinchey, & Aston-Jones, 2015). Hence, the IL cortex has been implicated in mediating response inhibition in behaviours involving fear, drug- and natural rewards (Moorman et al., 2015). Evidence for this is provided by extinction studies where IL lesions have been shown to increase reinstatement of an appetitive Pavlovian response after extinction learning, indicating that this region encodes the suppression of conditioned responses after extinction (S. E. V. Rhodes, 2004; S. E. V Rhodes & Killcross, 2007). Based on this framework in which decreased IL and concomitant potential decreases in NAc activity could increase cue-evoked responding, the reduced Pavlovian approach and NAc Fos, in our current study may seem at odds with the

aforementioned findings. However, these seemingly discrepant findings can be reconciled by findings from a recent study by Suto et al., in which they demonstrated that cues predictive of reward availability vs. unavailability activate distinct ensemble representations in this area. Hence, following reward devaluation instead of a smaller IL ensemble a new and different ensemble may be recruited in response to the cue. This in turn could activate local NAc inhibitory neurons, resulting a suppression of NAc ensemble recruitment and decreased cue-evoked sucrose seeking (Suto et al., 2016).

The PL cortex is traditionally thought to mediate opposing behaviours compared to the IL cortex, namely the execution of fear and reward related behaviours, such as the behavioural response to reward-associated cues (Moorman et al., 2015). This is supported by a study revealing attenuation of cue-responding and firing rate in the NAc core after inactivation of the PL cortex (A. Ishikawa, Ambroggi, Nicola, & Fields, 2008). More evidence comes from extinction studies, where the PL cortex may mediate the renewal of cue-evoked food seeking as Fos expression in this region has been shown to be elevated in association with renewal of food seeking after extinction learning in rats (L. C. Anderson & Petrovich, 2018; A. Ishikawa et al., 2008). Hence, in reference to the current study neuronal activity in the PL cortex may be reduced in response to outcome devaluation, providing decreased excitatory input to the NAc core ensembles, eliciting the observed reduction in Fos expression and decreases in cue-evoked sucrose seeking. Even though a clear functional Go/NoGo distinction between the anatomically distinct mPFC subregions seems tempting, it might not be as clear cut as assumed initially and a variety of opposing evidence has accumulated requiring further investigation (Moorman et al., 2015).

Robust glutamatergic inputs into the NAc arise from the ventral HP, possibly altering its activation patterns and eliciting the reduced Fos expression we observed after devaluation (Groenewegen et al., 1999; Voorn et al., 2004). The HP has long been implicated in memory

formation, first in early studies on human patients by Brenda Milner and colleagues (Scoville & Milner, 1957), and now its role specifically in episodic and spatial memory has been well-established (Spiers, Burgess, Hartley, Vargha-Khadem, & O'Keefe, 2001; Vargha-Khadem et al., 1997). A recent study using *in vivo* calcium imaging has confirmed the existence of reward-encoding neuronal ensembles in the HP which play a role in goal-directed navigation (Gauthier & Tank, 2018). These ensembles could project to the NAc, which is supported by the finding that ventral HP neurons active during goal-directed behaviours mostly project to the NAc (Ciocchi, Passecker, Malagon-Vina, Mikus, & Klausberger, 2015; Gauthier & Tank, 2018). Additionally, lesion studies have shown that the ventral HP plays a role in the acquisition, but not expression of Pavlovian appetitive conditioning in sign-trackers (Fitzpatrick, Creeden, Perrine, & Morrow, 2016). More specifically, a pathway specific lesion study has found that the projections from the ventral HP to the NAc shell are involved in mediating context dependent appetitive conditioning but not discrete cue-dependent conditioning (R. Ito, Robbins, Pennartz, & Everitt, 2008). Hence, one possibility is that in the current study the reduction in NAc Fos reflects reductions in contextual activation of the NAc originating in the HP following devaluation.

2.4.6.2 The ventral tegmental area

Although the role of NAc DA inputs originating from the VTA have been studied extensively, a non-DA cell type now identified as glutamatergic projects from the VTA to the NAc shell and has been shown to be active in response to aversive stimuli (Qi et al., 2016; Mark A Ungless et al., 2004; Yamaguchi et al., 2007). This excitatory signal has been shown to activate GABAergic interneurons in the NAc to decrease MSN activity, motivation and subsequent approach behaviours (Qi et al., 2016; Root, Estrin, & Morales, 2018). Consequently, an increase in glutamatergic VTA originating input could drive the decrease in NAc shell activation and approach behaviour we observed after reward devaluation, if the

previously rewarding sucrose acquired aversive properties in our paradigm. This cannot be excluded, however the fact that in the present study the *ad libitum* sucrose fed mice reached free feeding body weight despite food restriction suggests that these mice consumed relatively high-levels of sucrose, raising the possibility that it did not acquire aversive properties. However, in future experiments this possibility would have to be confirmed using measurement of orofacial reactivity, which has been established as a measure for hedonic impact for a wide range of species (Berridge, 2000; Jankunis & Whishaw, 2013; Kiefer, Hill, & Kaczmarek, 1998; Jacob E. Steiner, Glaser, Hawilo, & Berridge, 2001).

The VTA provides the major DA input to the NAc, where DA modulates neuronal inputs and consequently affects behavioural output (Cepeda & Levine, 1998). It is known to play a role in mediating motivational properties and is therefore directly involved in reward learning (for a review see Salamone & Correa, 2012). Lesion studies have shown that DA VTA neurons are involved in signalling drug- and sucrose reward (D. C. S. Roberts & Koob, 1982; Shibata, Kameishi, Kondoh, & Torii, 2009). Recently the specific DA VTA-NAc shell and VTA-NAc core projections have been shown to mediate reward in a self-stimulation paradigm (X. Han et al., 2017). Conversely, aversive stimuli have been shown to inhibit DA VTA neuronal activity (Brischoux et al., 2009; Mark A Ungless et al., 2004). It has been suggested though that this only represents a dorsal subset of DA VTA neurons, whereas a more ventral subset is activated in response to aversive but not rewarding stimuli (Brischoux et al., 2009). Therefore, besides the previously discussed glutamatergic inputs alterations in this, a decrease in reward-encoding dopaminergic input needs to be considered as a possible reason behind the decreased NAc activation pattern after devaluation we observed in the current study. A likely mechanism here would be via modulation of D2 receptors altering neuronal activity in the NAc directly or via modulation of intra NAc lateral inhibition, subsequently altering behavioural output (Dobbs et al., 2016; Soares-Cunha et al., 2018).

Alternatively, NAc D1 receptors could also play a role here as their antagonism has been shown to reduce cue-elicited food approach and cue-induced p-ERK expression in NAc and both receptor types are known to be involved in mediating self-stimulation of the DA VTA-NAc pathway (Dalley et al., 2005; Fricks-Gleason & Marshall, 2011; Steinberg et al., 2014). As mentioned before, the devalued sucrose is unlikely to have acquired aversive properties in the present study, hence dorsal VTA projections may not modulate NAc activity here.

2.4.7 Downstream effects of devaluation-induced reduced Nucleus accumbens shell and core activity

Finally, regardless of which of the discussed mechanisms is driving the decrease in NAc activity after devaluation, the result is a reduced output into areas such as the VP, LH and VTA.

Projection neurons from the medial NAc shell, and to a lesser extent the NAc core, the areas in which we observed a devaluation-induced activity reduction, back to the VTA seem to be activated in response to reward-associated cues (S. V. Mahler & Aston-Jones, 2012). It has recently been shown that the medial NAc shell controls VTA output driving motivated behaviours in an GABAA receptor mediated inhibitory feedback loop via direct inhibition and, to a lesser extent, disinhibition (Yang et al., 2018). Reward devaluation could therefore activate this feedback circuit, resulting in reduced activation of these specific outputs, encoding the decreased salience of the sucrose-associated cue.

Whereas this NAc shell-VTA feedback loop is based on general reward, the NAc shell-LH projections are more specific to appetitive reinforcers (Kelley, Baldo, & Pratt, 2005; Mogenson et al., 1983; R. H. Thompson & Swanson, 2010). Decreased NAc input into the LH after sucrose devaluation could lead to reduced appetite in the mice, as the LH is known to mediate seeking of highly palatable food even in the absence of caloric need, possibly via

opioid signalling in the NAc (Kelley et al., 2005; Tandon et al., 2017). Therefore, even though we have shown that caloric need is not the sole driving force behind the attenuation in food approach behaviour after reward devaluation, it is still possible that downstream areas such as the LH drive our behavioural observations here.

The NAc also projects to the VP, with projections from the core to the dorsolateral and projections from the shell to the medial VP (Heimer et al., 1991). The NAc core to dorsolateral VP pathway has been implicated in reward related behaviour, specifically the reinstatement of cocaine seeking (Stefanik, Kupchik, Brown, & Kalivas, 2013). The NAc to VP pathway is thought to be involved in feeding behaviour as well as hedonic liking of palatable foods, even though this might also rely in the LH or other areas (Daniel C Castro et al., 2015; Root, Melendez, Zaborszky, & Napier, 2015; K. S. Smith, 2005; Taha, 2005). Additionally, outcome-specific Pavlovian-to-instrumemtal-transfer (PIT) is attenuated by NAc shell lesions and the specific shell-VP projection neurons show increased levels of Fos expression after PIT, but not in association with just Pavlovian conditioning (Leung & Balleine, 2013). Waracznyski and colleagues concluded from their study examining the effect of VP lesions on different aspects of self-stimulation that it is reward-driven responding, rather than reward value, which is mediated by the VP (Waraczynski & Demco, 2006). Hence, this NAc output might be more important in behaviours involving instrumental action and may therefore not be altered by the devaluation paradigm in the current study.

2.4.8 Conclusion

In the present study, *ad libitum* sucrose exposure-induced reward devaluation attenuated cue-evoked sucrose seeking and NAc neuronal activity, as indicated by reduced Fos expression. These effects were driven by hedonic, not homeostatic factors, reflecting a decrease in the incentive properties of sucrose. This reduction in activity could occur through several possible mechanisms including lateral inhibition within the NAc, as well as

devaluation-induced alterations in inputs from upstream areas could lead to changes in output and elicit the observed behavioural changes. However, it is still unclear how the devaluation procedure influences physiological properties of neuronal ensembles to drive neuronal activity and the Fos expression presented here. Therefore, in chapter 3 we will establish a link between electrophysiological properties hinting at underlying changes in ion channels determining neuronal excitability and cue-evoked sucrose seeking with and without reward devaluation.

3 Devaluation eliminates differences in neuronal excitability between neuronal ensembles and non-ensembles following sucrose cue exposure in the nucleus accumbens shell

3.1 Introduction

In chapter 2 we demonstrated that reward devaluation via four days of *ad libitum* sucrose exposure attenuated cue-evoked sucrose seeking, which is likely due to the reduction in the hedonic properties of sucrose as *ad libitum* chow did not modulate this behaviour. Additionally, this behavioural attenuation was accompanied by a reduction in the number of Fos-expressing neurons in the Nucleus Accumbens (NAc) core and shell, but not dorsal striatum (DS). This supports the idea that these brain areas play a role in updating outcomes about reward value. We concluded that the incentive properties of sucrose might be encoded by modulation of the number of cue-activated neurons, which is either driven by modulation of incoming signals from upstream brain areas or by modulation of intraaccumbal signalling. These changes may in turn alter the functional properties of these cue-activated neurons. Thus, in this chapter, we further investigated whether reward devaluation could alter the physiology of cue-evoked neuronal ensembles following reward devaluation.

Neurons are highly plastic and adapt their responses to stimuli depending on past experiences (Bailey & Kandel, 1993; Bliss & Lømo, 1973; David H. Hubel & Wiesel, 1998; Katz & Shatz, 1996; Robert C. Malenka & Bear, 2004). Such adaptations are critical for normal brain function, and impairments in experience-dependent neuronal plasticity are associated with psychiatric and eating-related disorders (Kasanetz et al., 2010; S. Liu et al., 2016; Morin et al., 2017; Oginsky et al., 2016; K. L. Smith et al., 2015). Much attention on research in this area has been devoted to plasticity at the synapse, the key site of neuronal communication, in which neural transmission efficacy is modulated depending on the type

of afferent activity patterns. However, another form of experience-dependent plasticity, which occurs outside of the synapse at the membrane on the soma, dendrites and axons via changes in the functional properties of intrinsic factors (i.e. ion channels). These alterations result in altered neuronal excitability, thereby modifying neuronal firing properties and output to neurons in proximal or distal brain areas (Kourrich et al., 2015).

Changes in the relationships between cues and their outcomes have been shown to be accompanied by such changes in neuronal excitability in brain areas implicated in encoding emotionally salient experiences such as the hippocampus (HP), amygdala, infralimbic (IL) and piriform cortex (McKay et al., 2009; Motanis et al., 2014; Moyer, Thompson, & Disterhoft, 1996; D Saar et al., 1999; Sehgal et al., 2014; Song et al., 2015; Zelcer et al., 2006). However, instead of specifically targeting cue-evoked neuronal ensembles, which constitute only a small subset of neurons that fire in response to a given stimulus, these studies recorded excitability properties from a randomly selected population of neurons. Hence the relationship between neural excitability and changes in cue-outcome associations could not be directly assessed in these ensembles (Nicolelis et al., 1997; C. M. A. Pennartz et al., 1994). To address this issue, we and others have utilised the *Fos-GFP* mouse that expresses a Fos-GFP fusion protein in strongly activated neurons, thus allowing the excitability properties of behaviourally activated neurons to be studied (Barth, 2004; Ziminski et al., 2017b).

Moreover, in this study the authors investigated alterations in neuronal excitability after a change in cue-outcome association via extinction learning. They found that existing differences in neuronal excitability between cue-responsive and surrounding neurons in the NAc shell were attenuated (Ziminski et al., 2017b). Similarly, reward devaluation modulates a cue-outcome association, but instead of producing an inhibitory association (due to the cue predicting reward unavailability) it reduces the value of the outcome that is predicted by

the cue (P. C. Holland & Straub, 1979; P. Holland & Rescorla, 1975). Recently, Carelli and colleagues have found the number of cue-responsive neurons in the NAc shell to be reduced after reward devaluation, which may be modulated by adjusting neuronal excitability (E. A. West & Carelli, 2016).

Based on these findings we hypothesised that reward devaluation is mediated by changes in neuronal excitability on cue-activated neuronal ensembles in NAc shell. To that end, we measured changes in neuronal ensemble excitability following devaluation in this area in *Fos-GFP* mice that express a Fos-GFP fusion protein in behaviourally activated neurons (Barth, 2004). In order to assess whether neuronal ensembles differed in their excitability compared to surrounding non-activated neurons, we measured excitability in both cue-activated (GFP+, 'ensemble') and non-activated (GFP–, 'non-ensemble') neurons.

3.2 Material and Methods

3.2.1 Animals

Similar to Chapter 2, male heterozygous *Fos-GFP* mice were housed and food restricted under the same conditions. Mice were 9 - 10 weeks old at the beginning of behavioural testing. All experiments were conducted during the light phase and in accordance with the UK Animals (Scientific Procedures) Act of 1986 after ethical approval by the University of Sussex Animal Welfare and Ethical Review Body.

3.2.2 Behavioural experiment

Mice were trained using the same behavioural protocols for Pavlovian conditioning and devaluation as in Chapter 2.

3.2.3 Electrophysiology

3.2.3.1 ex vivo *brain slice preparation*

Ninety minutes after the start of Pavlovian approach testing, mice were anaesthetized using intraperitoneal injections of a mixture of ketamine and xylazine (150 mg/kg and 20 mg/kg, respectively; Anaesktin©, Dechra Veterinary Products; Rompun©, Bayer Healthcare) in saline, and then transcardially perfused with ice-cold aCSF (concentrations in mM: NaCl 126, KCl 4.5, $MgCl_2$ 1, $CaCl_2$ 2.5, NaH_2PO_4 1.2, D-glucose 11, $NaHCO_3$ 26, pH 7.4). Following perfusions, the brains were immersed in ice-cold filtered recovery solution (concentrations in mM: NMDG 93, KCl 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 30, HEPES 20, D-glucose 25, $C_6H_7NaO_6$ 5, $SC(NH_2)_2$ 2, $C_3H_3NaO_3$ 3, $MgSO_4H_20$ 10, $CaCl_2.2H_20$ 0.5, osmolarity 300 - 310 mOsm, pH 7.4) for 2 minutes. The cerebellum was removed and the brain was mounted onto a stage and placed in a slicing chamber filled with ice-cold recovery solution. 250 µm thick coronal slices were cut corresponding to approximately 1 – 1.5 mm

AP from Bregma. Slices were stored in recovery solution for 5 minutes at 32 °C and then transferred to artificial cerebrospinal fluid (aCSF) at room temperature until recording. Recovery solution and aCSF were continuously bubbled with a 95% O_2:5 % CO_2 mixture.

3.2.3.2 Electrophysiological recording

For NAc shell current clamp recordings, the slices were hemisectioned and transferred to the recording chamber continuously (flow rate approximately 2 ml/min) refilled with aCSF at 32 °C. GFP+ neurons were identified using a 488 nm laser and a Revolution XD spinning disk confocal system (Andor system) and differential interference contrast visualization was done using an Olympus BX51W1 microscope. Whole cell patch clamp recordings were performed using ICS (intracellular solution, concentrations in mM: K-gluconate 125, KCl 10, HEPES 10, $MgCl_2$*$6H_2O$ 2, EGTA 1, $CaCl_2$*$2H_2O_2$ 10mM 0.1, Mg-ATP 2, Na-GTP 0.2, pH 7.25)-filled borosilicate capillary glass-pipettes (inner diameter 0.86 mm, outer diameter 1.5 mm, resistance 5 - 9 MOhm) pulled from 1 mm capillary tubing (Sutter Instruments) using a horizontal electrode puller (Sutter Instrument). Alexa Fluor 568 dye (100 µM, A10437, Thermo Fisher Scientific) was added to the ICS to confirm patched cells by colocalization with GFP. medium spiny neurons (MSNs) were identified using morphology, resting membrane potential (RMP), and action potential (AP) waveform and held at -75 mV for the duration of the recordings. Liquid junction potential was -13.7 mV and was not adjusted for. The current clamp recording protocol consisted of 800 ms current injections starting at -60 pA and increasing in 4 pA steps. The bath solution consisted of standard aCSF.

Data were collected with a Multiclamp 700B amplifier (Molecular Devices), WinEDR (version 3.7.5) and WinWCP Software (version 5.2.2, courtesy of Dr. John Dempster, University of Strathclyde, Glasgow, UK; http://spider.science.strath.ac.uk/sipbs/software_ses.htm, RRID: SCR_014713). Signals were digitized at 10 kHz and filtered at 5 kHz (PCI 6024E; National Instruments) and 50 Hz noise was filtered out using a HumBug (Quest Scientific) module.

Rheobase and input resistance were calculated manually. The input resistance *(Ri)* was calculated as the slope of the I/V curve between -60 pA and 20 pA injections. Spike kinetics (amplitude and half width) and afterhyperpolarization (AHP) were calculated using Mini Analysis Software (version 6.0; Synaptosoft, RRID:SCR_002184) and spike counts were calculated using Stimfit 0.14 software (Python 2.7.9).

3.2.4 Experimental Design and Statistical Analysis

Data were analysed and visualized using GraphPad Prism 6 (Graphpad software, RRID:SCR_002798), SPSS (IBM SPSS statistics, RRID:SCR_002865), and Excel (Microsoft). All data is presented as mean ± SEM. ANOVAs with significant interactions or main effects were followed up by *Fisher's* LSD post-hoc test. Our lab and others in the field regularly use this test to compare between groups which show a significant main effect in an ANOVA, and/or significant interaction (Whitaker et al., 2016, 2017; Ziminski et al., 2017b)

In text and figures values of mean ± 2xSD were considered outliers. In chapter 3 there was one outlier for Rheobase (GFP+ Non-devalued), two for Input resistance (GFP+ Non-devalued, GFP- Devalued), one for AP half-width (GFP- Devalued), and one for AP amplitude (GFP- Non-devalued), no cells were identified as outliers in the spike counts. Normal distribution was assessed using visual inspection of the data and Kolmogorov-Smirnoff tests. For repeated measures data, normality testing was performed on the standardized residuals. If independent sample data sets or the majority of a repeated measures data set deviated from normal distribution, non-parametric tests were used to confirm the results of the parametric tests In data sets following a Gaussian normal distribution, an alternative method of outlier detection was used (Grubb's test). See Appendix 1 for normality testing and non-parametric tests, as well as reanalysis of data with altered number of outliers according to Grubb's.

Spike counts and I/V curves were first analysed using a three-way mixed ANOVA with devaluation (Non-devalued, Devalued) and GFP (+/–) as between-subjects factors and current step as within-subjects factor. This was followed up by two-way mixed ANOVAs using current step as within-subjects factor and GFP (+/–) or devaluation (Non-devalued, Devalued) as between-subjects factor. In appendix 2 an alternative analysis of spike counts and I/V curve using GFP as within-subjects factor is shown.

RMP, rheobase, input resistance (Ri), afterhyperpolarisation (AHP), spike amplitude and half-width were analysed using two-way ANOVAs with devaluation (Non-devalued, Devalued) and GFP (+/–) as between-subject factors.

3.3 Results

3.3.1 Ensembles activated following sucrose cue exposure are more excitable than surrounding cells, and this difference is attenuated by sucrose devaluation

Similar to Chapter 2, mice established a sucrose-cue association and underwent a Pavlovian approach test for cue-evoked sucrose seeking. We then assessed the excitability of cue-responsive, GFP+ ensembles and surrounding cue non-responsive GFP– cells. We injected increasing amounts of current into the cells and quantified the number of action potentials fired in response to assess the firing capacity of these cells (Figure 1). A mixed three-way ANOVA showed a three-way interaction of current step x devaluation x GFP ($F_{8, 312} = 2.711$, $p = 0.007$), an interaction of current step x GFP ($F_{8,312} = 6.349$, $p < 0.001$), as well as a significant main effect of current step ($F_{8,312} = 53.66$, $p < 0.001$) and GFP ($F_{1, 39} = 7.667$, $p = 0.009$). Further examination with a two-way ANOVA comparing the firing rates of GFP+ and GFP– cells within Non-devalued mice separately revealed an interaction of current step x GFP ($F_{8,160} = 11.03$, $p < 0.001$), as well main effects of current step ($F_{8,160} = 35.21$, $p \leq 0.001$) and GFP ($F_{1,20} = 16.64$, $p < 0.001$). Post-hoc tests revealed a significantly higher number of spikes in GFP+ cells compared to GFP– cells from 80 pA current injection onwards (Figure 1 A). A similar ANOVA within the Devalued group comparing GFP+ and GFP– cells yielded a main effect of current step ($F_{8,152} = 21.43$, $p < 0.001$) but no effect of GFP or interaction (Figure 1 B). These results indicate that differences in excitability between cue-responsive and non-cue responsive cells present in Non-devalued mice are eliminated by reward devaluation.

Changes in both ensemble and non-ensemble neurons underlie alterations in appetitive learning (Whitaker et al., 2017; Ziminski et al., 2017b, 2018). Therefore, we next compared

spike numbers of GFP+ and GFP– cells separately across conditions. Two-way mixed ANOVAs revealed a main effect of current step ($F_{8,160}$ = 39.22, $p < 0.001$) for the GFP+ ensemble but no interaction of current step x devaluation ($F_{8, 160}$ = 1.089, p = 0.3737) or main effect of devaluation ($F_{1, 20}$ = 0.8656, p = 0.3633). For the GFP– cells we discovered an interaction of current step x devaluation ($F_{8,152}$ = 20.48, p = 0.04), a main effect of current step ($F_{8,152}$ = 15.91, $p \leq 0.001$) but no main effect of devaluation ($F_{1, 19}$ = 3.271, p = 0.0864). Taken together, these results suggest that the lack of excitability differences observed following devaluation are not due to significant shifts in the excitability of ensemble and non-ensemble neurons following devaluation.

We also analysed the I/V curves and a mixed three-way ANOVA revealed a lack of a three-way interaction but a significant interaction of current step x GFP ($F_{20, 780}$ = 11.031, $p < 0.001$), as well as a significant effect of current step ($F_{20, 780}$ = 430.768, $p < 0.001$), GFP ($F_{1,39}$ = 16.829, $p < 0.001$), but not devaluation. Further analysis using a two-way ANOVA comparing GFP+ and GFP– cells separately within Non-devalued and Devalued groups revealed a significant interaction of current step x GFP ($F_{20,400}$ = 9.02, $p < 0.001$), as well as main effects of each factor (current step $F_{20,400}$ = 215.1, $p < 0.001$; GFP $F_{1,20}$ = 12.92, p = 0.002) in the Non-devalued group. Post-hoc tests revealed differences between GFP+ and GFP– cells in negative and positive potentials (Figure 1 A inlay). In the Devalued group, a two-way ANOVA comparing GFP+ and GFP– cells yielded an interaction of current step x GFP ($F_{20,380}$ = 2.931, $p < 0.001$), as well as main effect of both factors (current step $F_{20,380}$ = 217.6, $p < 0.001$, GFP $F_{1,19}$ = 5.189, p = 0.047). Post-hoc tests revealed pairwise differences for two current steps in the negative potential range only (Figure 1 B inlay). In summary, the differences in the I/V curves of GFP + and GFP– neurons observed in the Non-devalued group persist in the Devalued group, but less pronounced and restricted to negative potentials.

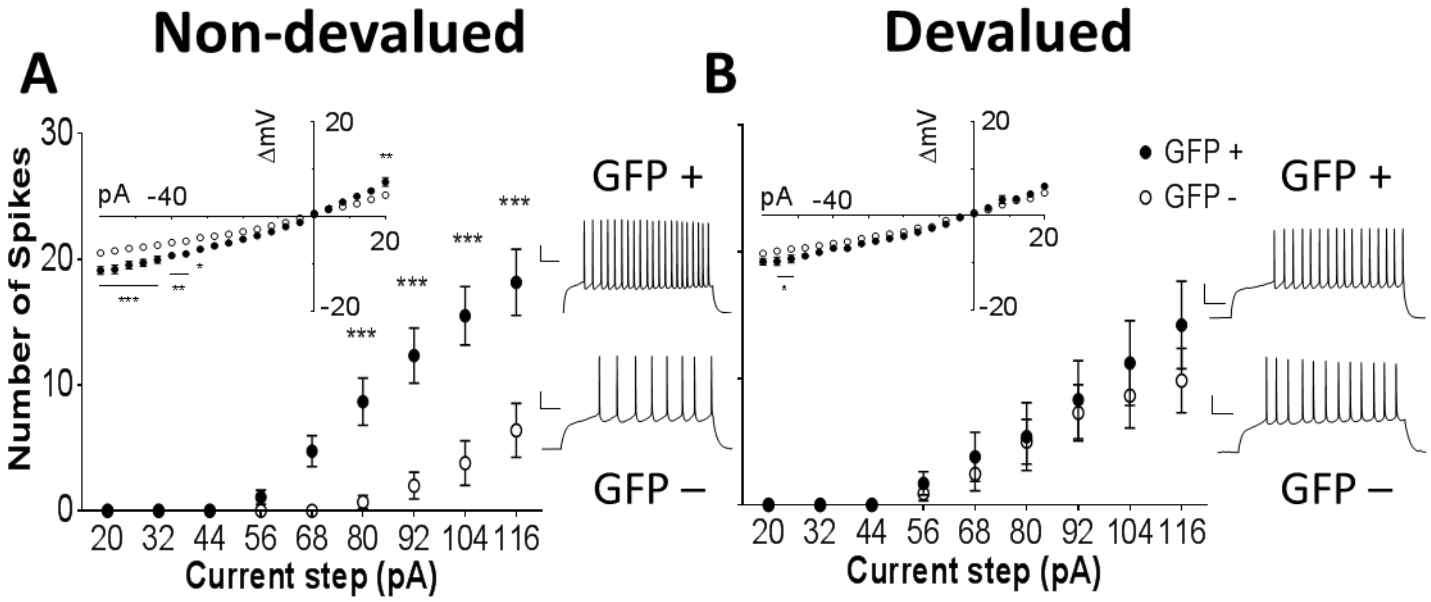

Figure 1: The increased excitability of GFP+ neurons compared to surrounding GFP – neurons in NAc shell is attenuated by reward devaluation. A: In the Non-devalued group, GFP+ cells exhibit increased spiking in response to increasing current injections compared to surrounding GFP – cells. The I/V curve (inlay) for GFP+ cells is shifted in positive and negative current steps, but not in the intermediate range (GFP – /+ *n* = 10/12). Representative traces from injections at 116 pA (right). B: After sucrose devaluation there is no difference in firing capacity between GFP+ and GFP– cells. Only a mild downward shift is observed for the I/V curves (inlay) from GFP+ and GFP – cells (GFP – /+ *n* = 11/10). Representative traces from injections at 116 pA (right). *p < 0.05, **p < 0.01, ***p < 0.001. All values are mean ± SEM. Scale bar in representative traces 20 mV and 100 ms.

3.3.2 Underlying changes in passive membrane and action potential properties

To investigate the source of the differences in firing capacity, we examined the RMP, rheobase, Ri, AHP, and AP half width and amplitude of GFP+ and GFP– cells from Non-Devalued and Devalued mice using two-way ANOVAs with devaluation (Non-devalued, Devalued) and GFP (+/–) as between-subjects factors (Figure 2, Table 1). Except for

rheobase ($F_{1, 37} = 4.572$, $p = 0.039$), we did not find any interactions between devaluation x GFP for these parameters. However, we found a main effect of GFP for Ri ($F_{1,38} = 13.47$, $p < 0.001$, Figure 2 C) and AP half-width ($F_{1, 37}$ 4.31, $p = 0.045$, Figure 2 D). An effect of devaluation was found for rheobase (besides the interaction, $F_{1, 37} = 7.012$, $p = 0.012$), AP half-width ($F_{1, 37} = 4.31$, $p = 0.045$) and AHP ($F_{1, 38} = 6.06$, $p = 0.018$, but no post-hoc differences, Figure 2 E). Post-hoc comparisons are indicated in Figure 2. For RMP and AP amplitude we did not find any effects or interactions to be significant (Figure 2 A, F). A full list of all effects and interactions can be seen in Table 1. In brief, here we are showing rheobase to be regulated differently in Devalued mice, as the difference between ensembles present in Non-devalued mice was abolished by Devaluation (Figure 2 B). For AP half-width there might be a differential regulation by Devaluation, however the interaction as found to be non-significant.

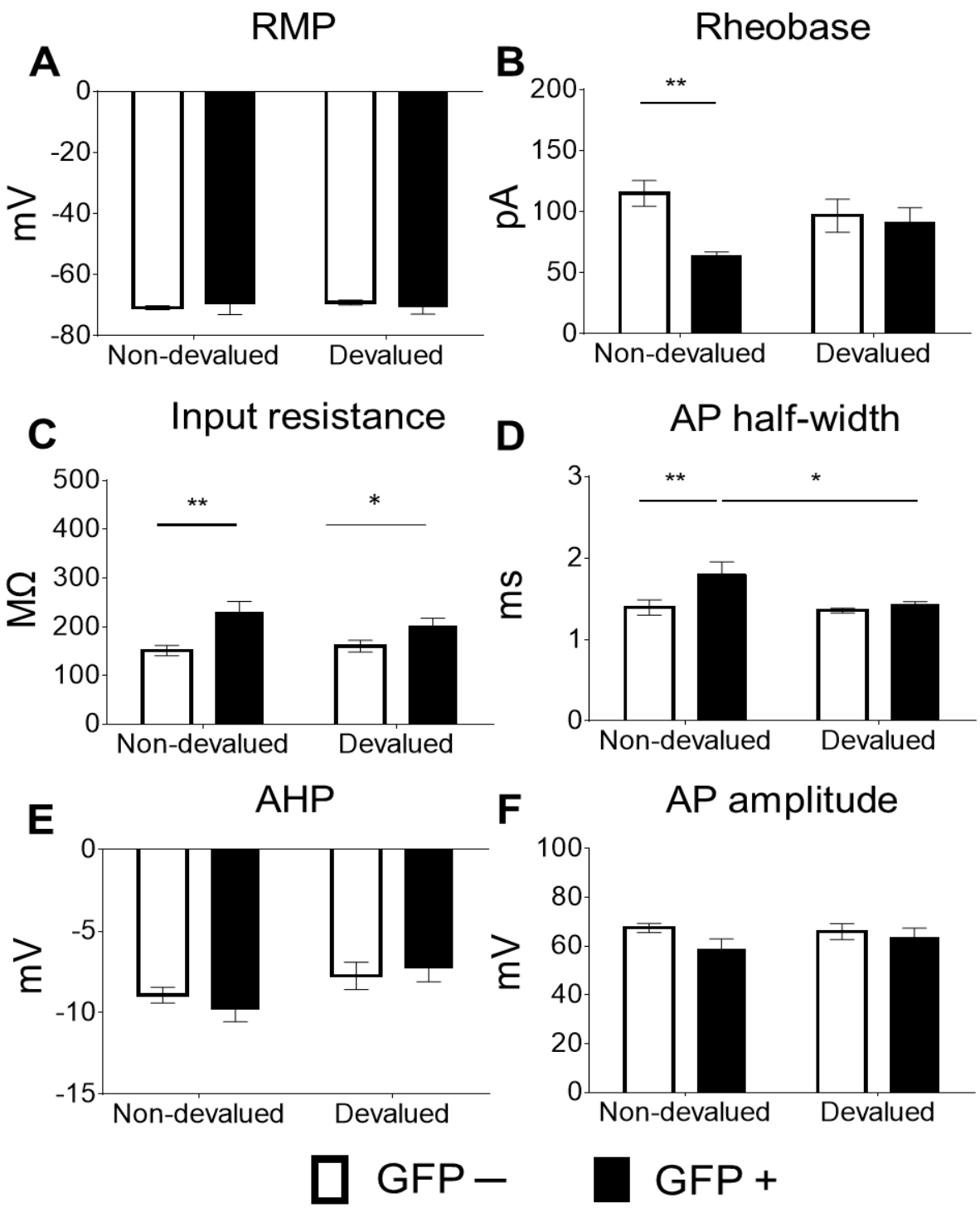

Figure 2: Basic passive membrane and action potential parameters in GFP+ and GFP – cells with and without devaluation. (A) RMP (resting membrane potential) was unchanged

in all groups. (B) Ensemble-specific differences in rheobase were eliminated by devaluation. (C) Input resistance was unaltered by Devaluation, displaying similar ensemble-specific differences in Devalued and Non-devalued groups. (D) AP half-width was increased in GFP+ neurons in Non-devalued, but not Devalued mice. (E) Afterhyperpolarisation (AHP) and AP amplitude (F) were unaltered in all groups. *n* = 9 – 14, All values are mean ± SEM, asterisk indicate post-hoc comaprisons after ANOVA, *p < 0.05, **p < 0.01.

Table 1: Basic membrane properties from the NAc shell in Non-devalued and Devalued mice

	Non-devalued		Devalued		Interaction	Main effect	
	GFP -	GFP +	GFP -	GFP +	GFP x Devaluation	GFP	Devaluation
RMP (mV)	-70.8 ± 0.7	-69.4 ± 1.1	-69.1 ± 0.8	-70.3 ± 0.8	F(1,38)=2.28, p=0.14	F(1,38)=0.19, p=0.66	F(1,38)=0.02, p=0.9
Rheobase (pA)	115.0 ± 10.5**	63.2 ± 4.0**	96.7 ± 13.5	91.2 ± 11.9	F(1,37)=4.57, p=0.04	F(1,37)=0.20, p=0.66	F(1,37)=7.02, p=0.01
Input resistance (MΩ)	151.2 ± 10.7**	246.5 ± 27.6**	160.2 ± 12.0*	200.6 ± 17.2*	F(1,38)=2.20, p=0.14	F(1,38)=0.99, p=0.33	F(1,38)=13.47, p<0.01
AHP (mV)	-8.9 ± 0.5	-9.8 ± 0.8	-7.7 ± 0.8	-7.3 ± 0.8	F(1,38)=0,78 p=0.38	F(1,38)=0.07, p=0.79	F(1,38)=6.07, p=0.02
AP half-width (ms)	1.4 ± 0.1**	1.8 ± 0.15**^	1.4 ± 0.03	1.4 ± 0.04^	F(1,37)=2.9, p=0.1	F(1,37)=6.0, p=0.02	F(1,37)=4.31, p=0.04
AP amplitude (mV)	67.4 ± 1.9	58.7 ± 4.2	65.9 ± 3.3	63.5 ± 3.8	F(1,37)=0.82, p=0.37	F(1,37)=2.53, p=0.12	F(1,37)=0.22, p=0.64

Data in first four columns are expressed as mean ± SEM. *p<0.05, **p<0.01 post-hoc comparison GFP+ vs GFP-. ^p<0.05 post-hoc comparison Non-devalued vs Devalued. RMP = resting membrane potential, AHP = afterhyperpolarisation.

Finally, we found no main effects of GFP ($F_{1,38}$ = 0.017, p = 0.897) or devaluation ($F_{1,38}$ = 0.195, p = 0.662) for RMP (Figure 2 A). Overall, these findings indicate that devaluation alters the active membrane properties without altering firing capacity in ensemble neurons. To reconcile these seemingly disparate results, we next examined whether the lack of change in firing capacity, despite decreased AP half-width and decreased AHP peak was due to changes in input resistance in GFP+ neurons in the Devalued group. A direct comparison of GFP+ neurons between the Non-devalued and Devalued groups revealed decreased input resistance following devaluation ($p < 0.05$, $t_{21} = 2.057$). Thus, the decreased input resistance may have counteracted potential differences in firing capacity which are normally associated with the observed alterations in action potential kinetics.

3.4 Discussion

We examined the effects of devaluation on the excitability properties of NAc shell neuronal ensembles following sucrose cue exposure. Confirming our recent study by Ziminski et al., NAc ensembles activated following sucrose cue exposure were more excitable than non-ensemble neurons in sucrose conditioned mice. Following devaluation, this difference was no longer observed. However, cue-activated ensembles exhibited altered rheobase and AP half-width after devaluation compared to non-ensemble neurons without impacting firing capacity. These findings suggest that devaluation alters the cue's ability to recruit an ensemble that is more excitable than its surrounding neurons in the NAc shell and modifies the properties of ion channels in these ensembles. In turn, these changes may modulate the attenuation of cue-evoked sucrose seeking.

We demonstrated that a neuronal ensemble activated following sucrose cue exposure was more excitable compared to its surrounding neurons before devaluation. Such an ensemble may encode a learned cue-outcome associations (Ziminski et al., 2017b). In addition to confirming our previous study by Ziminski et al., our findings add to a newly emerging body of studies that reveal how appetitive learning with food rewards recruits neuronal ensembles with altered intrinsic excitability. A recent study by Whitaker and colleagues revealed in *Fos-GFP* rats that operant food self-administration selectively increased excitability in activated neurons in the mPFC (Whitaker et al., 2017). Thus, a hyperexcitable ensemble was recruited as the relationship between a particular action and rewarding outcome was established. Although this study did not examine excitability in cue-activated neurons *per se*, together with this study it highlights the importance of examining activated neurons to reveal learning-relevant neurophysiological alterations. Collectively, they demonstrate the utility value of using *Fos-GFP* rodent lines to perform such investigations.

3.4.1 Devaluation limits the ability of the cue to recruit a hyperexcitable ensemble: Potential mechanisms

To our knowledge, so far there have been no direct investigations of excitability changes in cue-responsive ensembles recruited following reward devaluation. However, Carelli and colleagues did measure firing rates of NAc neurons after sucrose reward devaluation in an operant conditioning paradigm including a visual cue (E. A. West & Carelli, 2016). They demonstrated that reward devaluation reduced the percentage of cue-responsive neurons in the NAc shell, further supporting findings from Fos studies, including the present one, finding the cue-encoding ensemble in this region to be reduced in size after devaluation (E C Kerfoot et al., 2007; E. A. West & Carelli, 2016).

Additionally, we can discuss the present findings in the context of extinction learning that involve updating of cue-outcome associations. Extinction learning, which constitutes a CS-US contingency degradation via presentation of the CS alone, is associated with changes in neuronal excitability (McKay et al., 2009; Santini et al., 2008; Song et al., 2015). Similar to our current study, our recent study revealed that extinction learning eliminated differences in excitability between the cue-encoding ensemble and cue non-encoding neurons in NAc shell of sucrose conditioned mice (Ziminski et al., 2017b).

Our findings from chapter 2 (chapter 2 Figure 3) indicate that the cue-activated ensemble decreased in size after reward devaluation, however it is unclear if in the current experiments after devaluation we recorded from a small subset of the ‘existing’ ensemble that is normally activated under Non-devalued conditions during sucrose seeking or from a ‘new’ ensemble that is distinct from this existing ensemble.

The notion that we recorded from a small subset of the ensemble activated in response to the cue prior to devaluation supports the idea of an updated memory substituting and destroying the original memory. Traditional models such as the Rescorla-Wagner model support this idea that only the current CS-US association determines behaviour and that any previous value of a CS (in the case of extinction learning) or US (in the case of devaluation) is irrelevant and is overwritten (McCloskey & Cohen, 1989; Robert A Rescorla & Wagner, 1972). From this framework, devaluation may induce a LTD-like phenomenon at the intrinsic excitability level in neurons of the existing ensemble. In turn, this plasticity may eliminate any excitability differences with surrounding neurons. Correspondingly, it has been shown that Fos-expressing memory engrams in the hippocampus encoding a positive memory can later on encode a negative memory and vice versa (Redondo et al., 2014). This indicates that after a change in outcome value, similar to the current study, the same ensemble can remain behaviourally activated (Figure 3 A). However, this stands in contrast to phenomena such as reinstatement or spontaneous recovery of extinguished behaviours, as these require reactivation of the original memory (Yoshii, Hosokawa, & Matsuo, 2017). Therefore, we raise the second possibility that in the current study we may have recorded from a population of neurons from a new ensemble after devaluation that does not display any excitability differences with the surrounding neurons (Figure 3 B). This supports the idea of the formation of a new neuronal ensemble, potentially inhibiting but not erasing the original memory, when the cue or outcome value is altered (Bouton, 2002, 2004; Quirk & Mueller, 2008). In line with this, Warren and colleagues showed that in the ventromedial prefrontal cortex distinct neuronal ensembles encode food reward and extinction memory (Warren et al., 2016). Additionally, in the BLA memory engrams encoding memories of different valence have been shown to be separate and non-convertible (Redondo et al., 2014). Along the same lines Suto and colleagues showed that in the infralimbic cortex the influence of distinct cues promoting and inhibiting food seeking are mediated by distinct ensembles in the same

brain area (Suto et al., 2016). Hence, the possibility of a distinct ensemble encoding the association of the cue and the updated, devalued outcome is likely. A third option is that these ensembles activated in response to the same cue representing different values are overlapping and after devaluation a mixed population of previously cue-encoding and previously not cue-encoding neurons is recruited (Figure 3C). Partially overlapping ensembles have been shown to be recruited in response to different stimuli of the same or different modality, events closely linked in time, and different types of rewards (Corder et al., 2019; Pfarr et al., 2018; Rashid et al., 2016). If this is the case in the current study however it is possible that some of these overlapping neurons are activated in response to background stimuli such as the test environment which are common to both Devalued and Non-devalued conditions. An additional control group could help shed light on this.

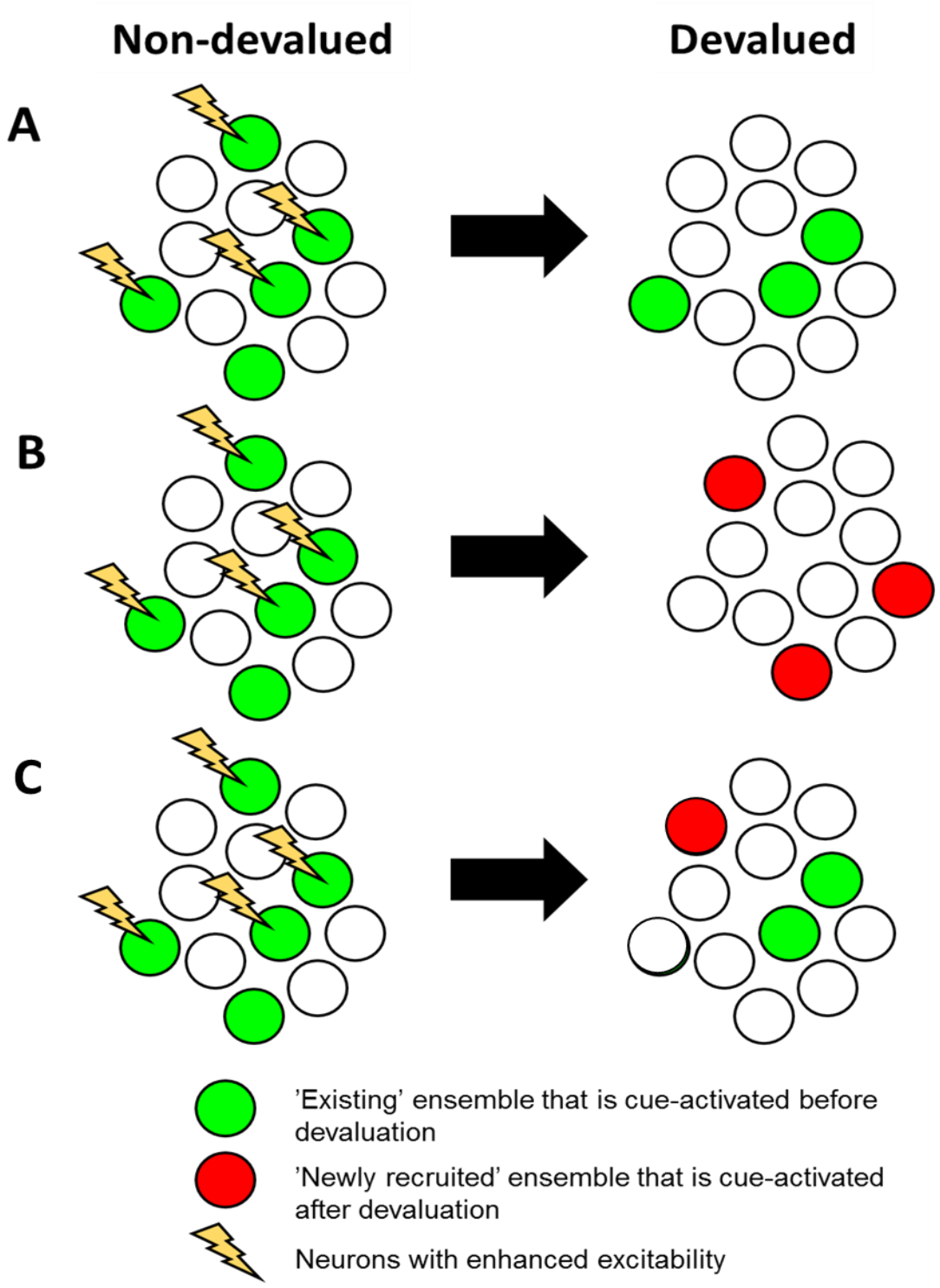

Figure 3: A hypothetical model of ensemble recruitment mechanisms following reward devaluation and reduction in conditioned approach. Following devaluation cues recruit a smaller ensemble_ A) with neurons from the 'existing' ensemble that was cue-activated during sucrose seeking before devaluation (green). These neurons no longer exhibit altered excitability compared to the surrounding neurons; B) with only newly recruited neurons that are distinct from the existing ensemble which do not exhibit altered excitability compared to the surrounding neurons; C) with a mixture of neurons consisting of previously cue-activated and previously not cue-activated neurons.

It is worth mentioning two points regarding the current experiment. First, in the current study only recently activated neurons were labelled with GFP and recorded following sucrose seeking after devaluation. Hence it is unknown whether coincidentally some of the GFP– neurons we recorded from were part of the GFP+, cue-activated ensemble prior to devaluation. Given the very small size of cue-encoding neuronal ensembles this seems unlikely and therefore it is possible that this "dormant" existing ensemble after devaluation persist to be highly excitable but not currently activated and GFP-expressing due to decreased excitatory input or increased local inhibition.

Second, it may be possible that the Pavlovian approach test itself elicited the observed physiological differences in Non-devalued animals, and not the training period. This would mean that the excitability difference is not present after Devaluation because here the Pavlovian approach test consists of a non-motivating cue presentation eliciting no behaviour, making it questionable if this "test" can be compared to the one conducted under Non-devalued conditions. Importantly however in Devalued conditions the cue is not meaningless, it still represents the sucrose, which in turn has lost its attractiveness, so the same cognitive processes are likely to be elicited by both tests, just with different behavioural outcomes. Future experiments using long-lasting markers labelling the original ensemble or already labeling the ensemble during training could help resolve both of these issues. The Fos-tTA x TRE-H2BGFP line allows for persistent labelling of activated ensembles and can be used alongside a *Fos*-driven marker for recent activation, such as GFP (Kanda, Sullivan, & Wahl, 1998; Tayler, Tanaka, Reijmers, & Wiltgen, 2013). Moreover, the time window for labelling can be tightly controlled via doxycycline allowing for an exact correlation of stages of behavioural tasks and occurrence of physiological changes.

3.4.2 Alterations in ion channel function as a potential mechanism for learning- and devaluation-induced changes in ensemble excitability.

3.4.2.1 Conditioning-associated changes in neuronal excitability: potential mechanisms

In a previous study from our lab, Ziminski et al. found the difference in neuronal excitability between GFP+ and GFP– neurons following sucrose cue exposure to be accompanied by an increase in input resistance (Ri) in GFP+ cells and a shift in the I/V curve in both depolarised and hyperpolarised potentials from rest, suggesting underlying modifications of intrinsic K^+ and Ca^+/Na^+ channels (Ziminski et al., 2017b). This is in line with our current findings.

The modification of inwardly rectifying potassium channels (Kir) could account for the shifts in the I/V curve in the negative potential range. The normal function of Kir channels, which open at hyperpolarised potentials, is to maintain the resting membrane potential via hyperpolarisation in MSNs (Hibino, 2010; Nisenbaum & Wilson, 1995; Uchimura, Cherubini, & North, 1989), and their modulation has been shown to regulate excitability in the NAc via CREB (Perez er al. 2006, Dong et al. 2006). In the retina MEK-ERK-CREB/Fos-mediated Kir inhibition led to loss of hyperpolarisation and increased excitability (Gao et al. 2017). Conversely, in the NAc, sucrose withdrawal induced downregulation of dopamine signalling and CREB in D1 receptor expressing neurons that was accompanied by increased Kir expression and concurrent decreased excitability (Kim, Shou, Abera, & Ziff, 2018). Moreover, there is one recent report of a decrease in excitability in NAc shell MSNs due to upregulation of Kir channels in a DA-independent mechanism induced by repeated cocaine . In Non-devalued mice, the observed shift in the I/V curve in positive potentials in ensemble neurons in the current study are likely to be based on modifications of voltage gated A-type potassium channels. Their normal function is to contribute to repolarisation after an AP and

these channels have been shown to regulate excitability in MSNs (Nisenbaum, Xu, & Wilson, 1994; Perez et al., 2006; D. James Surmeier, Stefani, Foehring, & Kitai, 1991; Wickens & Wilson, 1998). Additionally, modulation of leak potassium channels (K2P) could contribute to shifts in the I/V curve of GFP+ cells in all potentials as they are open permanently (Piechotta et al. 2011). These channels are known to normally counteract depolarisation and maintaining a hyperpolarized resting potential and have been shown to regulate excitability in MSNs (Enyedi et al. 2010, Perez et al. 2006). Similar changes in Ri associated with altered excitability after associative learning have previously been shown in the prelimbic cortex (Whitaker et al. 2017, Song et al. 2015). Hence in the current study, alterations in Kir and A-type potassium channels are likely to generate the observed differences in Ri accompanying differences in ensemble excitability following sucrose conditioning in Non-devalued mice.

Besides the changes in Ri confirming the results of Ziminski et al., excitability differences between GFP+ and GFP– neurons after associative learning in the current study were also accompanied by changes in rheobase and AP half width. These parameters are known to reflect changes in voltage-gated sodium, calcium, and potassium channels as the opening of these channels is responsible for the rapid increase and decrease in membrane potential during depolarisation and repolarisation, respectively. The physiological role of voltage-gated sodium channels is to depolarise the cell in the rising phase of an action potential, allowing sodium to enter the cell (Hille, 2001; Hodgkin & Huxley, 1952a). Inhibition of these sodium currents has been shown to increase the firing threshold and therefore decrease neuronal excitability in the striatum via the activation of D1 receptors (Schiffmann et al., 1995). This is reflected in the differences in rheobase and half width accompanying the differences in neuronal excitability between GFP+ and GFP– neurons after sucrose conditioning in the current study. Furthermore, alterations in calcium signalling are likely to contribute to the observed differences in AP half width here as blockage of calcium currents

is known to elongate APs due to decreased activation of voltage-gated calcium channels and big-conductance calcium-activated potassium channels (BK channels), which normally contribute to repolarisation (Bean, 2007; Bennett, Callaway, & Wilson, 2000; Sah, 1996). Modulation in BK channel function has previously been implicated in excitability-mediated alterations of reward signalling in the NAc (Ma et al., 2013). Finally, besides changes in the aforementioned BK, Kir, and A-type potassium channels, alterations in other voltage-gated potassium channels might account for the increased AP half width observed in the current study in GFP+ neurons after sucrose conditioning, as these channels are known to contribute to repolarisation after an AP (Bean, 2007). Taken together, the ensemble specific alterations in input resistance, AP half width and rheobase accompanying differences in neuronal excitability between GFP+ and GFP– neurons after sucrose conditioning are likely due to modulation of either potassium, sodium, and calcium currents or a combination of these.

3.4.2.2 Devaluation-associated lack of differences in neuronal excitability: potential mechanisms

Following devaluation firing capacity differences between GFP+ and GFP– neurons were no longer observed. Moreover, I/V curves from these neurons did not exhibit any shifts at deporalised potentials, but there was a slight downward shift at hyperpolarised potentials. This indicates devaluation-induced alterations in A-type channel expression or function, eliminating differences between GFP+ and GFP– neurons. Also, the ensemble-specific potential reductions in Kir expression or function persisted following devaluation, so are not altered by it. Interestingly, this differs from findings of a previous study from our lab investigating excitability modifications after extinction learning, hence emphasizing the existence of differences in ensemble recruitment between extinction learning and

devaluation, that produce similar behavioural results but utilise different learning mechanisms but yet different paradigms (Ziminski et al., 2017b).

Furthermore, in the current study after devaluation cue exposure recruited a neuronal ensemble with reduced AP half width and, even though the spike capacity remained unaltered. As previously discussed, currents mediating alterations in AP half width by broadening or narrowing the AP are dependent on voltage-gated sodium, potassium and calcium channels, as well as BK channels, indicating that these channels are altered in the devaluation-recruited neuronal ensemble. Additionally, ensemble-specific differences in rheobase present in Non-Devalued mice were eliminated by Devaluation. This suggests the selective alteration of voltage-gated sodium channels as discussed above.

Interestingly, in contrast to other conditioning studies, we did not observe a change in AHP peak in GFP+ neurons after devaluation. This suggesting a lack of modification of small-conductance calcium-activated potassium channels (SK channels). These channels open more slowly than BK channels and therefore their opening contributes to determining shape and size of the AHP (Blatz & Magleby, 1986; Kohler et al., 1996; Shepard & Bunney, 1991). Previous studies using fear conditioning and operant food self-administration have implicated altered AHP and SK channel signalling in ensemble recruitment and associated modification of neuronal excitability (Whitaker et al., 2017; Y. Zhou et al., 2009). However, in the current study AHP was measured as the negative peak after an AP, independent of time course and therefore not differentiating between fast, medium, and slow AHP, complicating comparisons with other studies. In summary, despite the lack of ensemble-specific changes in firing capacity, cue-exposure after reward devaluation is likely to recruit an ensemble with altered sodium, calcium and potassium signalling.

However, further studies are required to determine the precise identity of aforementioned ion channels and underlying mechanisms, such as whether they are due to changes in ion channel number and/or their functional properties.

3.4.3 Acute and chronic alterations of dopamine signalling as regulatory mechanism of neuronal ensemble excitability

Ion channel conductances are modified by dopamine in a complex way. Decreased dopamine signalling in the NAc has been associated with decreased neuronal excitability due to Kir channel upregulation, keeping the neuron in the down state (Kim et al., 2018). D1 and D2 activation however regulate A-type potassium current in opposing directions in order to promote the switch to the reactive up state (Kitai & Surmeier, 1993; H. Steiner & Tseng, 2010; D. J. Surmeier & Kitai, 1997). Dopamine has also been shown to reduce the depolarizing sodium current responsible for the rise phase of the AP, hence decreasing MSN spiking frequency (Schiffmann et al., 1995; H. Steiner & Tseng, 2010). Based on this, it is possible that the lack of an excitability difference between ensemble and non-ensemble neurons and Fos decrease following devaluation are based on altered dopamine signalling.

A series of studies conducted by the Carelli lab found devaluation of a palatable taste by pairing it with a negative outcome to elicit acute reductions in NAc shell dopamine levels, a general dampening in dopamine function, as well as a specific NAc firing pattern, all of which are typically observed in response to aversive stimuli (Regina M. Carelli & West, 2014; Roitman, Wheeler, Wightman, & Carelli, 2008). However, as the reward devaluation in the current study was satiety- and not aversion-based, it is unclear if these findings are applicable to the current results. The same group later also found the number of NAc shell neurons firing phasically in response to a food-associated cue to be reduced after satiation-based reward devaluation, but did not directly measure dopamine regulation (E. A. West & Carelli, 2016).

Adolescent chronic sucrose exposure has been shown to have a variety of detrimental effects on reward signalling in adulthood. Specifically, motivation and hedonic reaction for sweet taste have been shown to be decreased after 16 days of adolescent sucrose exposure (Naneix, Darlot, Coutureau, & Cador, 2016; Vendruscolo, Gueye, Darnaudéry, Ahmed, & Cador, 2010). This was based on a downregulation of D1 and D2 receptor expression, a decreased sensitivity of present dopamine receptors, and reduced Fos expression, leading to a depressive-like phenotype in these animals (Gueye et al., 2018; Naneix et al., 2016, 2018). Generally, dopamine has been implicated in modulating NAc activity bidirectionally and as a regulator of the signal-to-noise ratio between evoked and spontaneous NAc activity to guide appropriate action selection (Gonon & Sundstrom, 1996; Kiyatkin & Rebec, 1999; Nicola & Deadwyler, 2000; Nicola, Surmeier, & Malenka, 2000; C. M. Pennartz, Dolleman-Van der Weel, Kitai, & Lopes da Silva, 1992; Pierce & Rebec, 1995; Yim & Mogenson, 1988). This has been shown to be at least in part mediated by D1 and D2 receptor activation eliciting changes in non-synaptic ion channels, as reflected in altered intrinsic membrane properties (Perez et al., 2006; Podda, Riccardi, D'Ascenzo, Azzena, & Grassi, 2010a). In regard to the current study this indicates that chronic sucrose exposure during devaluation could have induced dopamine-dependent modifications to reward signalling, resulting in the decreased NAc shell Fos expression (Chapter 2 Figure 3) and concurrent decreased excitability in cue-encoding GFP+ cells after devaluation.

Besides direct effects on the NAc and reward signalling, chronic sucrose exposure in adolescence has also been shown to impair hippocampal neurogenesis (Gueye et al., 2018) and performance in hippocampus-dependent spatial learning tasks (Hsu et al., 2015). This could affect NAc reward signalling as the hippocampus sends direct projections to the NAc, as well as indirect inputs via dopaminergic cells in the VTA (Loureiro, Renard, Zunder, & Laviolette, 2015). In light of the present study this could indicate that four days of sucrose

consumption resulted in changes in glutamate and dopamine input into the NAc, resulting in a cue-encoding neuronal ensemble which no longer differs in excitability compared to surrounding neurons.

A point of consideration however is that unlike aforementioned studies on effects of adolescent chronic sucrose consumption, in the current set of experiments we used adult mice and the sucrose exposure only lasted four days, as opposed to 16 - 28 days of sucrose in aforementioned studies. Chronic sucrose consumption in adulthood has been shown to produce some motivational deficits, but they were less severe and transient and potentially dopamine-independent (Gueye et al., 2018; Vendruscolo et al., 2010). Severe hippocampus-dependent memory deficits however have been shown to arise even from adult sucrose exposure (Lemos et al., 2016). Hence, in combination with the age of our mice, it is likely that our devaluation procedure was insufficient to induce long-term alterations to mesolimbic dopamine signalling, even though hippocampal alterations might be present.

3.4.4 Consequences of the lack of excitability differences between ensembles after outcome devaluation

The excitability differences between the cue-responsive GFP+ neuronal ensemble and surrounding neurons we observed after Pavlovian learning were eliminated by devaluation. Changes in intrinsic excitability control neuronal firing properties. Thus, this elimination suggests that the likelihood of a cue recruiting and eliciting firing in an ensemble is similar to a non-ensemble neuron following devaluation. In support, recently Carelli and colleagues measured firing rates of NAc shell neurons after sucrose reward devaluation using an operant conditioning procedure in which lever pressing for sucrose was paired with a visual

cue (E. A. West & Carelli, 2016). They demonstrated that devaluation (using a satiation procedure) reduced the proportion of neurons in this area that modulated cue-evoked firing responses. Their findings are also in line with our current study, in which we observed decreases in the size of the cue-activated ensemble following devaluation. Taken together, our findings together with West and Carreli may indicate that alterations in excitability controls the number of neurons that are capable of interpreting cue-relevant information. (E C Kerfoot et al., 2007; E. A. West & Carelli, 2016). Future studies may directly assess this relationship by expressing excitatory opsins in Fos-expressing neurons and using optrodes to identify these neurons and record their firing rates in response to cue presentations.

By preventing recruitment of a hyperexcitable ensemble, devaluation may influence how information is sent between ensemble and non-ensemble neurons to other downstream areas. Fewer output signals will be sent to downstream areas such as the lateral hypothalamus, VTA and ventral pallidum. The details of this have already been discussed in chapter 2, but briefly the behavioural consequence will be reduced cue-evoked sucrose seeking (Chapter 2 Figure 2) after reward devaluation. As mentioned previously, impairments in experience-dependent neuronal plasticity are associated with psychiatric and eating-related disorders, which in turn have been associated with altered reactivity to reward-associated cues (Boswell & Kober, 2016; B. L. Carter & Tiffany, 1999; Kasanetz et al., 2010; S. Liu et al., 2016; Morin et al., 2017; Oginsky et al., 2016; K. L. Smith et al., 2015). Therefore, it can be speculated that the devaluation-induced impairments of a cue to recruit an ensemble with a different excitability phenotype may interfere with the regulation of appropriate responses to food and food-associated cues following satiety. This might have important implications for the understanding and ultimately treatment of eating-related disorders and comorbidities such as binge eating disorder and obesity. Indeed, individuals

suffering from eating disorders exhibit disturbances in the ability to flexibly control behaviour upon changes in the external or internal (e.g. satiety) environment.

3.4.5 Conclusion

In summary, here we present for the first time an investigation of the changes in intrinsic excitability properties of Pavlovian sucrose-cue encoding neuronal ensembles after satiety-based reward devaluation. After confirming the existence of excitability differences between cue-responsive and cue non-responsive neurons after learning, we found these differences to be abolished by reward devaluation. Moreover, devaluation recruited an ensemble with altered intrinsic properties at the level of action potential kinetics and basic membrane properties. These mechanisms are likely to drive the attenuation of cue-elicited sucrose seeking after devaluation we observed in Chapter 2, however it is also possible for specific synaptic alterations to play are role, which we will investigate in Chapter 4.

4 The effects of devaluation on synaptic alterations in a Nucleus accumbens shell neuronal ensemble

4.1 Introduction

In the previous chapters we used reward devaluation to investigate how alterations in outcome value alter cue-evoked sucrose seeking and underlying Nucleus accumbens (NAc) neuronal ensemble recruitment and intrinsic excitability. Devaluation attenuated the ability of the cue to evoke sucrose seeking and to recruit a hyperexcitable ensemble. Moreover, the size of the recruited ensemble was smaller compared to what was recruited under Non-devalued conditions. These mechanisms are likely to contribute to the attenuation of cue-evoked sucrose seeking observed following devaluation. In addition to changes in intrinsic excitability the fidelity of information transfer in neural networks is modulated by changes in synaptic transmission. Here we investigated whether devaluation would induce synaptic alterations on cue-activated NAc ensembles.

Synaptic plasticity is one of the crucial mechanisms of learning and memory processes in normal brain function and impairments have been associated with neuropsychiatric disorders such as drug addiction and binge-eating disorder, as well as with increased intake of highly palatable food, potentially leading to overeating and obesity (Kasanetz et al., 2010; S. Liu et al., 2016; Morin et al., 2017; K. L. Smith et al., 2015). Plasticity at the glutamatergic synapse can involve the formation of silent synapses, alterations in AMPA receptor subunit composition (altering conductance), changes of the AMPA- and NMDA receptor-mediated postsynaptic potentials or the ratio between them, as well as plasticity of synaptic spines (Namburi et al., 2015; Singer et al., 2016; Whitaker et al., 2016; Marina E. Wolf & Ferrario, 2010). Additionally, repositioning of AMPA receptors can act as a mechanism of synaptic

plasticity, as their efficiency depends on their location which can be at the postsynaptic density, extrasynaptically, or in internal storages (Esteves da Silva et al., 2015; Penn et al., 2017). Reward-related behaviours have been shown to be accompanied by an alteration of AMPA receptor subunit composition at NAc shell synapses, strengthening synaptic transmission and behavioural responses (McCutcheon, Wang, Tseng, Wolf, & Marinelli, 2011). Additionally, D1 receptor activation has been shown promote insertion of AMPA receptors to extra synaptic sites and hence facilitate their insertion into the synapse tas a form of synaptic plasticity (Marina E. Wolf, 2010). Studies investigating associative learning with drug rewards revealed that drug-associated cues induce synaptic adaptations at glutamatergic synapses in ventral tegmental area (VTA) and NAc (Chen et al., 2008; Degoulet, Stelly, Ahn, & Morikawa, 2016; Gipson et al., 2013; M. E. Wolf, 2003). In line with this, Suto and colleagues demonstrated glutamate levels in NAc to be elevated in response to drug-associated cues (Suto, Elmer, Wang, You, & Wise, 2013). Similarly to drug cues, cues associated with highly palatable appetitive rewards have been shown to induce adaptations in synaptic strength at glutamatergic synapses in areas relevant for mediating reward-associated cues such as NAc, amygdala, and VTA (Namburi et al., 2015; Stuber et al., 2008; Tye, Stuber, De Ridder, Bonci, & Janak, 2008). Conversely, optogenetic modulation of glutamatergic transmission in NAc has been shown to alter cue-evoked sucrose seeking behaviour (Stuber et al., 2011). Mostly due to technical limitations, most previous studies investigating synaptic alterations elicited by associative learning were conducted in a random selection of neurons or in a pathway specific manner and utilized a reward with a stable value. Therefore, little is known about glutamate synapse alterations specifically on neurons encoding cue-reward associations and whether this is modulated by changes in outcome value.

Based on this, we hypothesised that reward devaluation is mediated by adaptations of glutamatergic synapses on cue-activated neuronal ensembles in NAc shell. To that end, the aim of this chapter was to examine the effects of associative learning using sucrose as a natural reward followed by devaluation on pre- and postsynaptic parameters of synaptic plasticity in cue-activated compared to cue non-activated NAc shell neurons.

4.2 Material and Methods

4.2.1 Animals

Similar to Chapter 2 and 3, male heterozygous *Fos-GFP* mice were housed and food restricted under the same conditions. Mice were 9 - 10 weeks old at the beginning of behavioural testing. All experiments were conducted during the light phase and in accordance with the UK Animals (Scientific Procedures) Act of 1986 after ethical approval by the University of Sussex Animal Welfare and Ethical Review Body.

4.2.2 Behavioural experiment

Mice were trained using the same behavioural protocols for Pavlovian conditioning and devaluation as in Chapter 2 and 3.

4.2.3 Electrophysiology

4.2.3.1 ex vivo *brain slice preparation*

Brain slice preparation was similar to Chapter 3.

4.2.3.2 Electrophysiological recording

Similar to chapter 3, for NAc shell voltage clamp recordings, the slices were hemisectioned and transferred to the recording chamber. GFP+ neurons were identified using a 488 nm laser and a Revolution XD spinning disk confocal system (Andor system) and differential interference contrast visualization was done using an Olympus BX51W1 microscope. Whole cell patch clamp recordings were performed using ICS specific for voltage clamp recordings (intracellular solution, concentrations in mM: Spermine 4HCl 0.1, $CsCH_3SO_3$ 120, NaCl 5, TEA-Cl 10, HEPES 10, EGTA 1.1, Mg-ATP 4, Na-GTP 0.3, pH 7.2, lidocaine N-ethyl chloride 0.001) - filled borosilicate capillary glass-pipettes (similar to Chapter 3). The rationale for including the sodium channel blocker lidocaine was to avoid action currents

when the cells were clamped to +40mV during the recording for AMPA/NMDA ratios. This data was later excluded from this thesis but the recordings were performed together. Alexa Fluor 568 dye (100 µM, A10437, Thermo Fisher Scientific) was added to the ICS to confirm patched cells by colocalization with GFP. Medium spiny neurons (MSNs) were identified using morphology and resting membrane potential and held at -80 mV for the duration of the recordings.

Excitatory post-synaptic currents (EPSCs) were measured in continuously (flow rate approximately 2 ml/min) refilled aCSF at 32 °C containing picrotoxin (100 µM, Sigma) to block GABAA receptor-mediated inhibitory postsynaptic currents. Spontaneous EPSCs (sEPSCs) were obtained in the absence of stimulation for 60 s. Since sodium-channel blockers such as tetrodotoxin do not modulate sEPSC frequency nor amplitude in the NAc (Koya et al., 2012) these events are likely action-potential independent, and represent the postsynaptic response to the release of one single vesicle (Pinheiro & Mulle, 2008). Additionally, MSNs are known even *in vivo* to show very low spontaneous activity and their typical shift between up- and downstates do not occur in slices as they depend on excitatory inputs from upstream areas (C J Wilson & Kawaguchi, 1996; Charles J. Wilson, 1993). A frequency distribution plot for EPSC amplitude followed a Gaussian, but not a bimodal distribution further supporting that the sEPSCs in the current study were only mEPSCs and not a mixture of mEPSCs and larger action potential dependent EPSCs.

The evoked EPSCs (eEPSCs) were obtained by stimulating in and/or near the NAc shell 200-400 µm away from the recorded neuron with single pulses of 0.1 ms duration each were delivered using an isolated stimulator (DS2A, Digitimer) at 0.033 Hz using a concentric bipolar stimulating electrode (FHC). Series resistance was monitored using -10 mV voltage steps of 200 ms and only cells maintaining stable access (<20% change) were included in the analyses (Ji et al., 2015; Nagarajan, Jones, West, Marc, & Capecchi, 2017; Tsui,

Schwartz, & Ruthazer, 2010) . Paired Pulse Ratios (PPRs) were measured by delivering stimulus trains at 20, 40, 60, 80, 100, 150, and 200 ms interstimulus intervals with three responses per each train. To calculate the PPR, the peak mean EPSC of the second evoked response was divided by the peak mean EPSC of the first.

Data were collected with a Multiclamp 700B amplifier (Molecular Devices), WinEDR (version 3.7.5) and WinWCP Software (version 5.2.2, courtesy of Dr. John Dempster, University of Strathclyde, Glasgow, UK; http://spider.science.strath.ac.uk/sipbs/software_ses.htm, RRID: SCR_014713). Signals were digitized at 10 kHz and filtered at 5 kHz (PCI 6024E; National Instruments) and 50 Hz noise was filtered out using a HumBug (Quest Scientific) module.

4.2.4 Experimental Design and Statistical Analysis

Data were analysed and visualized using GraphPad Prism 6 (Graphpad software, RRID:SCR_002798), SPSS (IBM SPSS statistics, RRID:SCR_002865), and Excel (Microsoft). Additionally, EPSCs were analysed using Win EDR (version 3.7.5) and Mini Analysis Software (version 6.0; Synaptosoft, RRID:SCR_002184) and PPRs were analysed using WinWCP Software (version 5.2.2, courtesy of Dr. John Dempster, University of Strathclyde, Glasgow, UK; http://spider.science.strath.ac.uk/sipbs/software_ses.htm, RRID: SCR_014713). All data is presented as mean ± SEM. ANOVAs with significant interactions or main effects were followed up by Fisher's LSD post-hoc test. Our lab and others in the field regularly use this test to compare between groups which show a significant main effect in an ANOVA, and/or significant interaction (Whitaker et al., 2016, 2017; Ziminski et al., 2017b).

In text and figures values of mean ± 2xSD were considered outliers and non-transformed raw data is shown. For PPR no outliers were detected. For sEPSC frequency one outlier was removed (Devalued GFP- group), for amplitude three outliers were removed (Non-

devalued and Devalued GFP+, Non-devalued GFP-). sEPSCs below 5 pA were excluded to remove noise from the analysis. Then normal distribution was assessed using visual inspection of the data and Kolmogorov-Smirnoff tests. For repeated measures data, normality testing was performed on the standardized residuals. If independent sample data sets or the majority of a repeated measures data set deviated from normal distribution, non-parametric tests were used to confirm the results of the parametric tests. In data sets following a Gaussian normal distribution, an alternative method of outlier detection was used (Grubb's test). See Appendix 1 for normality testing and non-parametric tests, as well as reanalysis of data with previous log transformation.

sEPSC frequency and amplitude were analysed using two-way ANOVAs with devaluation (Non-devalued, Devalued) and GFP (+/–) as between-subjects factors. PPRs were analysed using a three-way mixed ANOVA with devaluation (Non-devalued, Devalued) and GFP (+/–) as between-subjects factors and interstimulus interval as within-subjects factor.

4.3 Results

Similar to Chapters 2 and 3, mice underwent a Pavlovian approach test following sucrose conditioning. We then assessed synaptic alterations of cue-responsive, GFP+ ensembles and surrounding cue non-responsive GFP– neurons.

4.3.1 Devaluation did not modulate Paired Pulse Ratios in cue-activated neurons

We calculated and compared the PPRs and a three-way mixed ANOVA showed no significant interactions (interval x GFP x devaluation $F_{6, 126} = 1.716$, $p = 0.122$; interval x GFP: $F_{6, 126} = 0.137$, $p = 0.991$; interval x devaluation: $F_{1, 126} = 0.485$, $p = 0.819$; GFP x devaluation: $F_{1, 21} = 0.01$ $p = 0.919$), no effect of devaluation ($F_{1,} 21 = 0.002$, $p = 0.963$) or GFP ($F_{1, 21} = 0.013$, p = 0-909) but an effect of within-subjects factor interstimulus interval ($F_{6, 126} = 9.471$, $p < 0.001$) (see Figure 1A, B). This indicates that neither reward devaluation nor ensemble identity modulates presynaptic release probability.

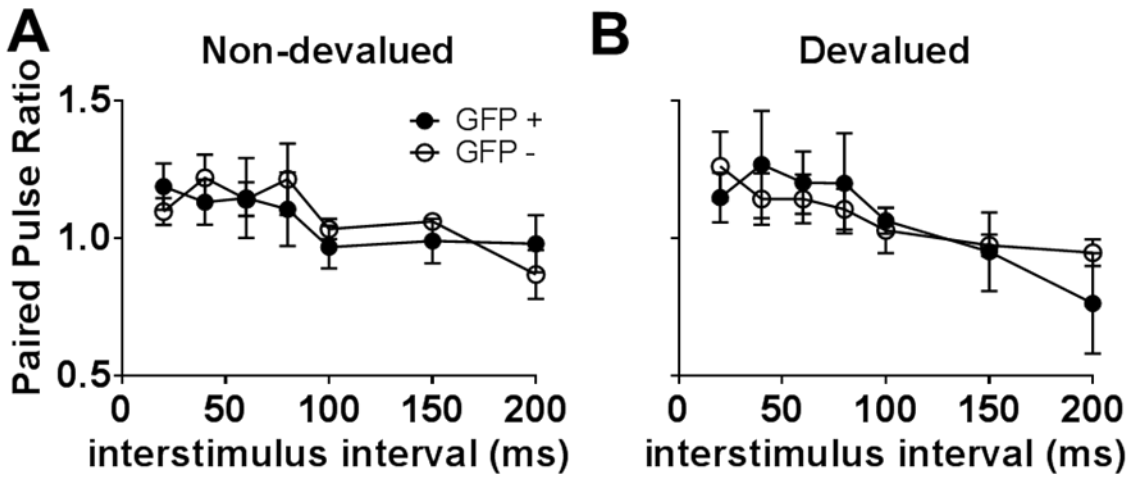

Figure 1: Paired Pulse Ratio did not differ between GFP+ and GFP– neurons and was not modulated by reward devaluation. A: Paired Pulse Ratios in the Non-devalued group did not differ between GFP+ and GFP– neurons. (GFP – /+ *n* = 9/3). B: Paired Pulse Ratios in the Devalued group did not differ between GFP+ and GFP– neurons. (GFP – /+ *n* = 8/5). All values are mean ± SEM.

4.3.2 Appetitive associative learning and reward devaluation did not induce any ensemble-specific differences in sEPSC amplitude or frequency, but sucrose exposure elicited a non ensemble-specific modulation of sEPSC frequency

Next we examined sEPSCs and analysed frequencies and amplitudes to determine devaluation-induced alterations in pre- and postsynaptic properties (Figure 2). A two-way ANOVA of the sEPSC frequency revealed no interaction ($F_{1,66}$ = 0.1183, p = 0.732) or effect of GFP ($F_{1,66}$ = 0.1145, p = 0.736) but a significant overall effect of devaluation ($F_{1,66}$ = 5.719, p = 0.0196) (Figure 2 A). Post-hoc tests reveal a non-significant trend between GFP– cells in Devalued and Non-devalued groups (p = 0.051). Based on this and on previous findings indicating a dependency of sEPSC frequency in NAc synapses on satiety level (Namburi et al., 2015; Ouyang et al., 2017) we performed an additional comparison of pooled, non

ensemble-specific sEPSC frequencies from Devalued and Non-devalued groups using a t-test revealed a significant overall difference ($t = 2.458$, $p = 0.0165$). The cumulative probability plots of the sEPSC interevent interval (Figure 2 C,D) confirm this result of no significant ensemble specific difference in sEPSC frequency.

Next, we examined postsynaptic changes and a two-way ANOVA of sEPSC amplitude showed no interaction between devaluation and GFP ($F_{1,68} = 0.8192$, $p = 0.3686$) and no main effects (GFP $F_{1,68} = 0.3376$, $p = 0.5631$; devaluation $F_{1,68} = 0.112$, $p = 0.7389$) (Figure 2 B). The cumulative probability plots of peak amplitude (Figure 2 E,F) confirm that there are no differences in sEPSC amplitude induced by devaluation or associative learning.

Taken together, this indicates that neither reward devaluation nor associative learning induced ensemble-specific alterations in the number of active or silent synapses or in the number or composition of postsynaptic receptors.

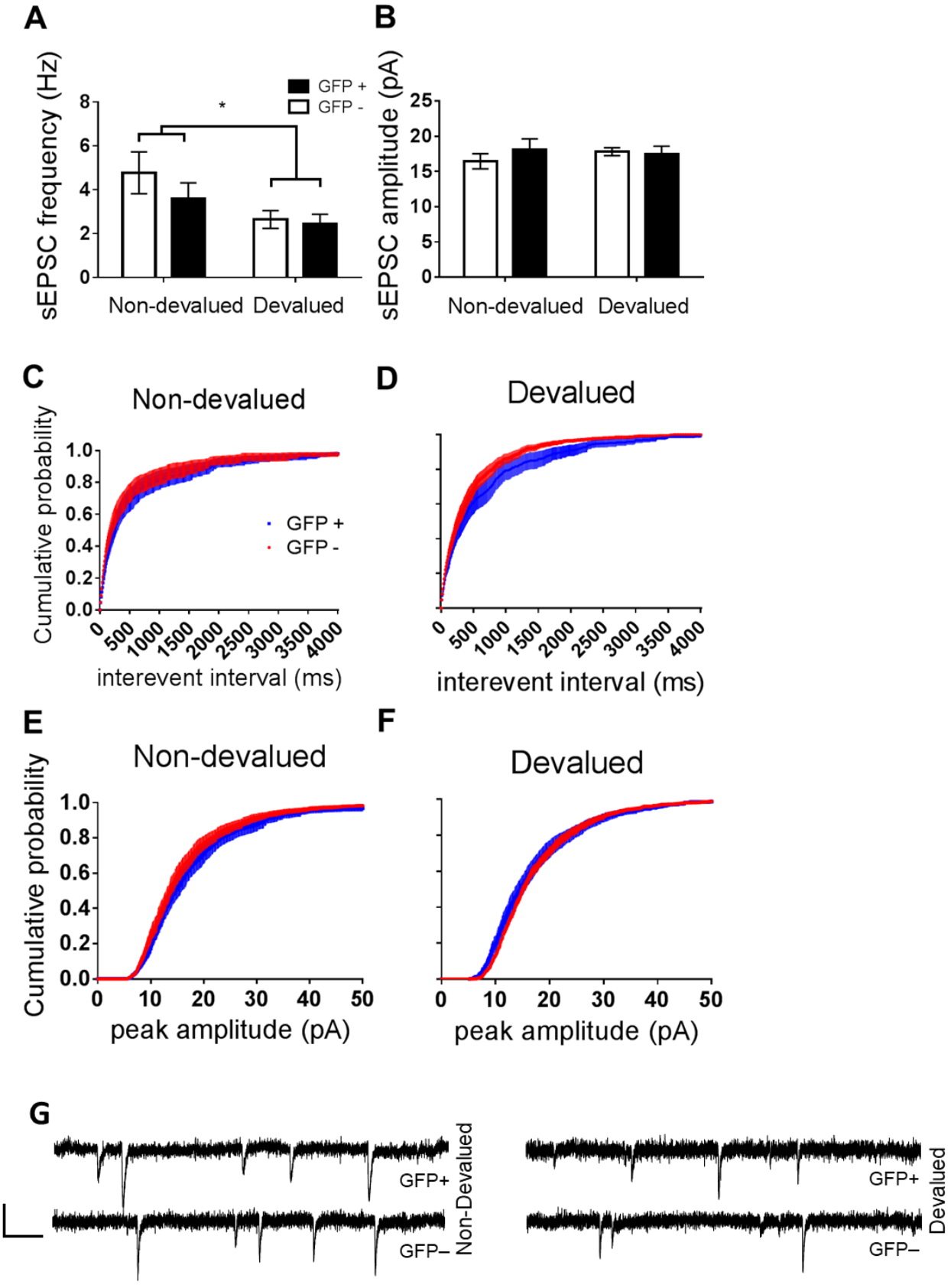
A
sEPSC frequency (Hz)
GFP +
GFP -
*
Non-devalued
Devalued
B
sEPSC amplitude (pA)
Non-devalued
Devalued
C
Non-devalued
Cumulative probability
GFP +
GFP -
interevent interval (ms)
D
Devalued
interevent interval (ms)
E
Non-devalued
Cumulative probability
peak amplitude (pA)
F
Devalued
peak amplitude (pA)
G
GFP+
GFP–
Non-Devalued
GFP+
GFP–
Devalued

Figure 2: No ensemble-specific synaptic plasticity was observed after reward devaluation or associative learning. (A) After devaluation sEPSC frequency was decreased in all neurons. (B) sEPSC amplitude was unaltered by associative learning, or devaluation. (C) Cumulative probability plot for interevent interval in Non-devalued and (F) Devalued groups. (E) Cumulative probability plot for mEPSC amplitude in Non-devalued and (F) Devalued groups. (G) Representative sEPSC traces. All values are mean ± SEM. Non-Devalued n (+/-) = 16/20, Devalued n (+/-) = 17/19, *$p<0.001$ GFP pooled analysis. Scale bar 20 pA, 100 ms.

4.4.4 Discussion

We examined the effects of reward devaluation on synaptic plasticity on cue-activated NAc shell neurons. Despite the role of glutamate synapse alterations in appetitive learning, we found no devaluation- nor conditioning-induced alterations in sEPSC frequency and amplitude and PPR. We however observed a generalised reduction in sEPSC frequency, indicating widespread synaptic alterations that were induced by four days of *ad libitum* sucrose consumption. Taken together, our findings raise the possibility that other plasticity mechanisms may play a role in the establishment and updating of appetitive cue-outcome associations. Below we discuss the measures used and possibilities for why we failed to observe the aforementioned indices of synaptic plasticity.

Electrophysiological recordings are a widely used technique to research synaptic plasticity. Alterations in PPR can provide information about changes in presynaptic vesicle release probability, a form of short term synaptic plasticity (Citri & Malenka, 2008; Gina G. Turrigiano & Nelson, 2004; Zucker & Regehr, 2002). In contrast, sEPSC amplitude and frequency changes indicate changes in postsynaptic receptors and synaptogenesis/activation or formation of silent synapses, respectively, constituting mechanisms of long term plasticity (Citri & Malenka, 2008; Gina G. Turrigiano & Nelson, 2004). Besides electrophysiological parameters, measurements of spine dynamics can provide information about long term synaptic alterations as the formation or elimination of a spine is usually associated with the formation or elimination of the according synapse (Becker, Wierenga, Fonseca, Bonhoeffer, & Nägerl, 2008; Trachtenberg et al., 2002). Additionally, spine types with bigger heads are known to have a larger postsynaptic density and more AMPA receptors compared to smaller spines, indicating a strengthening of synaptic transmission (K M Harris & Stevens, 1989; Kristen M Harris, Jensen, & Tsao, 1992; M Matsuzaki et al., 2001).

4.4.1 Devaluation did not alter pre- and post-synaptic indices of plasticity on cue-activated ensembles

In the current study, we did not observe any devaluation- nor conditioning-induced differences in PPR, sEPSC frequency or amplitude between the cue-activated ensemble and surrounding neurons. Based on this, synaptic alterations on NAc shell ensembles may not play a role in encoding cue-outcome associations or reward value for natural rewards in our procedure. This stands in contrast to studies using drugs as reward in conditioning paradigms to investigate ensemble-specific synaptic alterations. Thus, NAc neuronal ensembles selectively activated in response to cocaine- and amphetamine-associated cues have been demonstrated to exhibit increased formation of silent synapses and spine dynamics, respectively (Singer et al., 2016; Whitaker et al., 2016).

This difference between conditioning paradigms using natural and drug rewards in their ability to generate synaptic alterations in NAc may be due to natural rewards being relatively weak compared to drugs. In support, Tunstall and colleagues revealed that cue-induced cocaine seeking is more robust than food seeking despite food being a better reinforcer than cocaine (B. J. Tunstall & Kearns, 2016). Moreover, incubation of cue-induced reinstatement of reward seeking appears to be more robust for cocaine than for sucrose, and some researchers report a complete lack of this phenomenon with sucrose (Grimm et al., 2003; Nugent, Anderson, Larson, & Self, 2017). Correspondingly, molecular and structural alterations associated with overall synaptic plasticity in NAc and VTA underlying these and similar behavioural paradigms have been shown to only be induced when cocaine is used as a reward, not sucrose, suggesting that the weak behavioural effects of sucrose may be mediated by different mechanisms (Gipson et al., 2013; Grimm et al., 2003; L. Lu, Grimm, Shaham, & Hope, 2003). Finally, also sucrose self-administration and cue-evoked approach produced only transient, reversible synaptic potentiation in the VTA, while the same

paradigm with a cocaine reward produced long lasting, persistent alterations, further supporting the notion of natural rewards being less powerful than drugs and hence not altering synaptic parameters in the current study (Chen et al., 2008; Stuber et al., 2008).

Alternatively, the lack of synaptic alterations in cue-encoding neuronal ensembles in NAc using appetitive conditioning in the current study may be based on a difference in neuronal pathways mediating associations of cues and natural compared to drug rewards. The Carelli lab conducted a series of studies in rats using *in vivo* electrophysiology to demonstrate that NAc neurons exhibit different firing patterns in response to natural and drug rewards, extending previous findings from rhesus monkeys, indicating the existence of separate functional circuits for these two types of rewards (Bowman, Aigner, & Richmond, 1996; R M Carelli & Deadwyler, 1994; R M Carelli, Ijames, & Crumling, 2000; Hollander, Ijames, Roop, & Carelli, 2002). Accordingly, food- and drug-associated cues have been found to act on overlapping but partially different receptors, offering further explanation for differences in synaptic plasticity depending on the type of reward (Wickham, Solecki, Nunes, & Addy, 2015). Furthermore, the fact that sucrose only indirectly alters DA transmission whereas cocaine acts directly on DA receptors may result in the recruitment of only a subset of neuronal pathways by sucrose and hence be responsible for the differences in these reinforcers' abilities to induce synaptic alterations. Cocaine as a reinforcer acts relatively unselectively on both D1 and D2 receptors in associative learning paradigms to produce behavioural effects, whereas for sucrose or food this is much less clear, which may further contribute to its potency in inducing synaptic plasticity (Bachtell, Whisler, Karanian, & Self, 2005; Bari & Pierce, 2005; Hubner & Edward Moreton, 1991; James, McGlinchey, Vattikonda, Mahler, & Aston-Jones, 2018; Spealman, 1990).

In the current study we used 12 days of training, however there is evidence that for associative learning paradigms using natural reinforcers a longer training period is needed

to induce synaptic alterations in cue-activated ensembles. In support of this, a pair of studies investigating synaptic plasticity after operant conditioning in rats using highly palatable food as reward revealed an increase in spine density and extracellular signal–regulated kinase (ERK) signalling in the NAc shell after 41 days, but not 10 days of training (Guegan, Cutando, Ayuso, et al., 2013; Guegan, Cutando, Gangarossa, et al., 2013). It has previously been suggested by Koya and Whitaker that repeated activation of the conditioned stimulus (CS) and unconditioned stimulus (US) encoding afferents, as would be the case in an increased number of training sessions, induces ensemble specific synaptic alterations (Koya et al., 2012; Whitaker et al., 2016). Accordingly, there has been a report of synaptic plasticity induced in behaviourally activated medial prefrontal cortex (mPFC) neurons after stress-induced reinstatement of operant food seeking (Cifani et al., 2012). As inherently in operant paradigms there are more cue-reward pairings per training session compared to Pavlovian conditioning, this further supports the idea of the induction of synaptic plasticity by extended training with natural rewards. However, we cannot excluded that the effects found by Cifani and colleagues are due to differences in the specific brain area (mPFC instead of NAc) or paradigm (stress-induced reinstatement of operant responding after extinction instead of Pavlovian approach test and reward devaluation) under investigation.

Finally, the length of the incubation period may impact the development of synaptic adaptations in neuronal ensembles encoding sucrose-associated cues in the current study. Evidence for this comes from Counotte and colleagues who were able to demonstrate a decrease in NAc synaptic strength 21 days but not 1 day after cessation of a sucrose self-administration paradigm (Counotte, Schiefer, Shaham, & Donnell, 2014). Therefore, an extension of the 7 – 9 day long period used in the current study may be able to induce synaptic alterations following sucrose conditioning.

Taken together, the lack of conditioning- and devaluation-induced indices of plasticity at glutamatergic synapses we demonstrate in cue-activated neuronal ensembles may reflect inherent differences of natural compared to drug rewards and the way their behavioural outcomes are manifested in NAc shell cue-activated neuronal ensembles. This suggests that conditioned behaviours with natural rewards may be mediated via different mechanisms or require modulation of the behavioural paradigm to evoke synaptic alterations.

4.4.2 Reward devaluation by *ad libitum* sucrose exposure induced a generalised, non ensemble-specific modulation of presynaptic properties

Here we observed a general decrease in sEPSC frequency in Devalued mice, indicating that removal of synapses or transformation into silent synapses took place in the majority of NAc shell neurons following four days of *ad libitum* sucrose (Gina G. Turrigiano & Nelson, 2004). An additional interpretation for changes in sEPSC frequency has been suggested by the MacGillavry lab, stating that a decrease in AMPA receptor cluster number could be reflected in a decrease in sEPSC frequency, based on the previous finding that spontaneous neurotransmitter release sites are not aligned with postsynaptic AMPA receptor clusters (W. Lu et al., 2009; Scheefhals & MacGillavry, 2018; Tang et al., 2016). In line with the current results, a previous study revealed an increase in sEPSCs frequency in NAc shell MSNs after food restriction compared to *ad libitum* fed conditions, indicating that in this region satiety state modulates synaptic transmission (Ouyang et al., 2017). Along the same lines, synaptic strength was reduced in glutamatergic synapses from basolateral amygdala (BLA) onto NAc in food restricted compared to non-restricted mice (Namburi et al., 2015). Thus, in the present study, 4 days *ad libitum* sucrose in the Devalued group, which reached 100% bodyweight, might have counteracted the food restriction induced increase of sEPSC frequency and elicited synaptic plasticity via removal of active synapses or a decrease in

AMPAR clusters. Due to the ubiquitous location of these alterations they are unlikely to drive the devaluation-induced attenuation in cue-evoked sucrose seeking observed in chapter 2.

4.4.3 Technical/methodological considerations

In the current study we measured PPR of eEPSCs as well as sEPSC amplitude and frequency. While spontaneous action potential dependent activity might also contribute to sEPSC frequency and amplitude in the current study, this is unlikely due to the known absence of spontaneous activity in MSNs, especially in slice (C J Wilson & Kawaguchi, 1996; Charles J. Wilson, 1993). Additionally, a previous study using tetrodotoxin to block APs during recording did not detect any differences in EPSC amplitude or frequency compared to pre-tetrodotoxin (Koya et al., 2012). In future this may be repeated for the specific experimental conditions in order to confirm unequivocally that the measured sEPSCs are miniature events and hence caused by the release of a single quanta if needed.

Changes in sEPSC amplitude are widely believed to reflect postsynaptic changes, such as altered number or function/subunit composition of postsynaptic receptors (Gina G. Turrigiano & Nelson, 2004). As the release of one quanta of neurotransmitter however may not saturate the postsynaptic receptors, a change in sEPSC amplitude can additionally indicate a change in the amount of neurotransmitter released (Takamori, 2016). Furthermore it has been remarked that only the postsynaptic receptors located closely to neurotransmitter release sites will be activated by diffusing neurotransmitters elicited by presynaptic activity, making it impossible to draw conclusions from sEPSC amplitude about total postsynaptic receptor numbers (Biederer et al., 2017; Lisman, Raghavachari, & Tsien, 2007). This seems to be true for synapses in brain regions with a high degree in adult plasticity such as the hippocampus, as for more rigid synapses in other regions there seems indeed to be a correlation of response amplitude and postsynaptic number of receptors (Tarusawa et al., 2009). As the NAc is a brain are with a high degree of adult plasticity, in the current study

sEPSC amplitude should only be used to draw conclusions about receptor function based on subunit composition, receptor density and position of receptor clusters close to the specific vesicle release site (Biederer et al., 2017; Lisman et al., 2007; MacGillavry, Kerr, & Blanpied, 2011). A better measure for the number of postsynaptic receptors is the AMPA/NMDA ratio, as it is calculated using eEPSCs which follow the release of a larger amount of vesicles and in close vicinity to postsynaptic receptor clusters (Biederer et al., 2017; Tang et al., 2016). Hence, the released glutamate is more likely to activate more AMPA receptors and therefore this parameters is giving more reliable information about synaptic strength (Biederer et al., 2017).

Moreover, this measure could provide additional information in future studies in relation to satiety induced effects on synaptic plasticity. Besides the aforementioned effects of satiety state on sEPSC frequency, which has been shown to be independent of calcium permeable GluR2 lacking AMPA receptors (CP AMPARs), eEPSC amplitude also seems to be modulated by food restriction, and this effect seems to be mediated by a shift in the number of CP AMPARs (Ouyang et al., 2017; Peng, Ziff, & Carr, 2011; Scheefhals & MacGillavry, 2018). A change in AMPA receptor subunit composition resulting in up- or downregulation of CP AMPARs is indicated by the AMPA rectification index, which can then be used to draw conclusions about functional alterations (Jonas & Burnashev, 1995; Seung, Hyeong, Ki, Qing, & Sung, 2009). Differential regulation of evoked and spontaneous EPSCs has previously been demonstrated (Kavalali, 2015; Ouyang et al., 2017; Owen et al., 2013; Scanziani, Capogna, Gähwiler, & Thompson, 1992) and might possibly be due differences in alignment of evoked and spontaneous neurotransmitter release site and postsynaptic receptor cluster (Tang et al., 2016) . Hence, AMPA/NMDA ratios and AMPA rectification indices could provide valuable information about diet induced postsynaptic alterations as well as give closer insight in the mechanisms behind potential postsynaptic alterations.

Besides the aforementioned 'classical' interpretations of sEPSC frequency changes, namely synaptogenesis or formation of silent synapses, a change in presynaptic release probability or an amplitude change close to noise levels should be considered. The connection between a change in miniature sEPSC frequency and presynaptic release probability has been shown before, however in the current study this is unlikely to drive the observed non ensemble-specific effects of devaluation, as the PPR was similar in all groups (E. B. Han & Stevens, 2009). Additionally, alterations in sEPSC amplitude near the noise level may have been filtered out as noise and hence these events may not been detected as sEPSCs, passively changing the frequency. This is a risk which we minimized by methods reducing noise, such as grounding all electrical equipment, insulating cables, and regular bleaching of electrodes. An additional measure for noise reduction is placing the electrophysiology rig in a Faraday cage, but this was not done here due to practical limitations.

In the current study we obtained aforementioned eEPSCs for calculation of PPR by stimulating 200-400 µm away from the recording site. Hence, we did not control where these inputs were originating. In order to determine if activation of inputs from specific upstream areas may have different effects on cue-activated neurons in future studies optogenetics could be used specifically activate one input by light and measure effects in NAc ensembles.

Formation of silent synapses has been associated with repeated drug exposure as well as associative learning using drug rewards specifically in behaviourally activated NAc neurons (Koya et al., 2012; Whitaker et al., 2016). To our knowledge it is currently unknown if sucrose reward learning or sucrose consumption alone can elicit similar increases in silent synapses. In the current study we observed a non ensemble-specific decrease in sEPSC frequency indicating the removal of synapses or a transformation of existing synapses into silent synapses by AMPA receptor endocytosis. In future experiments, this could be elucidated using a minimal stimulation assay (Koya et al., 2012; Liao, Hessler, & Malinow, 1995).

In the current study we recorded EPSCs from MSNs which *in vivo* originate from glutamatergic inputs, however MSNs also receive GABAergic input from local interneurons, causing inhibitory postsynaptic potentials (IPSPs). Whereas the synaptic plasticity associated with excitatory inputs on MSNs is well researched, studies researching synaptic plasticity of IPSPs in MSNs are rare. It has been shown that this type of plasticity plays a role in cocaine self-administration, in which cue-reward associations are established similar to the current study (Otaka et al., 2013). In future studies these currents and their plasticity could be measured in MSNs, which will provide valuable information about synaptic plasticity of inhibitory inputs on MSNs and their role in reward devaluation.

Not only principal neurons such as MSNs, but also interneurons can undergo synaptic plasticity. In the VTA and NAc GABAergic and cholinergic interneurons have been shown to display synaptic plasticity (Fino, Deniau, & Venance, 2008; Ostroumov & Dani, 2018). In parvalbumin expressing interneurons in the NAc this plasticity has been shown to be involved in regulating cue-elicited cocaine seeking in the case of (Yu et al., 2017). The involvement of NAc interneuron synaptic plasticity in appetitive conditioning and reward devaluation could be elucidated by recording specifically from GABAergic or cholinergic interneurons. To this end, using transgenic animals and/or viral vectors based on Cre-recombinase activity or double labelling using the *Fos* promoter would be feasible, making it possible to visualize recently activated specific subtypes of interneurons (Camillo et al., 2018; DeFalco et al., 2001; S. Gong et al., 2007; Kaiser, Ting, Monteiro, & Feng, 2016; Neske, Patrick, & Connors, 2015; Tanahira et al., 2009; Taniguchi et al., 2011).

Finally, the modulatory effects of neuropeptides such as orexins, endocannabinoids and opioids on cue-evoked sucrose seeking may be addressed in future studies. These peptides mediate motivation for food in the NAc with a specific hotspot in the medial NAc shell mediating palatability or hedonic liking (D. C. Castro & Berridge, 2014; Stephen V. Mahler

et al., 2007; Pecina, 2005; K. S. Smith, 2005). Therefore the amount or efficacy of these neuromodulators may be regulated differentially depending on reward value. This may impact ensemble recruitment and activity, as well as neuronal plasticity ultimately determining behavioural output in the Devalued group of the current study. Future studies could investigate these questions first by measuring levels of the endogenous peptides in Devalued and Non-devalued groups, and subsequently performing direct microinfusions in the NAc in order to see behavioural consequences of the peptide action in the NAc without side effects of activation of peptide producing upstream areas.

4.4.4 Conclusion

In the current chapter, we investigated synaptic plasticity of glutamatergic synapses on MSNs in the NAc shell following appetitive Pavlovian conditioning and subsequent satiety based reward devaluation. Our results indicate that neither appetitive conditioning nor devaluation induced ensemble-specific alterations in PPR or sEPSC frequency or amplitude at glutamatergic synapses, which stands in contrast to similar paradigms using drug rewards. This indicates that cue-outcome associations and their updating are mediated by a different mechanism when natural rewards are used. However, a generalized decrease of sEPSC frequency was observed after four days of sucrose which might have elicited a decrease in functional synapses or AMPA receptor clusters.

5 General discussion

5.1 Summary of results

In the current study we examined the effects of reward devaluation on ensemble plasticity at the levels of recruitment, excitability, and synaptic physiology in sucrose conditionined *Fos-GFP* mice that express green fluorescent protein (GFP) in recently activated neurons. In chapter 3, after appetitive conditioning we provided mice with 4 days of *ad libitum* sucrose or standard chow. Sucrose access, but not caloric satiation alone attenuated cue-evoked sucrose seeking and hence led to reward devaluation. This indicates that changes in the hedonic, incentive value of sucrose, and not caloric need alone drove cue-evoked sucrose seeking. Additionally, immunohistochemical staining revealed that sucrose reward devaluation reduced the size of the cue-activated neuronal ensemble in NAc, but not ds. In chapter 3, we used slice electrophysiology in NAc shell to record from cue-activated, GFP+ neurons and surrounding, non-activated GFP– neurons. In line with our recent study by Ziminski and colleagues cue-activated neurons were more excitable than non cue-activated neurons after appetitive conditioning, and reward devaluation eliminated this excitability difference (Ziminski et al., 2017b). Interestingly, in chapter 4 reward devaluation did not alter the physiology of excitatory synapses on cue-activated neurons.

In conclusion we achieved our aim to elucidate how devaluation modulates recruitment in terms of ensemble size as well as underlying intrinsic and synaptic neuroplasticity in the NAc shell. We confirmed our hypothesis that non-aversive devaluation of a sucrose reward decreases cue-evoked sucrose seeking and that this is reflected in alterations in activity patterns as well as neuronal plasticity in NAc shell. This hints at a causal relationship between these changes and the behavioural outcome however this needs confirmation in further experiments (using functional manipulations such as DREADD or optogenetics). Our findings provide new insights into how updates in the hedonic value of sucrose critically

modulates the flexibility of cue-evoked sucrose seeking and recruitment of ensembles with an altered excitability phenotype in the NAc shell.

5.2 Potential role of intrinsic excitability alterations in cue-activated neuronal ensembles

In the current study we found neuronal excitability but not excitatory synaptic physiology to be altered by sucrose reward devaluation. In contrast, in studies examining associative learning paradigms employing fear conditioning, both, intrinsic excitability and synaptic physiology are concomitantly altered and with overlapping signalling pathways (Antonov, Antonova, Kandel, & Hawkins, 2003; Daoudal & Debanne, 2003; Li et al., 2004; Rosenkranz & Grace, 2002; Xu, 2005). These types of plasticity can be regulated in the same or opposing directions (Antonov et al., 2003; Daoudal & Debanne, 2003; Fan et al., 2005; Lorenzetti, Mozzachiodi, Baxter, & Byrne, 2006; Rosenkranz & Grace, 2002). Hence, it has previously been suggested that alterations in excitability serve as a transient priming mechanism for memory formation before synaptic changes take place (Janowitz & Van Rossum, 2006; Mozzachiodi & Byrne, 2010). Evidence for this stems from studies showing that excitability changes occur early on during learning of a conditioning procedure and return to baseline even though the memory persists, pointing at a different, potentially synaptic mechanism for the later phases of memory consolidation (Moyer et al., 1996; Drorit Saar & Barkai, 2003; L. T. Thompson, Moyer, & Disterhoft, 1996). In regard to the current study, this could indicate that the alterations in excitability we observed are transient priming mechanisms active during rule learning of the updated reward value and synaptic alterations consolidating the memory might be detectable later on.

In contrast to aforementioned studies of concurrent synaptic and intrinsic plasticity, excitability changes have also been shown to exist without synaptic activation (Labno, Warrier, Wang, & Zhang, 2014; Wu, Chan, Surmeier, & Disterhoft, 2008). Such alterations

in neuronal excitability independent of synaptic activity have been suggested to be long-lasting and might therefore play a different role than the priming role of aforementioned transient excitability changes (Cudmore, 2004; Egorov, Hamam, Fransén, Hasselmo, & Alonso, 2002). Further research is needed to elucidate the exact role and time scale of the devaluation-induced alterations in excitability we observed here. To examine how long-lasting changes in intrinsic excitability are, a technique incorporating a long-term marker for neuronal activity could be used, such as the *Fos-tTA* x *TRE-H2BGFP* mouse line (Kanda et al., 1998; Tayler et al., 2013). This approach allows persistent tagging of behaviourally-activated neurons for several weeks after activation. Additionally, the time window for labelling can be narrowly regulated by administration of doxycycline making it possible to correlate neuronal activation patterns and electrophysiological parameters with stages in behavioural tasks.

5.3 Devaluation induced alterations in neuronal excitability are likely to be non-homeostatic

Homeostatic neuronal plasticity is necessary to maintain neuronal responsivity to relevant stimuli, or Hebbian learning, in an ever changing environment (Miller, 1996; Gina G. Turrigiano & Nelson, 2004). Intrinsic and synaptic homeostasis are the two major forms of homeostatic plasticity, altering the input-output relationship or synaptic strengths of a neuron, respectively (G. Turrigiano, 2011). In NAc, DA depletion has been shown to induce a homeostatic increase in neuronal excitability counterbalancing a decreased synaptic excitatory input to maintain firing rates in a functional range (Azdad et al., 2009). Accordingly, a study by Ishikawa and colleagues demonstrated that MSNs in NAc shell in acute and organotypic slices exhibited a homeostatic increase in excitability in response to a decrease in excitatory synaptic function (M. Ishikawa et al., 2009). Hence, it might be possible that in the current study the devaluation-induced decreased excitability of NAc cue-activated

ensembles was of homeostatic nature. This appears unlikely however as we did not see an opposing increase in excitatory transmission or neuronal activation patterns and therefore we favour the explanation that the observed alterations in excitability were due to a decrease in reward value.

5.4 Methodological considerations

5.4.1 Fos-based labelling of cue-activated ensembles

The immediate early gene *Fos* has extensively been used to label recently activated neurons, alongside with *Arc* and *Zif268* (Cruz et al., 2015, 2013a; Guzowski, Setlow, Wagner, & McGaugh, 2001; Koya et al., 2009a; Lonergan, Gafford, Jarome, & Helmstetter, 2010). Whereas *Zif268* has been shown to have a high baseline expression (Knapska & Kaczmarek, 2004; Penke et al., 2014), potentially leading to an undesired ceiling effect, *Arc* expression has been shown to correlate with *Fos* expression and neuronal activity (Minatohara, Akiyoshi, & Okuno, 2016; Nakagami, Watakabe, & Yamamori, 2013; C. L. Thompson et al., 2010). Additionally, Fos has causally been linked to conditioned behaviours using food and drug rewards (Cruz et al., 2013a; Koya et al., 2009a; Warren et al., 2016). However, *Fos* expression requires sustained neuronal activity and calcium influx via NMDA receptors and therefore only labels a subset of strongly activated neurons (Cruz et al., 2013a). More specific, spike activity has been shown to not be sufficient to induce Fos expression, and as transcription appears to be needed for its expression, Fos may be better described as a marker of plasticity (Luckman, Dyball, & Leng, 1994). Additionally, the temporal resolution is low as *Fos* has an expression time of approximately 90 minutes (Cruz et al., 2015; Sheng & Greenberg, 1990; Xiu et al., 2014). Hence, in the current study it cannot be excluded that GFP+ cells were activated in response to other events within the same

time window besides the cue, such as the environment, handling, or stress. This could be controlled for to a certain extend by an unpaired control group, which we previously performed for Non-devalued mice, confirming that external non-cue factors alone do not drive the observed effects (Ziminski et al., 2017a). Additionally, the Fos-GFP fusion protein in the current study, similar to Fos itself, starts degrading after a few hours in the neurons, limiting recording time (besides cell death) and experimental designs (Xiu et al., 2014). It has to be noted that this time line allows for the possibility that we might have recorded from neurons activated shortly before the Pavlovian approach test when the animals were still in the home cage. These cells would be mostly visible early on during recording immediately after the approach test. This activation could be due to external factors or the acute *ad libitum* sucrose exposure in Devalued animals. This paradigm has been shown to not elicit other plasticity-related parameters in DA transmission or synaptic changes (see 5.4.2 for more detail), but to fully confirm that Fos-expression is not triggered by *ad libitum* sucrose further investigations are needed as this would represent a different population from cue-activated neurons and hence potentially impact our data interpretation (V. Bassareo et al., 2015; Valentina Bassareo & Di Chiara, 1997, 1999; Rada, Avena, Barson, Hoebel, & Leibowitz, 2012; Tukey et al., 2013). To circumvent these downfalls a new method has recently been developed, a light- and calcium dependent form of GFP that turns red when activated, termed CaMPARI2 (Moeyaert et al., 2018). This method would allow researchers to narrow the time window for tagging of neuronal ensembles activated exclusively during cue exposure and hence exclude confounding factors which could trigger *Fos*-expression in the current study.

As Fos expression requires calcium influx, it is conceivable that in a heterogenous cell population Fos is expressed more readily in a proportion of neurons with differing calcium buffer capabilities or other relevant biochemical or physiological differences (Trejo & Brown,

1991). MSNs and the very diverse interneuron populations in NAc differ in many physiological and morphological properties, some of which can alter calcium buffering (such as calbindin or parvalbumin as calcium binding proteins) (J.M. Tepper & Koós, 2016; James M. Tepper & Bolam, 2004). However, as in the current study the electrophysiological recordings were selectively performed on MSNs and the proportion of interneurons in NAc is less than 5% of the total neurons, they may be disregarded for the Fos quantification (Freund et al., 1984; Garas et al., 2018; Rymar, Sasseville, Luk, & Sadikot, 2004; J.M. Tepper & Koós, 2016; James M. Tepper & Bolam, 2004). Within the population of striatal MSNs a dichotomy in excitability and total dendritic area between D1 and D2 expressing neurons has been found, however if this is restricted to the dorsal striatum or can if it may be extended to the NAc is unclear (Gertler, Chan, & Surmeier, 2008; Planert, Berger, & Silberberg, 2013; Ziminski et al., 2017a). D1 and D2 expressing MSNs do however as mentioned previously differ in their expression of biochemical components as well as in their inputs (C R Gerfen et al., 1990; Charles R. Gerfen & Scott Young, 1988; Yasuo Kawaguchi et al., 1990; Kupchik et al., 2015). Additionally, there is evidence that Fos expression is not only mediated via slightly different signaling cascades in D1 vs. D2 MSNs, but also that the underlying calcium maybe be regulated differentially by dopamine (Cruz et al., 2015; Swapna, Bondy, & Morikawa, 2016). Even though we previously determined that the cue-activated ensemble consists of equal amounts of D1 and D2 expressing neurons, it is advisable for future studies to identify ensemble neurons according to their dopamine receptor expression as well after devaluation (Ziminski et al., 2017a).

5.4.2 Possible baseline effects of sucrose consumption itself

As discussed in chapter 4, chronic sucrose consumption is known to have a variety of effects including anhedonia, decreased motivation for sweet taste, a depressive-like phenotype, as well as cognitive deficits (Gueye et al., 2018; Reichelt, Killcross, Hambly, Morris, &

Westbrook, 2015) . Even though the literature shows that motivational deficits are most likely to occur with adolescent sucrose exposure of at least 16 days, as opposed to 4 days of sucrose exposure during adulthood in the current study, it is still possible that acute sucrose effects affect the current results.

Acute sucrose consumption in sucrose-trained rats has been shown to increase locomotion, therefore if sucrose consumption itself would have influenced our behavioural results in Devalued mice, we would expect an increased number of head entries, however we observed a decreased number of head entries compared to Non-devalued mice (Tukey et al., 2013). Additionally, it has been shown that only intermittent, repeated sucrose access increased DA signalling permanently, whereas *ad libitum* sucrose failed to do so (Rada et al., 2012). In line with this, DiChiara and colleagues reported one previous exposure to highly palatable food or sucrose to be sufficient to induce habituation to increased DA transmission in NAc shell, but this was not the case for daily sucrose self-administration (V. Bassareo et al., 2015; Valentina Bassareo & Di Chiara, 1997, 1999). Hence, it is unlikely that the decreased NAc activation after devaluation we observed in the current study is due to acute effects of sucrose consumption on DA modulation of NAc activity. Moreover, acute sucrose exposure in sucrose-trained rats has been reported to not be sufficient to elicit alterations in excitatory synaptic signalling in the NAc shell, which is in line with our results (Tukey et al., 2013). Finally, a study has found that acute consumption of a sweet solution is not sufficient to induce Fos and that Fos habituates rapidly (in our Devalued group) (Duncan, Knapp, & Breese, 1996; Struthers, DuPriest, & Runyan, 2005).Taken together, this indicates that the effects we observed in our Devalued group were due to a decreased hedonic value of sucrose rather than acute or chronic effects of 4 days of sucrose consumption. In order to experimentally control for the effects of acute sucrose consumption in the current experimental design it would be ineffective to introduce an unpaired devalued group, as the

unpaired and the Devalued condition both decrease the measured parameters, resulting in a floor effect making it impossible to distinguish the source or extent of the reduction. One measure we took in the current study to avoid the influence of very short-term acute sucrose consumption on experimental measures was to conduct the Pavlovian approach test under extinction conditions.

5.4.3 Specificity of behavioural measures

In the current study, we use cue-evoked sucrose seeking as a measure for motivation for a sucrose reward. As mentioned above, the experimental design requires us to use an extinction test to measure this, in an effort to avoid the effects of sucrose consumption immediately before sacrificing the animals to impact the Fos quantification and electrophysiological recordings. This implies that we here measured the motivation to obtain a reward, but not the palatability of the reward itself. This could be done by measuring orofacial expressions during sucrose consumption in Devalued and Non-devalued groups, as performed by Kerfoot and colleagues after reward devaluation (Erin C Kerfoot et al., 2007). Additionally, the amount of sucrose consumed in this test could provide information about perceived palatability but also in potential future inactivation studies help tear apart the role of NAc shell cue-activated ensembles in the updating of the reward value itself compared to the updating of the cue value to the new reward value (Johnson et al., 2009; Simmons & Neill, 2009; Wellman, 2005; Elizabeth A. West et al., 2012).

When faced with a Pavlovian conditioning paradigm in which a cue, the CS, is associated with a US, a rewarding outcome, animals tend to express one of two main anticipatory behavioural phenotypes: sign-tracking or goal-tracking (Shelly B. Flagel, Akil, & Robinson, 2009; Robinson & Flagel, 2009). Sign-trackers engage with the cue, which acquires incentive motivational properties itself, whereas goal-trackers engage with the reward delivery site and the cue only acquires reward predictive properties (Robinson & Flagel,

2009). It has been shown that these conditioned responses of sign-trackers, but not goal-trackers depend on mesolimbic DA signalling (Danna & Elmer, 2010; Shelly B. Flagel et al., 2011; Shelly B. Flagel, Watson, Robinson, & Akil, 2007; Saunders & Robinson, 2012). As for sign-trackers the cue itself becomes rewarding, in contrast to goal-trackers they have been demonstrated to be insensitive to reward devaluation and extinction, a disruption of the CS-US contingency (Ahrens, Singer, Fitzpatrick, Morrow, & Robinson, 2016; Beckmann & Chow, 2015; Morrison, Bamkole, & Nicola, 2015). As in any random sample of mice, in the current study we would expect a mixture of sign- and goal-trackers, but surprisingly in the current study all mice were sensitive to outcome devaluation. This suggests that our cohort of mice consisted exclusively of goal-trackers. This is evident by the decrease in cue-evoked sucrose seeking in all mice, following a homogenous distribution of responses, as opposed to a bimodal distribution that would be expected from a cohort with sign- and goal-trackers. One caveat here is that we only measured the number of head entries into the food magazine, whereas latency and cue-approach behaviour would provide more reliable information about the identity of goal- and sign-trackers. Importantly, the modality of the CS has been shown to greatly influence its ability to acquire incentive salience and hence to elicit sign-tracking behaviour in Pavlovian conditioning paradigms, as well as DA dependency of this behaviour (Cheng, De Bruin, & Feenstra, 2003; Meyer, Cogan, & Robinson, 2014; Wassum, Ostlund, Balleine, & Maidment, 2011). Hence, the reason why we exclusively observed goal-tracking behaviour in the current study might be due to the fact that we used an auditory cue, which is neither manipulatable or easily localiseable (Meyer et al., 2014).

5.5 Open questions and future outlook

5.5.1 Neuronal ensemble formation

In the current study we observed cue-activated ensembles in NAc shell to be more excitable than surrounding neurons after appetitive conditioning, and this difference was attenuated by reward devaluation. However, it is unclear if the associative learning induced the initial difference in excitability between the ensembles or if this difference is an inherent quality present before learning. A few studies have investigated this specific question with mixed results.

On the one hand, using fear conditioning and conditioned taste aversion it has been shown in the amygdala and insular cortex, respectively, that differences in CREB levels and excitability of neurons before learning determine the recruitment of ensembles that encode learned associations (Gouty-Colomer et al., 2015; Sano et al., 2014; Yiu et al., 2014; Y. Zhou et al., 2009). Similarly, Han and colleagues reported that in the lateral amygdala artificial upregulation of CREB in a subset of neurons increases the likelihood of these neurons to be included in the fear memory trace (J. H. Han et al., 2007). Conversely, when neurons with increased CREB expression were deleted or inactivated after fear conditioning, these animals were unable to express the conditioned fear response (J. H. Han et al., 2009; Rogerson et al., 2016). Similar results have been found using cue-cocaine associations in the lateral amygdala (Hsiang et al., 2014). It is important to note here that increased levels of CREB are known to increase excitability (Dong et al., 2006). These studies suggest that increased excitability is an inherent property of a subset of neurons which are therefore more likely to be recruited in memory traces.

On the other hand however, there is evidence for excitability increasing gradually during associative learning via repeated activation of neuronal ensembles. It has been shown previously that the same neuronal ensembles are activated during learning and memory recall in fear conditioning in the BLA, as well as in NAc during context-specific cocaine sensitization (Mattson et al., 2008; Reijmers, Perkins, Matsuo, & Mayford, 2007). However,

to date there are few studies examining the excitability of these repeatedly activated ensembles throughout learning. One of these studies reported that in the prelimbic cortex at day 1 compared to day 10 of an appetitive operant learning paradigm, intrinsic excitability was initially the same in all neurons and the ensembles modified their excitability only during training (Whitaker et al., 2017). This supports the idea of a form of learning where cells repeatedly firing together will adapt their excitability accordingly (Whitaker et al., 2017).

Taken together it appears that neuronal ensemble recruitment might be based on different mechanisms depending on brain area and behavioural paradigm. Additionally, our lab previously found increased *Fos* expression in paired mice after sucrose conditioning in the OFC and after cocaine conditioning in the NAc shell without ensemble specific increases in excitability, suggesting the existence of additional ensemble recruitment mechanisms not based on preconditioning excitability (Ziminski et al., 2017b, 2018). Further research is needed to tear apart the specific mechanisms in place for cue-evoked sucrose seeking and the effects of reward devaluation. To this end, a technique incorporating a long-term marker for neuronal activity could be used, such as the aforementioned *Fos-tTA* x *TRE-H2BGFP* mouse line (Kanda et al., 1998; Tayler et al., 2013).

This mouse line could also be used to explore another option, namely as mentioned in Chapter 3 that the physiological changes observed in the Devalued group were not present before the Pavlovian approach test but may have been induced by it. An increased prediction error in the Devalued group may play a role in this test-induced difference, as after four days of constant sucrose access they are exposed to a test under extinction conditions. Therefore an increased prediction error due to an unexpected outcome may have altered NAc physiology via altered DA input from the VTA (Keiflin, Pribut, Shah, & Janak, 2019). It has to be noted however that the sucrose access during devaluation is presented *ad libitum* in the home cage, so outside of the test context and in a different pattern. During acquisition,

in all groups the cue is paired with sucrose delivery in the conditioning context and similarly in all groups, the next time the animals encounter this cue and context is during the test, when (for the first time) they do not receive sucrose. Hence, as the sucrose exposure in the Devalued group happens outside of the test context, it is unlikely that the prediction error varies between the groups in the current study (Nakahara, Itoh, Kawagoe, Takikawa, & Hikosaka, 2004). Alternatively, the idea of test-induced differences may be based on the assumption that the test in the Devalued group, as it consists of the presentation of a cue which does not elicit approach behaviour, is to an extend different regarding the animals' cognitive processes compared to the test in the Non-devalued group (cue eliciting approach behaviour). However, the cue in the Devalued group is still associated with the sucrose reward, it is just the reward that has decreased in value, hence the animals are likely to undergo similar cognitive processes in both tests, making it unlikely that the test elicited the observed changes after Devaluation. Whitaker and colleagues investigated the effect of the test itself on excitability changes in PL after operant food self-administration and found the excitability in the non-activated (GFP-) neurons to be unaltered by the test (GFP+ neurons were not compared) (Whitaker et al., 2017). In order to confirm this for the experimental setup of the current study, the aforementioned *Fos-tTA* x *TRE-H2BGFP* mouse line will be of use (Kanda et al., 1998; Tayler et al., 2013).

5.5.2 Identity of neuronal ensembles

GABAergic MSNs, the principal NAc shell neurons comprise over 95% of the local neuronal population and can further be divided into classes according to their DA receptor expression and projection areas (C R Gerfen et al., 1990; Rymar et al., 2004). There are two major pathways, the direct pathway consisting of D1 expressing MSNs projecting to the output neurons in the ventral mesencephalon and the indirect pathway consisting of D2 expressing

MSNs projecting to the globus pallidus and ventral pallidum first (Burke et al., 2017). However, it is important to keep in mind that this division is not as clear cut as thought traditionally, with 14.6% of shell MSNs expressing both receptor types, a high degree of inhomogeneity of receptor distribution and according functional consequences (Gagnon et al., 2017; Gangarossa et al., 2013). In the current study we examined MSNs in NAc shell without directly testing the receptor expression of these neurons. However previous results from our lab indicate that after sucrose conditioning, cue-activated ensembles contain similar amounts of D1 and D2 expressing neurons with no difference between paired and unpaired groups, a negligible amount of neurons expressing both receptor types and no shift in this expression pattern after extinction learning (Ziminski et al., 2017b). In line with this, a recent study has shown that optogenetic inhibition of either MSN subpopulation in the ventrolateral striatum lead to disruption of an appetitive goal-directed task (Natsubori et al., 2017; but also see Yawata, Yamaguchi, Danjo, Hikida, & Nakanishi, 2012). Hence, even though the current study was conducted in a neighbouring striatal area, it is likely that the proportion of cue-activated D1 and D2 expressing MSNs remained unchanged after reward devaluation.

Finally, in addition to the different projection pathways of these MSNs future studies need to identify the detailed molecular composition and transcriptional response of these activated ensembles to gain further insight into the mechanisms underlying the formation of neuronal ensembles as functional engrams and their plasticity (Jaeger et al., 2018; Lacar et al., 2016).

5.6 Conclusion

In this study we have revealed decreased intrinsic excitability and ensemble size to be mechanisms taking place in sucrose cue-activated neuronal ensembles in NAc shell during reward devaluation-induced reductions of cue-evoked sucrose seeking. Further questions, such as the longevity of these changes, specific characterization of ensembles,

as well as the exact mechanisms underlying the observed changes as well as the potential existence of synaptic alterations not investigated in the current study may be addressed in future studies taking advantage of new technological advances. The current and future studies may contribute to a better understanding of how cues such as TV advertisements or logos associated with highly palatable and highly caloric unhealthy snacks promote food seeking independently of caloric need leading to adverse health outcomes such as obesity.

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Appendix 1 Non-parametric tests, Grubb's outliers, log transformation and reanalysis

The data presented in the figures is analysed using parametric tests to correspond to submitted versions of this work and also as in small sample sizes normality tests are unreliable and non-parametric tests have low power (Ghasemi & Zahediasl, 2012; Potvin & Roff, 1993).

Chapter 2

Outliers:

In chapter 2 Grubb's test did not detect any outliers, which corresponds to the number of outliers found by our original criterion for the data in Figure 1. For the Fos data, this differs from the number of outliers detected by the previous criterion. Therefore, the t-tests with corrections for multiple testing were repeated and the

previously significant differences between Devalued and Non-devalued groups in rostral NAc core (adjusted p = 0.1) and shell (adjusted p = 0.52) are now reduced to non-significant trends. Importantly, a targeted comparison of the rostral NAc shell Fos expression between Devalued and Non-devalued groups using a one-tailed t-test is significant $t_{28} = 2.037$, p = 0.086.The rationale for this is provided by a study revealing a significant reduction in the number of phasically firing NAc shell neurons after reward devaluation, hence in the current study a similar reduction in Fos expression is to be expected in the NAc shell (E. A. West & Carelli, 2016).

Normal distribution and non-parametric tests:

Figure 1 A, C, E:

All data is normally distributed.

Figure 1B:

The group paired control is not normally distributed, therefore a non-parametric two-tailed Mann-Whitney U (MWU) test was performed to compare the Control group data (unpaired and paired pooled) with the *ad libitum* chow data (paired and unpaired pooled) in order to confirm the main effect of caloric satiation shown by the parametric tests. The MWU test indicated that the body weight was greater for mice consuming *ad libitum* chow (median = 1.040) than for animals in the Control group (median = 0.91), U = 8, p < 0.001.

Figure 1F:

The group Unpaired *ad libitum* chow ITI deviates from a Gaussian normal distribution, therefore two non-parametric tests were performed to confirm the negative findings from the parametric tests. A two-tailed MWU test indicated that head entries made during ITI did not differ between Control (median = 5) and *ad libitum* fed animals (median = 5), U = 103, p = 0.717. Furthermore, the number of head entries in *ad libitum* fed animals during ITI compared to CS did not differ, p = 0.845, as indicated by a two-tailed Wilcoxon signed rank test.

Figure 1D:

The group Paired Non-devalued ITI deviates from a Gaussian normal distribution, therefore two non-parametric tests were performed to confirm the negative findings from the parametric tests. A two-tailed Wilcoxon signed rank test indicated that head entries made by Non-devalued animals during ITI and CS did differ significantly, p < 0.001. Furthermore, the number of head entries during ITI did not differ between Non-devalued (median = 7) and Devalued mice (median = 7), U = 106, p = 0.813, as indicated by a two-tailed MWU test.

Figure 2:

Al the data from the Fos quantification is normally distributed.

Chapter 3

Outliers:

Using Grubb's test we detected an outlier for AP amplitude in the GFP- Non-devalued group, which corresponds to the outlier we found with our previous criterion. For RMP and AHP, no outliers were detected, which is in line with our previous outlier analysis. For Input resistance, no outlier was found, whereas the previous criterion excluded two values. A rerun of the ANOVA including this data point reveals a significant effect of ensemble $F_{1,38} = 13.47$, $p = 0.0007$, no interaction $F_{1,38} = 2.203$, $p = 0.146$, and no effect of devaluation $F_{1,38} = 0.9941$, $p = 0.3251$. No cells were identified as outliers using Grubb's test for the spike counts and I7V curves, which corresponds to our previous exclusion criterion.

Normal distribution and non-parametric tests:

Figure 1:

The majority of the data is normally distributed.

Figure 2 A, C, E:

All data is normally distributed.

Figure 2B:

The group GFP+ Non-devalued deviates from the Gaussian normal distribution, therefore two-tailed MWU tests were performed to confirm the significant difference in Rheobase between GFP+ (median = 60) and GFP- (median = 108) cells in the Non-devalued group $U = 11$, $p = 0.0009$ as well as the absence of a difference between GFP+ Non-devalued (median = 60) and Devalued (median = 86) groups $U = 38$, $p = 0.249$ previously found using a parametric test.

Figure 2D:

The groups GFP- Devalued (median = 1.282) and Non-devalued (median = 1.282) showed no difference in AP half-width using parametric tests, and this was confirmed using a two-tailed MWU test U = 48, p = 0.626. The significant difference between Devalued GFP+ (median = 1.465) and GFP- (median = 1.282) groups in AP half-width revealed using a parametric test was confirmed by a two-tailed MWU test U = 43, p = 0.4377. However, the difference between GFP+ Devalued (median = 1.465) and Non-devalued (median = 1.648) groups, which was significant using a parametric test is only a trend when using a two-tailed MWU test U = 29.5, p = 0.0599.

Figure 2F:

The GFP- Devalued group deviated for AP amplitude from a Gaussian normal distribution, hence we performed two-tailed MWU tests to confirm the lack of differences between GFP- Devalued (median = 67.32) and Non-devalued (median = 68.12) U = 46, p = 0.832, as well as GFP+ Devalued (median = 64.88) U = 49, p = 0.705 groups.

Chapter 4

In addition to the raw data used in Chapter 4, here the data was log transformed before any outlier removal and analysis in order to ensure that outlier removal does not enforce a normal distribution. Therefore here we present the numbers of outliers, distribution and analysis results after log transformation.

Outliers:

In the PPR data set no cells were excluded as outliers after log transformation following Grubbs's test, which is in line with our original exclusion criterion. In the sEPSC frequency and amplitude data according to Grubbs' test no outliers were excluded after log transformation.

Normal distribution and non-parametric tests:

Figure 1

The majority of residuals of log transformed PPR values in most groups followed a Gaussian normal distribution, however in the Non-devalued GFP- group the *n* is too small for normality testing. For an analysis as complex as a mixed three-way ANOVA there is no appropriate non-parametric equivalent. But as non-parametric tests generally have less power than parametric tests and the original ANOVA we performed on the log transformed data did not reveal any meaningful significant differences, any non-parametric alternative will likely have the same outcome. As an alternative way of accounting for a non-normal distribution, we log transformed the PPRs and compared them using a three-way mixed ANOVA. This revealed a significant three-way interaction of interval x GFP x devaluation: $F_{6,126} = 2.493$, $p = 0.026$, but no other interactions (interval x GFP: $F_{6, 126} = 0.444$, $p = 0.848$; interval x devaluation: $F_{6, 126} = 0.790$, $p = 0.58$; GFP x devaluation: $F_{1,21} = 0.039$, $p = 0.845$). There was no effect of devaluation ($F_{1,21} = 0.075$, $p = 0.787$) or GFP ($F_{1,21} = 0.07$, $p = 794$) but an effect of within-subjects factor interstimulus interval ($F_{6,126} = 9.624$, $p < 0.001$). To follow up on the three-way interaction, we next performed post-hoc two-way mixed ANOVAs yielding no significant interactions or main effects of GFP or devaluation (GFP+ only: interstimulus interval x devaluation $F_{6,36} = 1.097$, $p = 0.383$, interstimulus interval $F_{6, 36} = 2.872$, $p = 0.022$, devaluation $F_{1,6} = 0.049$, $p = 0.831$; GFP+ only: interstimulus interval x devaluation $F_{6, 90} = 1.432$, $p = 0.211$, interstimulus interval

$F_{6, 90} = 7.193$, $p < 0.001$, devaluation $F_{1, 15} = 0.006$, $p = 0.937$; Non-devalued only: interstimulus interval x GFP $F_{6, 60} = 0.814$, $p = 0.563$, interstimulus interval $F_{6, 60} = 3{,}587$, $p = 0.004$, GFP $F_{1, 10} = 0.004$, $p = 0.952$; Devalued only: interstimulus interval x GFP $F_{6, 66} = 2.111$, $p = 0.064$, interstimulus interval $F_{6, 66} = 6.973$, $p < 0.001$, GFP $F_{1, 11} = 0.088$, $p = 0.772$). Due to the lack of post-hoc differences, this indicates that reward devaluation or ensemble identity does not modulate presynaptic vesicle release probability and hence confirms our initial results.

Figure 2 A:

After log transformation, in the sEPSC frequency data only the GFP+ Non-devalued group deviated from a Gaussian normal distribution. Next we used a parametric two-way ANOVA on the log transformed data and we found no significant interaction of GFP x devaluation $F_{1, 67} = 0.3183$, $p = 0.575$, main effect of GFP $F_{1, 67} = 0.1318$, $p = 0.7177$ or devaluation $F_{1, 67} = 0.9848$, $p = 0.3246$. An alternative analysis using a non-parametric MWU test between the pooled Devalued (median = 1.9) vs. Non-devalued (median = 2.9) groups without log transformation was non-significant as well $U = 467$. $P = 0.1257$. This deviates from our original analysis as these test have less power. This requires more investigation, however as in this study we investigated devaluation-induced effects on ensembles it does not change our main findings.

Figure 2B:

The sEPSC amplitude data was normally distributed after log transformation. We used a parametric two-way ANOVA on the log transformed data and revealed no

significant interaction of GFP x devaluation $F_{1, 66} = 1.223$, $p = 0.273$, main effect of GFP $F_{1, 66} < 0.001$, $p = 0.9817$, or devaluation $F_{1, 66} = 1.184$, $p = 0.281$. This is in line with our previous negative findings.

Appendix 2 Reanalysis of data using GFP as within-subjects factor

The data from chapter 3 were reanalysed using GFP as within-subjects factor as sometimes GFP+ and GFP- cells were from the same animal.

For the I/V curve data shown in Chapter 3, Figure 1 inlay this three way mixed ANOVA with current step and GFP as within-subjects factors and Devaluation as between-subject factor revealed a three way interaction of current step x GFP x Devaluation ($F_{20, 360} = 1.679$, $p = 0.035$),as well as a two way interaction of GFP x current step ($F_{20, 360} = 11.187$, $p < 0.001$). There was also a significant main effect of GFP ($F_{1, 18} = 22.893$ $p < 0.001$) and current step ($F_{20, 360} = 337.156$, $p < 0.001$). All other main effects and interactions were non significant: current step x Devaluation $F_{20, 360} = 0.240$, $p = 1$, GFP x Devaluation $F_{1, 18} = 3.605$, $p = 0.074$; Devaluation $F_{1, 18} = 0.399$, $p = 0.536$. These results confirm some of the effects and interactions from the ANOVA performed in Chapter3 but importantly add a three way interaction, interaction and a main effect of GFP, now allowing for post-hoc comparisons between Devalued and Non-devalued groups. To this end, we used additional two-way ANOVAs to separately compare the impact of devaluation on GFP+ and GFP– cells. For

both, GFP+ and GFP– cells, no interaction or effect of devaluation, but an effect of current step (GFP+: $F_{20,400} = 208$, $p < 0.001$, GFP–: $F_{20,380} = 267.7$, $p < 0.001$) were revealed.

For the excitability data shown in Chapter 3, Figure 1 a three-way mixed ANOVA with current step and GFP as within-subjects factors and Devaluation as between-subject factor reproduces the results found using the original ANOVA. A three way interaction $F_{8, 144} = 4.913$, $p < 0.001$, the GFP x Devaluation interaction $F_{1, 18} = 5.774$, $p = 0.027$ and the current step x GFP interaction $F_{8, 144} = 7.726$, $p < 0.001$, were found to be significant, as well as the main effects of GFP ($F_{1, 18} = 9.535$, $p = 0.006$) and Devaluation ($F_{1, 18} = 63.214$, $p < 0.001$), and current step ($F_{8, 144} = 62.229$, $p < 0.001$). The interaction of current step x Devaluation was not significant ($F_{8, 144} = 0.113$, $p = 0.999$). This confirms the previous results and extends them by adding a significant GFP x devaluation interaction and the Devaluation main effect..

This altered analysis is strengthening the results of the current study, however in the majority of cases GFP+ and GFP- cells were not recorded from the same animal and therefore GFP may not be used as within-subjects factor (which is in line with our previous analyses Ziminski et al., 2017b, 2018). To strengthen statistical results in future studies these recordings could be done in pairs and then GFP can be used as a within-subjects factor in all analyses.