A thesis submitted for the degree of

Doctor of Philosophy

Arc and *homer 1a* expression following intravenous administration of heroin and cocaine: a novel application of the catFISH technique

Philip Vassilev

School of Psychology

University of Sussex

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Declaration

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.

Signature: 

Name: Philip Vassilev

Summary

This thesis applies catFISH, a variant of the standard fluorescence *in situ* hybridisation technique, to study the neuronal ensembles activated by heroin and cocaine across brain structures involved in motivated behaviour, in the Sprague-Dawley rat.

The first chapter reviews the pharmacology of heroin and cocaine, rodent models of drug-related behaviours, and heroin and cocaine's ability to trigger immediate-early gene expression when administered acutely or chronically. It is suggested that the empirical evidence points towards a significant separation between the neuronal systems engaged by the two drugs. The main goal of this thesis was to test whether this separation can be seen within brain areas playing a key role in motivation and reward (e.g. the nucleus accumbens). Since immediate-early genes serve as markers of neuronal activity, and catFISH is a technique which can detect the expression of such genes in response to two separate stimuli, the technique was chosen as the best method to test if heroin and cocaine activate the same neuronal ensembles when administered acutely.

The second chapter summarises the methods used across experiments described in following chapters.

The third chapter presents an experiment addressing the methodological issues associated with using catFISH to study pharmacological stimuli. The technique was originally used to study the hippocampus and brain activity triggered by stimuli with well-controlled on- and offset (e.g. exposure to a novel environment or discrete cues). Arguably, acute drug injections comprise a stimulus with an on- and offset which can only be controlled indirectly – they depend on the drug dose and route of administration, among other factors. It was shown that acute intravenous injections of heroin and cocaine at doses usually self-administered by animals are suitable stimuli to use with catFISH.

Chapter four describes an experiment showing that, across the striatum, the neuronal ensembles activated by an injection of cocaine followed by an injection of heroin overlap significantly less than the neuronal ensembles activated by two consecutive injections of cocaine. This is interpreted as direct evidence for a significant separation between

the neuronal pathways activated by heroin vs. cocaine, even in brain areas often considered a common neurobiological substrate for the effects of the two drugs. It must be noted that the magnitude and the nature of this separation depends on the particular part of the striatum and the order in which drugs are administered.

Chapter five describes a pilot experiment attempting to incorporate the logic of the catFISH technique into a rodent drug self-administration paradigm. It was found that the rats preferred self-administering heroin over cocaine, and that there was no detectable expression of the *homer 1a* gene across the striatum in response to acute heroin and cocaine after extended experience with the two drugs. There was also no change from baseline expression of the *homer 1a* and *arc* genes in areas of the prefrontal cortex.

Finally, chapter six summarises the main findings and the key methodological considerations from all three experiments. As a whole, it is suggested that the experiments in this thesis provide a proof of concept that heroin and cocaine are processed differently by the brain, even within brain areas considered to be common substrates for the reinforcing and addictive properties of the two drugs. Future studies should take this separation into account, as it may have important implications for understanding drug addiction as a whole.

The appendices contain representative fluorescence microscopy images of brain tissue analysed for catFISH.

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List of abbreviations

3’ UTR	3 prime untranslated region of a mRNA molecule
5-HT	5-hydroxytryptamine (serotonin)
6-MAM	6-monoacetylmorphine
A(SP)A	Animal (scientific procedures) act
ACd	Anterior cingulate cortex, dorsal part
ADHD	Attention deficit hyperactivity disorder
ALDH	Aldehyde dehydrogenase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
arc	Activity-regulated cytoskeleton-associated gene
BBB	Blood-brain barrier
BLA	Basolateral amygdala
c	Cocaine
CaMK	Ca2+/calmodulin-dependent protein kinase
cAMP	Cyclic adenosine monophosphate
catFISH	Cellular compartment analysis of temporal activity by FISH
coc	Cocaine
CPP	Conditioned place preference
CREB	cAMP response element binding protein
D1	Dopamine D1 receptor (family)
D2	Dopamine D2 receptor (family)
DA	Dopamine
DAPI	4',6-diamidino-2-phenylindole
DLS	Dorsolateral striatum
DMS	Dorsomedial striatum
DNA	Deoxyribonucleic acid
DSM-V	Diagnostic and statistical manual 5th edition
egr1	Early growth response protein 1 (another name for *zif268*)
FACS	Fluorescence-activated cell sorting
FISH	Fluorescence *in situ* hybridisation
fmi	Anteriror forceps of the corpus callosum
FR1	Fixed ration 1 schedule of reinforcement
GFP	Green fluorescent protein
h	Heroin
h1a	Homer 1a
HCl	Hydrochloride
her	Heroin
i.p.	Intraperitoneal
i.v.	Intravenous
ICSS	Intracraneal self-stimulation
IEG	Immediate-early gene
IL	Infralimbic (cortex)
LC	Locus coeruleus
LED	Light-emitting diode
LTD	Long-term depression

LTP	Long-term potentiation
MAPK/ERK	Mitogen-activated protein kinase/extracellular signal–regulated kinase
MFV	Microscope field of view
mGluR5	Metabotropic glutamate receptor 5
Mkp-1	Mitogen-activated protein kinase 1
mPFC	Medial PFC
mRNA	Messenger ribonucleic acid
NA	Noradrenaline
Nacc	Nucleus accumbens
NMDA	N-methyl-D-aspartate recpetor
NOP-R	Nociceptin receptor
PAG	Periaqueductal grey
PCR	Polymerase chain reaction
PFC	Prefrontal cortex
PKA	Protein kinase A
PL	Prelimbic (cortex)
ROI	Region of interest
s	saline
SNc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
SRF	Serum response factor
TAI-FISH	Tyramide-amplified immunohistochemistry FISH
TO	Time-out (between self-administration trials)
UV	Ultraviolet
vmPFC	Ventromedial PFC
VP	Ventral pallidum
VTA	Ventral tegmental area
zif268	Zinc finger protein 225

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Chapter 1 – Introduction

1. Why study heroin and cocaine?

Recreational drug use and addiction

For the year 2015, estimates of drug use and abuse across the world are proportionally low, but numerically high – an estimated 5% of people have used illicit drugs at least once in that year, and 29.5 million of those people have a diagnosed drug use disorder. The highest level of harm in health terms is caused by opioids, including heroin, and psychostimulants (methamphetamine and cocaine) follow after. Overall, 70% of total drug-induced health problems is attributable to opioids. This is in great part the result of rising misuse of pharmaceutical opioids and the risks associated with injectable drugs (United Nations Office on Drugs and Crime, 2017). Overall, heroin and cocaine still deserve their place at the top of the harm scale (D. Nutt, King, Saulsbury, & Blakemore, 2007).

Heroin and cocaine produce feelings of intense pleasure as well as other desirable effects (e.g. overcoming tiredness or alleviation of negative emotions) making the two drugs very attractive for recreational users. From a certain perspective, heroin, cocaine and other psychoactive substances can be used for self-medication: many recurrent drug users are struggling with some kind of a psychological issue such as aggression, depression or social anxiety, and drugs of abuse can ameliorate such problems (Khantzian, 1985). Alternatively, recreational drug use can be understood in terms of "drug instrumentalisation" where psychoactive substances are used as a means to control mental states at the will of the user. In the modern-day social environment humans often have to quickly switch between micro-environments demanding very different mental states (e.g. work vs. leisure). Drugs can provide such rapid changes in mental states in different ways such as enhancing social interaction, combating fatigue or enhancing cognitive performance (Muller & Schumann, 2011).

However, prolonged use of some drugs under certain circumstances and by certain individuals may transition to pathological use; i.e. addiction. Addiction is characterised, above all, by loss of control over drug intake, drug-taking despite negative consequences and at the expense of other pleasurable activities. Drug addiction can be very costly to both individuals and society.

Prevention and treatment of addiction could benefit from the study of the neural circuitry engaged by both acute and chronic administration of drugs, as well as the study of the changes in this circuitry which are believed to mark the transition from recreational use to pathological use. Such has been the aim of both psychiatric and psychological research over the past few decades, but only recently scientists and medical practitioners have begun to acknowledge the complexity and diversity of drug abuse disorders.

Diagnosis and theory of substance use disorders (drug addictions)

As discussed above, addiction can be broadly defined as compulsive drug-seeking and -taking despite negative consequences, and at the expense of other recreational activities. Within the mental health practice, this phenomenon has been termed substance use disorder, and the problematic use of different classes of drugs has been classified into a number of substance use disorders. The latest edition of the DSM-V classifies substance use disorders into 10 separate categories encompassing alcohol, cannabis, some hallucinogens, inhalants, opioids, sedatives, stimulants, tobacco and other/unknown (APA, 2013). There are 11 diagnostic criteria to describe these substance use disorders, which generally cover three aspects of problematic behaviour surrounding the use of the substance in question: *loss of control, social impairment,* and *problematic use*, plus two *pharmacological criteria*. The first grouping of criteria, *loss of control*, refers to the inability to stop or reduce use of the substance despite wanting to do so, using in higher quantities than intended, and craving – the intense desire to take the drug. *Social impairment* criteria cover aspects of substance use which interfere with the ability of the user to fulfil social roles or invest time in activities other than drug taking. *Problematic use* includes criteria describing the use of the substance in hazardous situations (e.g. while driving), or despite known physiological or psychological

problems caused by the substance. Finally, *pharmacological criteria* include tolerance and withdrawal.

Although most diagnostic criteria can apply to all problematic use of substances, the separation of substance use disorders in the latest edition of the DSM-V comes from the recognition of important differences between the brain mechanisms underlying the abuse potential of different substances (Badiani, Belin, Epstein, Calu, & Shaham, 2011). This separation is also a step away from the commonly used term "addiction" which can refer to behavioural addictions such as pathological gambling. Regardless of the separation of drug abuse diagnoses, currently prominent psychobiological theories explaining the mechanisms behind substance abuse still revolve around the idea of a common mechanism for substance abuse (Piazza & Deroche-Gamonet, 2013).

For example, Homeostatic Dysregulation Theory (Koob & Le Moal, 1997) suggests that pathological substance use arises from a persistent change in the set point of the reward circuitry of the brain – the dopamine system. That is, with prolonged use of substances that directly activate the brain's reward system, the system becomes less sensitive (manifesting as tolerance to the acute effects of the drug and reduced sensitivity to other rewards). That, in turn, leads to a negative affective state in the absence of the drug (i.e. withdrawal) rendering drug-taking necessary to maintain a functional level of motivation. Central notions for this theory are the withdrawal syndrome (both physiological and psychological) and reduction in the sensitivity of the reward system.

Another theoretical framework is set out by Incentive-Sensitization Theory (Berridge & Robinson, 2016; Robinson & Berridge, 1993) which emphasizes the difference between liking and "wanting" of abused substances, and sensitization of the mechanisms underlying the latter (the dopamine system). "Wanting" within this framework refers to the motivational drive elicited by substance-associated stimuli through classical conditioning, while liking refers to the hedonic responses elicited by the consumption of the substance (the "high"). According to the theory, prolonged drug use and individual genetic predisposition renders the dopaminergic system hyper-reactive to drug-associated stimuli in such a way that exposure to these stimuli precipitates further drug use at the expense of other activities, and promotes relapse following abstinence.

Finally, substance abuse has also been viewed from the perspective of compromised executive control (Jentsch & Taylor, 1999) and aberrant learning (Everitt, Dickinson, & Robbins, 2001). Theory focusing on executive dysfunction emphasises the role of the prefrontal cortex (PFC) in the inhibition of impulsive responses, and the role of the amygdala in the influence of conditioned stimuli on behaviour. For example, impaired function of the PFC humans leads to impairments in tasks that tap into decision making and inhibitory control (Goldstein & Volkow, 2011). On the other hand, the amygdala is responsible for the suppression of behaviour by fear-conditioned stimuli and invigoration of behaviour in the presence of reward-associated stimuli (Everitt et al., 1999). Thus, from this point of view, addiction arises in part because the individual is highly responsive to drug-associated cues because of amygdala hyperfunction, and less able to withhold engaging with these stimuli due to PFC hypofunction. On the other hand, aberrant learning theory emphasises the difference between outcome-sensitive behaviour (i.e. goal-directed behaviour) and stimulus-response (i.e. habitual behaviour), which are mediated by separate brain mechanisms involving the ventral vs. dorsal striatum dopamine, respectively (Everitt & Robbins, 2005). The mechanism underlying substance abuse proposed by such theories is that adaptive learning mechanisms are altered in substance abusers so that behaviour becomes less dependent on its outcome and more dependent on impulses and conditioned responses.

There is no definite consensus yet as to which of the hitherto mentioned theories focuses on the key mechanisms by which substance abuse disorders are developed and maintained. However, aberrant learning theory has recently been challenged by one study clearly showing that addictive behaviour can arise even when drug-seeking is not habitual, and when it is controlled by dopamine in the ventral striatum (Singer, Fadanelli, Kawa, & Robinson, 2018). Nevertheless, it is safe to say that there are separate brain circuits engaged and altered by different classes of drugs. More importantly, in some cases, even when circuits overlap between drugs, drug effects go in opposite directions. This is particularly true for opiates and psychostimulants, and therefore – heroin and cocaine. If any psychobiological theory was to explain drug abuse, it would have to account for the mechanisms involved in each of the stages of development of the disorder. First, the acute rewarding effects of the drug, which drive initial recreational

use. Second, the physiological and psychological changes which accompany the transition to compulsive (pathological use), taking into account that such changes occur only in some individuals. Third, the mechanisms underlying the propensity to relapse after long periods of abstinence. Understanding these mechanisms requires manipulation of brain function which is currently possible only in the field of animal research. Rodent models have been especially useful in that endeavour, and have served as the basis for all currently available psychobiological theories of drug addiction. In many cases, however, such theories have overlooked some important differences between the mechanisms of heroin and cocaine which can have implications for the prevention and treatment of substance abuse disorders.

Modelling drug reward and relapse into drug-seeking

Before reviewing any animal models of drug reward and other aspects of substance abuse, it must be noted that the concept of reward in psychology is multifaceted. First, rewards serve as reinforcers. Second, rewards can include a hedonic/pleasure aspect; i.e. they produce positive affect when obtained. The definition of rewards as reinforcers is grounded in classical and operant conditioning: it refers to stimuli that elicit approach and consummatory behaviours, or to outcomes of actions that increase the probability of those actions occurring again. Reward, therefore, can take the form of "natural" reinforcers such as food and sex, or can be more abstract – as an extreme example, recently, it was shown that social interaction and aggression can be reinforcing in the mouse (Golden et al., 2017). Rewarding effects can also be implicit or explicit, in the sense that learning, positive affect and motivation can occur outside of conscious awareness. A good example of such a case is self-administration of morphine by humans at doses which do not differ from placebo in their subjective effects (Lamb et al., 1991). Thus, reward has motivational, cognitive and hedonic components, which are separable not only conceptually, but also in terms of the neurobiological mechanisms underlying them (Berridge & Robinson, 2003).

There are numerous animal models capturing different aspects of drug reward, and some are specifically designed to measure these aspects separately. However, by and large, animal models of drug reward are variations of the operant self-administration and conditioned place preference (CPP) procedures.

The self-administration model is based on operant conditioning where an animal (usually a rat) is given the opportunity to interact with a manipulandum in order to receive an intravenous infusion of a drug. This is achieved through the implantation of an intrajugular catheter connected to an in infusion pump that is activated by pressing a lever or by a nose-poke. In this case drugs such as heroin and cocaine act as positive reinforcers, because the frequency of lever-presses or nose-pokes increases as the animal learns the contingency between the action and the outcome (Gardner, 2000). Often there is a control lever or a control hole present to show that the action is specifically oriented to the drug-paired manipulandum and is not just the result of general activity of the animal.

In the CPP procedure, a compartment within a chamber is paired with the administration of a drug by the experimenter, while a different compartment is paired with the administration of a vehicle (e.g. saline). In this case, following repeated parings, the animal develops a preference for the drug-paired compartment when given the option to choose in drug-free conditions. The logic behind this paradigm is that if a drug such as cocaine or heroin induces a pleasant experience (i.e. acts as an unconditioned reward/reinforcer), this pleasantness will be associated with the environment through classical conditioning. Then, the environment will act as a conditioned stimulus which elicits approach (Tzschentke, 1998). Indeed, it is well-established that pairing of both heroin and cocaine effects with an environment make it preferable over a vehicle-paired environment (Brenhouse & Andersen, 2008; Schlussman et al., 2008).

The self-administration paradigm has the advantage of modelling self-initiated drug-taking, which arguably makes it a more ecologically valid model. However, the CPP procedure is also relevant since it suggests that even experimenter-administered drugs can have rewarding properties.

The associative processes seen in CPP are also relevant in the sense that they relate to a key aspect of the relapse mechanism. Not only the environment, but also discrete cues can be associated with drug administration so that they themselves acquire rewarding properties. For example, laboratory animals that have learned to associate a light cue with the delivery of a drug infusion will later lever press for the presentation of the light (Di Ciano & Everitt, 2004a). This conditioned reinforcement is at the basis of modelling

drug seeking in general and plays a key role in models of relapse. In such models, rats learn to lever press for a drug after which the behaviour is extinguished through presentation of the lever, but no contingent drug delivery. With time, the animal decreases responding on the lever in the absence of the reinforcer. However, if a light is presented contingently on pressing of the lever during training, when the drug was available, and then this light was presented contingently again in a test session (in the absence of the drug), animals would reinstate lever pressing for the presentation of the light, even though the drug is not present (Grimm, Shaham, & Hope, 2002). Thus, presentation of a drug-associated stimulus can provoke a behaviour oriented towards drug-taking through a motivational process engaged thanks to classical conditioning. In addition, lever pressing can be reinstated through exposure to stress or administration of a small dose of the drug (de Wit & Stewart, 1981; Shaham & Stewart, 1995). The logic behind this model is that a similar process occurs in human substance abusers whereby drug-associated stimuli such as drug-taking paraphernalia, or seeing other people take the drug, can promote craving – a desire to take the drug (Epstein, Preston, Stewart, & Shaham, 2006).

Similarly, CPP can be used to model reactivation of the memory of rewarding drug effects. In this paradigm, CPP is first established and then extinguished by pairing the drug-context with the delivery of a vehicle or simply exposing the animal to the drug-paired context repeatedly without the drug. Then, once preference for the drug-paired environment has disappeared, this preference can be reinstated by exposing the animal to a small dose of the drug (Mueller, Perdikaris, & Stewart, 2002; Mueller & Stewart, 2000).

To summarise, both the rewarding properties of drugs and the propensity to relapse can be understood at least in part in terms of classical and operant conditioning. Therefore, any understanding of the mechanisms underlying drug reward driving recreational use, transition to pathological use, and relapse would require an understanding of the neurochemistry and neuroanatomy of brain circuits involved in these forms of learning.

Neuroanatomical substrates of reward and motivation

Previous sections introduced major current theories of drug abuse and mentioned some key brain areas and neurotransmitter systems involved in the development of the disorder. This section will describe the connectivity and neurochemistry of brain areas mentioned throughout this thesis as targets of heroin and cocaine action in the brain, and as areas of interest in experimental chapters. These brain areas include the striatum and several of its input structures (PFC, amygdala, hippocampus, thalamus, ventral tegmental area (VTA) and substantia nigra), as well as some output structures.

The striatum receives glutamatergic inputs from the thalamus, basolateral amygdala, hippocampus and prefrontal/sensorimotor cortices (Voorn, Vanderschuren, Groenewegen, Robbins, & Pennartz, 2004). These inputs can be divided in a dorsolateral to ventromedial direction, such that the ventromedial striatum receives input predominantly from limbic structures, while the dorsolateral striatum receives input mostly from the thalamus and sensorimotor cortices (fig. 1.1, p.10). Note, however, that only cortical inputs to the ventral striatum (Nacc) distinguish between core and shell – basolateral amygdalar and thalamic inputs have a complex organisation and project to both core and shell (Wright, Beijer, & Groenewegen, 1996). More specifically, the Nacc shell receives input from the infralimbic cortex, ventral portions of prelimbic area, and ventral agranular insular cortex (lateral shell). The Nacc core receives some input from the ventral prelimbic area as well, but is mostly targeted by the dorsal PL, and (the lateral core) from dorsal agranular insula (Groenewegen, Wright, Beijer, & Voorn, 1999). The dorsomedial striatum receives input from the dorsal prelimbic and ventral orbital cortices, while the dorsolateral striatum receives input mostly from the anterior cingulate dorsal cortex and sensorimotor cortices (Berendse, Galis-de Graaf, & Groenewegen, 1992; Voorn et al., 2004). The striatum also receives dense dopaminergic innervation from the VTA and substantia nigra pars compacta (SNc). The former targets primarily the ventral striatum, while the latter provides dopaminergic input mostly to the dorsal striatum. However, there are some sparse reciprocal projections between SNc and core (Beckstead, Domesick, & Nauta, 1979). Finally, there are some GABAergic inputs to the ventral striatum, for example from the ventral pallidum (VP) forming

reciprocal connections with the Nacc. The ventromedial VP projects to the shell, while the dorsolateral VP – to the Nacc core (Brog, Salyapongse, Deutch, & Zahm, 1993).

The main targets of striatal efferent projections are other regions of the basal ganglia: ventral pallidum, globus pallidus, substantia nigra pars compacta/reticulata, and the VTA. As already mentioned, the Nacc core projects to the dorsolateral VP, and the shell – to the ventromedial VP (Zahm & Heimer, 1990). From the VP, projections continue to the thalamus, either directly (for the circuit passing through the Nacc shell) or via the substantia nigra pars reticulata, SNr (for the circuit passing through the Nacc core). The thalamus then sends projections back to the cortex to form the so-called cortico-basal ganglia-thalamocortical loops (Groenewegen, Galis-de Graaf, & Smeets, 1999). The dorsal striatum projects to the dorsal rather than ventral pallidum, and either directly to the GP internal/SNr, or indirectly through the GP external and STN. The direct and indirect pathways are characterised by predominantly dopamine D1 or D2-receptor-expressing MSNs, respectively (Groenewegen, 2003; Maurin, Banrezes, Menetrey, Mailly, & Deniau, 1999; Nagy, Carter, & Fibiger, 1978).

To summarise, the striatum receives glutamatergic input from several brain areas including the amygdala and PFC, and dopaminergic input from the SNc and VTA. The afferent projections to the striatum are not uniformly distributed, but are organised in a ventromedial to dorsolateral fashion: ventral and medial striatal regions receive dense innervation from limbic structures and the VTA, while dorsolateral regions receive somatosensory inputs and projections from the SNc. The striatum itself sends mainly GABAergic projections to its output targets.

The striatum is comprised predominantly (95%) of GABAergic medium spiny neurons (MSNs), plus some GABAergic and cholinergic interneurons (Lobo, 2009). The GABAergic interneurons can be further subdivided in calbindin-, parvalbumin-, or neuropeptide-Y-expressing ones (Tepper & Bolam, 2004). Contrary to the segregated topography of striatal glutamatergic inputs, these cell type distributions are generally uniform across the striatum. The MSNs can be further subdivided in dopamine D1- or D2-receptor expressing neurons (direct/indirect pathway MSNs), co-expressing either substance P or preproenkephalin A, respectively (Le Moine & Bloch, 1995). The dorsal striatum exhibits a prominent separation between D1-expressing and D2-expressing MSNs, while the

ventral striatum (especially Nacc shell) contains a relatively greater proportion of MSNs expressing both types of receptor (Bertran-Gonzalez et al., 2008).

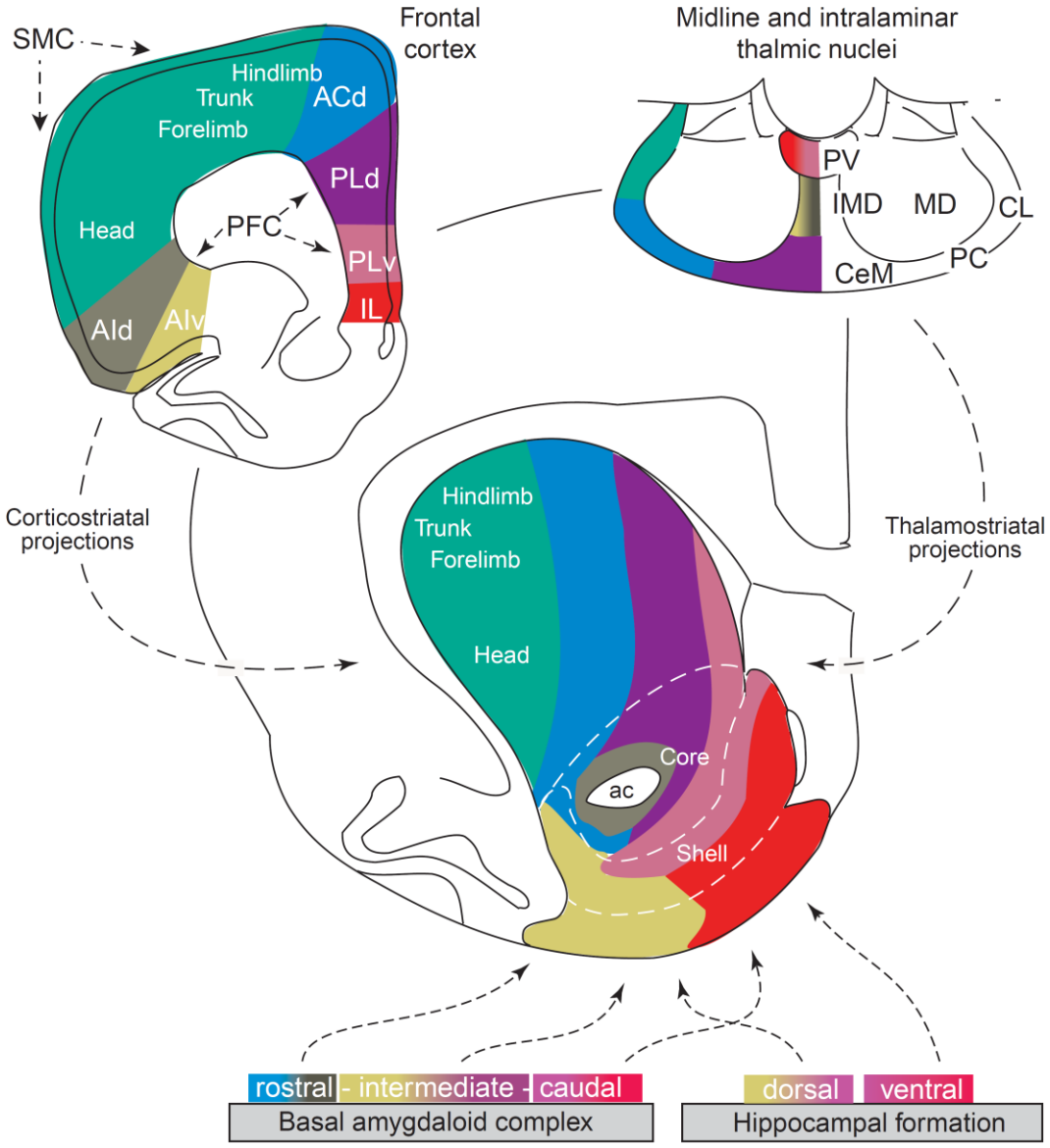

Fig. 1.1. Schematic representation of the major afferent glutamatergic projections of the striatum originating from the prefrontal cortex, thalamus, amygdala and hippocampus. Subdivisions of these four areas and their corresponding projections areas in the striatum are coloured the same. This is a simplified schematic representing the densest projections. Adapted from Voorn et al. (2004). Note the dorsolateral-ventromedial distribution of somatosensory vs. limbic inputs, with the prefrontal cortex inputs being the leading organisational principle. Cortical subdivisions as follows - SMC: somatosensory; ACd: anterior cingulate dorsal; PLd and PLv: prelimbic dorsal and ventral, respectively; IL: infralimbic; AId and AIv: agranular insular dorsal and ventral, respectively. Thalamic subdivisions – PV: paravetricular; IMD: intermediodorsal; CeM: central medial; MD: mediodorsal; PC: paracentral; CL: central lateral.

Neural circuits engaged by heroin vs. cocaine reward and relapse

So far, it was suggested that heroin and cocaine are two drugs with well-established addictive properties, and that according to current psychobiological theories drug abuse arises from the interaction of drugs with an interconnected system of brain areas with a key role for the striatum, amygdala, PFC and dopamine. This section will elaborate on the historical origins of current theories of drug abuse and discuss some key differences between heroin and cocaine suggesting that a unified view of addiction should be called into question.

Historically, studies of reward circuits of the brain were influenced by research going back as far as the 1950s. At that time, intracranial electric stimulation was used to identify the role of different brain areas in particular behaviours. The discovery of the so called "pleasure centre" through this method can be attributed to James Olds and colleagues.

In a somewhat serendipitous manner, Olds and Milner found that electrical stimulation of the medial forebrain bundle produced what is now referred to as CPP (Olds, 1956). That is, rats would develop a preference towards a particular place where that brain area was stimulated. Olds and Peters also demonstrated an operant reinforcement effect of this stimulation (Olds & Milner, 1954). The obvious question then was why stimulation of particularly that area produces rewarding effects. It was suggested that the result comes from the involvement of dopamine release produced by the stimulation, and the effect that dopamine has on target areas such as the Nacc. This was in part because the Nacc receives input from projections from the VTA, and these projections are embedded in the median forebrain bundle. In support of this claim, firing of VTA neurons was found to be necessary for maintenance of intracranial self-stimulation (ICSS) (You, Chen, & Wise, 2001).

Thus, the euphorigenic effects of drugs, which were believed to be the driving force behind drug-taking at least initially, were hypothesised to be mediated by the dopaminergic system. Indeed, drugs of abuse, including heroin and cocaine, were found to interact with the ICSS and the dopamine system (Bozarth, Gerber, & Wise, 1980; Fish et al., 2010; Wauquier & Niemegeers, 1974). However, their effects on dopaminergic

transmission and associated long-term changes can differ between drug classes, specifically opiates and psychostimulants. It is also the case that both increases and decreases in dopaminergic transmission may be associated with vulnerability to addiction, and ratings of drug-induced high are independent of dopamine release (for a review see D. J. Nutt, Lingford-Hughes, Erritzoe, and Stokes (2015)). For the sake of this introduction, only several key studies will be summarised suggesting that opiates and psychostimulants are processed differently by the brain and produce different behavioural effects.

For example, it has been shown that heroin and cocaine self-administration are mediated by separate neurotransmitter systems. Rats treated with opioid receptor antagonists tend to compensate for reduced opioid neurotransmission by increasing their operant responding for heroin. In contrast, such manipulation has no effect on cocaine self-administration. The opposite is true for the effect of pre-treatment with dopamine antagonists which leads to a compensatory increase in operant responses for cocaine but not heroin (Ettenberg, Pettit, Bloom, & Koob, 1982). These findings clearly show that cocaine exerts its reinforcing effects through direct interaction with the dopamine system, while heroin might have either indirect effect on dopaminergic release or operate through an entirely separate mechanism. It has been shown that it is the latter case, rather than the former, at least as far as DA transmission in the Nacc is concerned. Destruction of dopaminergic terminals within that brain area reduces cocaine self-administration by 70% within 5 trials post-lesion. In contrast, the same manipulation has little effect on heroin self-administration which recovers to more than 70% of its pre-lesion levels within the same amount of time (Pettit, Ettenberg, Bloom, & Koob, 1984). If heroin was producing its reinforcing effects through disinhibition of VTA dopamine neurons projecting to the Nacc, as some suggest (Devine & Wise, 1994), this would not have been the case. Accordingly, reducing DA D1-receptor function in the Nacc shell through RNA interference has been shown to impair acquisition of cocaine, but not heroin self-administration in rats (Pisanu et al., 2015).

It must be noted, however, that there is some evidence for opioid receptor activation in the VTA being rewarding (Devine & Wise, 1994). In addition, intracranial injections of morphine into the VTA lead to release of DA in the NAcc (Leone, Pocock, & Wise, 1991).

One possibility is that the projections from VTA have diverse targets, and DA release in the Nacc, although a consequence of VTA neuron firing, is not what drives opiate reinforcement. One way to examine this hypothesis through existing research (albeit indirectly) is to look for patterns of neuronal activity and morphological changes following drug administration. That is, if the rewarding effects of both opiates and psychostimulants are encoded in the same way, then neural activity in the reward circuitry should be similar. Empirical evidence suggests this is not the case either. In vivo multichannel singe-unit recordings during cocaine and heroin self-administration has revealed that the majority of neurons respond differently to the two drugs in the Nacc and the medial prefrontal cortex. That is, out of all neurons that showed an inhibitory or excitatory response to at least one of the drugs, only 25% or less showed the same response for the other drug as well. This was true for both brain areas and regardless of the order in which the drugs were self-administered (Chang, Janak, & Woodward, 1998).

Even long-term effects of opiates and psychostimulants differ. Repeated exposure to psychoactive substances including but not limited to heroin and cocaine can lead to structural changes in neurons. These changes are likely to be related to synaptic reorganization that serves as the biological basis for learning and memory, and are likely to underlie maladaptive behaviour such as drug abuse. It has been found that while cocaine increases dendritic branching and spine density in the Nacc core and shell and medial PFC, morphine has the opposite effects in these brain areas except Nacc core where such data is not available (Robinson & Kolb, 2004). It has been shown that DA is involved in this type of plasticity in the PFC (Reynolds et al., 2018), so these findings add further evidence against common DA mechanisms for opiates and psychostimulants. Also relevant is the finding that prolonged abstinence from cocaine fails to produce many of the behavioural changes associated with withdrawal from morphine, nicotine, alcohol and THC in mice. Cocaine withdrawal does not produce the impairments in direct social interaction, the increase in motor stereotypy, and the exacerbated conflict anxiety produced by withdrawal from the rest of the drugs mentioned. These differences in behavioural changes are also accompanied by a decrease in markers of neuronal activity in the VTA for all other drugs except cocaine. Finally, morphine and cocaine abstinence

produced opposite transcriptional changes in the extended amygdala for a set of HTT-related genes involved in striatal neurotransmission (Becker, Kieffer, & Le Merrer, 2017).

The differences between heroin and cocaine extend beyond their direct pharmacological actions on cell function. The environmental context can modulate the reinforcing and subjective effects of opiates and psychostimulants in opposite directions (Caprioli, Celentano, Paolone, & Badiani, 2007). For example, the novelty of the environment in which the two drugs are administered can be manipulated by either keeping rats in the drug-administration chambers at all times (resident rats) or moving rats to a physically identical chamber only during testing (non-resident rats). Studies using this paradigm have shown that residents will press more for heroin over a range of doses, while non-residents will press more for cocaine and amphetamine (Caprioli et al., 2008; Caprioli, Paolone, et al., 2007). The same studies show that, as measured by a progressive-ratio schedule, residents are more motivated for heroin than non-residents, while the opposite is true for cocaine and amphetamine (at least at certain doses). Non-residents also reinstate cocaine-seeking after a cocaine priming, while residents do not, and the opposite is true for heroin-seeking after a heroin priming (Montanari et al., 2015). Therefore, the environment can modulate the reinforcing properties of opiates and psychostimulants in opposite ways, as well as the propensity to relapse to drug seeking. There is also some evidence that these environmental effects may extend to the subjective/pleasurable effects of the heroin and cocaine. For example, residents are better at discriminating morphine from saline than non-residents, while the latter are better at discriminating amphetamine from saline than residents (Caprioli, Celentano, et al., 2007; Paolone, Palopoli, Marrone, Nencini, & Badiani, 2004), suggesting that the salience of the interoceptive effects of the two drugs are modulated in opposite ways by the environmental context. Non-residents also emit more 50 kHz ultra-sonic vocalisations than residents in response to cocaine, while the opposite is true for heroin. Rats emit this type of vocalisations when exposed to rewarding stimuli, suggesting they reflect positive affective states (Avvisati et al., 2016).

Human studies have provided further support for the findings from rodent studies. Both rats and humans prefer taking heroin in familiar/home environments, while cocaine is taken outside the home (Caprioli et al., 2009). That is, given the choice, resident rats

prefer to self-administer heroin, while non-residents prefer cocaine, and experienced human addicts report taking each drug predominantly in different environments. Finally, humans also report a pleasant experience of the cocaine high in a novel environment, but an unpleasant one at home, while the opposite is true for heroin. These self-reports are accompanied by opposite changes in activity of the left middle frontal gyrus, left dorsal caudate and cerebellum during cocaine vs. heroin imagery at home and outside (De Pirro, Galati, Pizzamiglio, & Badiani, 2018).

It has been suggested that the opposite modulatory effects of novelty on all these effects of opiates and psychostimulants results in part from an interaction between the interoceptive stimuli produced by heroin and cocaine (e.g. changes in blood pressure, heart rate) and the sensory input from the environment (e.g. visual/auditory stimuli) which modulate the acute effects of the two drugs (Badiani, 2013).

In summary, there is ample evidence that the acute reinforcing effects of opiates and psychostimulants are processed very differently within the putative reward circuitry. Long-term drug-induced neurobiological and behavioural changes also differ between drug classes, and the setting of drug-taking can modulate the subjective and rewarding effects of heroin and cocaine in opposite directions.

Finally, there are known differences between circuits engaged in rodent models of heroin and cocaine relapse which shouldn't be overlooked. Drug seeking can be reinstated through exposure to drug-associated context and stimuli or the drug itself (Crombag, Bossert, Koya, & Shaham, 2008; Stewart, 2000). The circuitry involved in this process involves projections from the medial PFC to the Nacc shell and core for both heroin and cocaine. However, while activating the projection from ventromedial PFC to Nacc shell promotes context-induced relapse to heroin-seeking (Bossert et al., 2012), activating this projection seems to inhibit drug-primed relapse to cocaine-seeking (Peters, LaLumiere, & Kalivas, 2008).

To summarise, a vast amount of empirical evidence points towards a separation of the neuronal circuits governing heroin vs. cocaine reward and the propensity to relapse. Importantly, the separation between these circuits is not absolute in the sense that, indeed, heroin and cocaine do engage the same neuromodulators and brain areas to a

significant extent. However, there is also significant variation within these brain areas and neuromodulator systems in terms of the information being encoded. It is necessary to emphasise this point for the sake of improving both the theory and clinical practice within the field of substance abuse. For this reason, the project described within this thesis aimed to add further evidence for the different ways in which the brain responds to heroin and cocaine.

2. Pharmacology of heroin and cocaine

Although heroin and cocaine can both lead to the development of pathological drug-seeking and drug-taking behaviour, their pharmacological properties are very different. To some extent, many differences in the acute behavioural and physiological effects of the two drugs can be attributed to their interaction with distinct neurotransmitter systems. In addition, heroin and cocaine differ greatly in terms of their metabolism and pharmacokinetics.

Chemical structure and metabolism of heroin and cocaine

Heroin is synthesised through the addition of two acetyl groups to the molecule of morphine (fig. 1.2, p. 17). This change in structure brings about significant changes to the pharmacokinetics of the drug, making heroin much more lipid soluble and therefore able to cross the blood-brain barrier (BBB) more efficiently (Pardridge, 2012). However, apart from this improved ability to enter the central nervous system, heroin is not much different from morphine in its pharmacological actions. In fact, heroin is rapidly metabolised to morphine after crossing the BBB. Of course, the change in pharmacokinetics is not irrelevant, because it means that equal doses of the two drugs would have very different efficacy, with heroin being much more potent in producing analgesia and euphoria when injected. It should be noted that, once heroin enters the brain, it is first metabolised into 6-monoacetylmorphine (6-MAM) before it is metabolised to morphine. Morphine itself is further broken down to morphine-3- and morphine-6-glucuronide. All these metabolites possess their own pharmacological effects. The peak concentration of 6-MAM in the blood and brain following a heroin injection is much higher than that of morphine, suggesting that many of the immediate effects of heroin might in fact be due to the pharmacology of 6-MAM rather than

morphine (Gottas et al., 2013). Thus, the physiological and psychological effects of heroin are anything but straightforward, and are heavily dependent on route of administration and individual differences in metabolism.

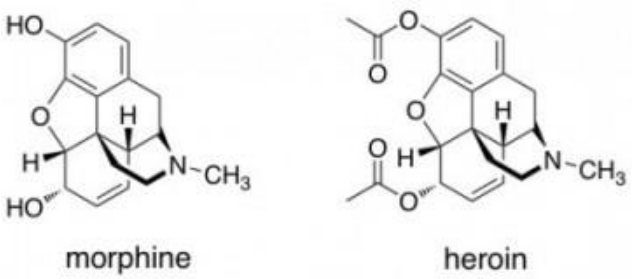

Fig. 1.2. Chemical structures of morphine and heroin.

Cocaine, on the other hand, is most commonly found with its natural chemical structure present in the coca leaf. The cocaine molecule is comprised of a six-carbon phenyl ring and a nitrogen-containing ring, both necessary for its biological activity (fig. 1.3, below).

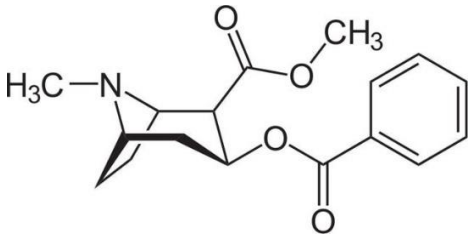

Fig. 1.3. Chemical structure of cocaine.

Cocaine is lipid-soluble and can get into the bloodstream and pass the BBB easily. For this reason, it is a very potent drug especially when injected or snorted. There are several known cocaine metabolites amongst which are norcocaine, benzoylecgonine and benzoylnorecgonine. It is suggested that individual differences in cocaine metabolism is an important factor for both the acute and long-term effects of the drug, since it determines the duration of cocaine action in the brain (Schindler & Goldberg, 2012; Schuelke, Konkol, Terry, & Madden, 1996). Thus, similarly to heroin, the effects of cocaine depend on metabolism just as much as they do on the chemical properties of the substance.

Even a brief glance at their chemical and metabolic properties, reveals some important differences between heroin and cocaine. The ability of the two drugs to reach the brain, together with their metabolism and rate of elimination, point to potential important

differences in their psychobiological effects. The speed at which they reach the brain, the amount of time they spend there and exert their effects, and the targets upon which they act all combine to produce their effects in the short and long term.

Heroin and its metabolites interact with the opioid receptors

Due to its metabolism (as described above), the effects of heroin are in great part the result of its metabolites 6-MAM and morphine binding to opioid receptors in the brain (Inturrisi et al., 1983). There are four known types of opioid receptor found through the mammalian nervous system – mu (μ), delta (δ), kappa (κ) and NOP-R. Out of these, the μ- and δ-receptors are perhaps most relevant to the analgesic, euphorigenic and addictive properties of heroin for two reasons: they are found in brain areas involved in pain processing, motivation, reward and learning (Mansour, Khachaturian, Lewis, Akil, & Watson, 1988), and they are readily bound by 6-MAM and morphine (Mignat, Wille, & Ziegler, 1995). It has been suggested that the primary effects of heroin are the result of 6-MAM and morphine binding to μ-receptors, and 6-MAM having higher efficacy at that receptor than morphine (Selley et al., 2001).

Opioid receptors are metabotropic, coupled to inhibitory G-proteins (G_i), so that activity at these receptors usually leads to decreased excitability and neurotransmitter release in the neurons carrying them. This is achieved through several mechanisms. The first one is opening of potassium channels which promotes depolarization of the cell membrane and thus decreases the probability of action potentials occurring (Torrecilla et al., 2002). Activity at kappa opioid receptors can also lead to closing of voltage-gated calcium channels which reduces pre-synaptic release of neurotransmitter triggered by action potentials (Rusin, Giovannucci, Stuenkel, & Moises, 1997). Finally, G_i proteins coupled to the receptors inhibit adenylyl cyclase and reduce the synthesis of cyclic adenosine monophosphate – cAMP (Minneman & Iversen, 1976). The consequences of this reduced synthesis are complex and beyond the scope of this section, but they have to do with chronic effects of heroin use such as tolerance and withdrawal. It happens when cAMP levels in the locus coeruleus (LC) become elevated after morphine/heroin administration has ceased as a compensatory response to cAMP reduction by those drugs. This renders the neurons more excitable, leading to more noradrenaline release and, presumably, a heightened state of arousal and negative affect (Nestler, 1996). This

process is a good example of how the location of μ-opioid receptors can determine the action of heroin – discussed in more detail in the following section.

Opioid receptors are located in circuits which process autonomic functions, pain, and reward

The various opioid receptor types are distributed inhomogeneously across the central nervous system (Mansour et al., 1988). The particular region of the CNS where opiates (including heroin and its metabolites) will act on the receptors can define particular behavioural and physiological effects of this class of drugs.

To begin with, heroin has profound depressant effects on the respiratory and cardiovascular systems, causing decreased respiration rate (Pattinson, 2008), decrease in blood pressure and heart rate (Thornhill, Townsend, & Gregor, 1989) and changes in body temperature (Chen, Geller, DeRiel, Liu-Chen, & Adler, 1996). Opiate administration also causes constriction of the pupils (Larson, 2008). Activation of μ-opioid receptors located in the respiratory centres of the brainstem can suppress breathing, which represents one of the primary causes of deaths from illicit drug overdose (Eigner et al., 2017). Heart rate and blood pressure are also regulated by μ- and δ-receptors in the brainstem (Sun, Liu, Li, & Ingenito, 1996). Due to its effects on the respiratory and cardiovascular systems, heroin can produce parasympathomimetic-like effects (that is, effects mimicking the activation of the parasympathetic nervous system).

It is worth mentioning that a major effect of heroin is pain relief which involves inhibitory action mostly through μ-opioid receptors both at the spinal and the supraspinal levels. In the spinal cord, μ-opioid receptors are present at projection neurons which transmit pain signals from nociceptors to higher brain centres. Supraspinally, periaqueductal grey (PAG) neurons synapse onto inhibitory interneurons in the Raphe nuclei and the LC. These synapses are rich in opioid receptors too, since the PAG contains endogenous opioid-releasing neurons, and disinhibition of the Raphe and the LC leads to release of serotonin and noradrenaline that can modulate pain signals. Finally, opioid receptors in the thalamus can prevent pain signals from reaching limbic regions such as the anterior limbic cortex, which is involved in the affective aspect of pain (Yaksh, 1997). The ability of opiate drugs such as heroin to engage pain-controlling systems is remarkable mainly

because it is unmatched by other known substances. The “Holy Grail” of opioid research has been the search for analgesics with comparable strength, but lower abuse potential, tolerance effects and withdrawal symptoms (Kieffer & Evans, 2002).

The mechanisms underlying both the abuse potential and the tolerance effects of opioids are still disputed amongst scientists (Badiani et al., 2011; Bailey & Connor, 2005), and a full review on that topic is beyond the scope of this section. However, within this context, it is worth mentioning that μ-opioid receptors are also located within the ventral tegmental area (VTA) and the nucleus accumbens (Nacc). The former contains a significant proportion of all dopamine-releasing neurons in the brain, and the latter is believed to be a point of convergence for circuitry involved in the processing of learning, motivation and pleasure (i.e. reward). The action of heroin and morphine on receptors in these areas has been shown to be necessary for certain types of behaviour in animal models of reward (e.g. drug self-administration). A more detailed explanation of these actions of opiates and animal models of reward are provided in following sections. At this point, it suffices to mention two receptors within this circuit. First, activity at μ-opioid receptors located on GABAergic inhibitory interneurons within the VTA results in disinhibition of dopamine-releasing neurons (Jalabert et al., 2011). Second, κ-receptors can inhibit dopamine release at terminals in the Nacc (Britt & McGehee, 2008). Supposedly, the first effect has rewarding properties (Devine & Wise, 1994), while the latter leads to negative affect (S. R. Ebner, Roitman, Potter, Rachlin, & Chartoff, 2010). Note, however, that rats may self-administer κ-opioid receptor agonists (Marinelli et al., 1998).

Cocaine blocks monoamine re-uptake transporters

Cocaine acts by blocking the re-uptake transporters of dopamine (DA), noradrenaline (NA) and serotonin (5-HT). It has the highest affinity for the 5-HT transporter, followed by the DA and then the NA transporters (Ritz, Cone, & Kuhar, 1990). Contrary to heroin, cocaine achieves its effects mainly through its primary structure rather than through its metabolites. By blocking the monoamine transporters, cocaine prolongs the presence of already released neurotransmitter in the synaptic cleft and thus prolongs its binding to receptors. One additional effect of cocaine, in large doses, is the inhibition of voltage-gated sodium channels which prevents the propagation of action potentials along axons

(Matthews & Collins, 1983). This effect is at the base of cocaine's local anaesthetic properties, as it prevents pain-receptor cells from transmitting signals to the brain.

Considering that cocaine interacts with not one but three neurotransmitter systems, and that those neurotransmitter systems involve several types of receptors each, it is safe to say that the effects of cocaine are just as multifaceted as those of heroin. Similarly to opioids, DA, 5-HT and NA act on metabotropic receptors. However, these receptors are organized in groups with often opposing actions in terms of cellular activity. To begin with, DA receptors can be subdivided to D1- and D2-like types. The D1-type are coupled to Gs proteins which stimulate cAMP and can be said to increase excitability of the cell containing them, while D2 inhibit cAMP and decrease excitability through Gi proteins similarly to opioid receptors (Vallone, Picetti, & Borrelli, 2000). Receptors for NA are also subdivided in two main groups, alpha (α) and beta (β), each of which have several members. Generally, however, postsynaptic receptors are involved in signalling cascades that stimulate cAMP formation and calcium build-up inside the postsynaptic cell (Strosberg, 1993). Finally, 5-HT receptors are the most diverse with at least 15 different members across 7 main groups. All of these are metabotropic except the 5-HT3 which is an ionotropic receptor (Kroeze, Kristiansen, & Roth, 2002). Among all 5-HT metabotropic receptors exist both inhibitory receptors which function similarly to opioid and D2 receptors (e.g. the 5HT1a receptor) but also receptors that engage the phosphoinositide second messenger system like adrenergic receptors (e.g. 5HT2a receptors). As already shown for the opioid receptor system, the location of all these receptors within the central nervous system defines the psychoactive properties of cocaine.

Cocaine enhances monoamine action both in the peripheral and central nervous systems

The acute effects of cocaine include increased blood pressure and heart rate (Foltin, Fischman, & Levin, 1995), increased respiration rate (Trippenbach & Kelly, 1994), hyperthermia (Crandall, Vongpatanasin, & Victor, 2002) and dilation of the pupil (Pitts & Marwah, 1988). Note that the effects on the cardiovascular and respiratory systems are in most part opposite to those of heroin, and resemble activation of the sympathetic nervous system (i.e. cocaine can be defined as a sympathomimetic drug). The somatic

effects of cocaine are likely to be linked to its effects on the noradrenergic and serotonergic systems, which are involved in regulation of the cardiovascular system. For example, vasodilation is in part regulated through adrenergic receptors on blood vessels, and that serves as a primary mechanism for temperature regulation. There is also evidence for dopaminergic and serotonergic mechanisms as well, since mice lacking the dopamine and serotonin transporters show lower hyperthermia in response to methamphetamine (Numachi et al., 2007).

Sympathetic effects of cocaine and other stimulants notwithstanding, by far the most researched effects of cocaine result from its interaction with the dopaminergic system. Primary recipients of dopaminergic inputs are the basal ganglia, including the Nacc and dorsal striatum, the prefrontal cortex (PFC) and the amygdala (Arias-Carrion, Stamelou, Murillo-Rodriguez, Menendez-Gonzalez, & Poppel, 2010). Cocaine increases dopamine levels at synapses in all these areas (Hurd, McGregor, & Ponten, 1997; Sorg & Kalivas, 1993; Stuber, Roitman, Phillips, Carelli, & Wightman, 2005). Dopaminergic activity in the amygdala and the Nacc regulates the motivating properties of cocaine; i.e. seeking and taking of cocaine are mediated by dopamine D1 receptors in these areas (Hurd et al., 1997; Maldonado, Robledo, Chover, Caine, & Koob, 1993). It is noteworthy that cocaine also has euphorigenic properties, but these are not mediated by dopamine (Leyton, Casey, Delaney, Kolivakis, & Benkelfat, 2005). On the other hand, dopaminergic activity in the prefrontal cortex has diverse functions depending on the particular circuitry in which DA receptors are embedded and the type of receptor in question. A full review of PFC dopaminergic function is beyond the scope of this section, but in general, cognitive deficits associated with ADHD and schizophrenia both involve PFC dopamine (Arnsten & Li, 2005; Knable & Weinberger, 1997).

Last but not least, cocaine's action on the dopaminergic system stimulates locomotor behaviour, and is central to the attribution of incentive salience to stimuli by which they acquire attention-grabbing and motivational properties (Berridge, 2007).

3. Arc and homer 1a – two genes related to neuronal activity and drug effects in the brain

The literature reviewed so far describes heroin and cocaine as two substances that differ greatly in their chemical properties, as well as physiological and psychoactive effects. Both drugs engage highly distributed neurotransmitter systems, and thus modulate fundamental functions of the nervous system (pain, motivation, executive function, etc.). Therefore, the study of heroin and cocaine has implications for understanding both normal and pathological function of the brain, as well as the use of opioids for analgesia. In addition to all that stands a number of drug-use-related disorders, amongst which is that of drug addiction – a concept with which heroin and cocaine are often associated. One approach to understanding how drug use leads to the development of addiction is the study of the neuronal circuitry involved in the transition from recreational to pathological drug use. Some of the most useful scientific tools in this area of research take advantage of immediate-early genes and their connection to neuronal activity and plastic changes in the brain.

Immediate-early genes

Immediate-early genes (IEGs) are genes that are transcribed in response to particular intra- or extracellular events, but otherwise have low expression levels. These properties distinguish them from constitutively expressed genes which are transcribed at constant levels and maintain basic cellular function. In addition, translational inhibitors do not prevent the expression of IEGs; i.e. they are transcribed without the need for new protein synthesis, and are therefore the first response of the genetic machinery of the cell to external stimuli. IEGs often encode for transcription factors and can regulate a great deal of transcriptional events with long-term consequences. The types of external stimuli that can trigger IEG expression include stress and UV light, as well as hormonal, growth factor and some neurotransmitter signalling. As their name suggests, IEGs are transcribed quickly after the triggering event and their expression is elevated only for a relatively short period of time. This is in part due to their shorter length and fewer exons relative to other types of genes (Healy, Khan, & Davie, 2013).

In the brain, IEG expression often begins after a ligand binds to a receptor in the cell membrane and leads to the activation of an intracellular signalling cascade (fig. 1.4, next page). One such cascade is the MAPK/ERK pathway, which is involved in the expression of some of the most well-known IEGs: *c-fos, egr1/zif268,* and *arc* as well as *homer 1a*. The MAPK/ERK pathway is activated by mitogens and growth factors and leads to activation of CREB, a transcription factor that promotes IEG transcription (Davis, Vanhoutte, Pages, Caboche, & Laroche, 2000). CREB can also be activated following calcium release within (Hardingham, Arnold, & Bading, 2001) or influx to the cell, which links expression of the IEGs mentioned to excitatory neuronal signalling through glutamatergic NMDA receptors (Riccio & Ginty, 2002; Xia, Dudek, Miranti, & Greenberg, 1996). For example, it has been shown that both normal and seizure-induced activity can lead to expression of IEGs in the brain (Link et al., 1995; Lyford et al., 1995). Other means by which CREB can be activated is the cAMP/PKA pathway which is linked to activity at G-protein coupled receptors such as dopaminergic receptors (Dudman et al., 2003).

Since IEGs are transcribed in response to neuronal activity, and often encode for transcription factors or effector proteins which can have long-term consequences for cellular functioning, they have been implicated in molecular events associated with experience-dependent plasticity and learning. Indeed, electrophysiological studies have shown that IEGs are expressed following stimulation of neurons which strengthens synaptic connections – e.g. long-term potentiation (LTP) (Link et al., 1995). Further support for the role of IEGs in learning comes from studies where interference with IEG transcription has been shown to affect performance on long-term memory tests and maintenance of LTP (Guzowski et al., 2000), as well as Pavlovian fear conditioning (Mahan et al., 2012).

In summary, IEGs are a major player in the functioning of the nervous system thanks to their link with excitatory neurotransmission, experience-dependent plasticity and learning. IEGs represent one mechanism by which neuronal cell functioning responds to changes in the environment, and IEGs are therefore major players in adaptive behaviour. They are also an important tool for many molecular biology techniques used to study the functioning of the nervous system. For example, they have been used as markers of

activity, and, more recently, as drivers of expression for artificially introduced fluorescent-protein-encoding genes (transgenes) that allow tagging of neuronal populations involved in specific functions – i.e. neuronal ensembles (Cruz et al., 2013; Kawashima, Okuno, & Bito, 2014).

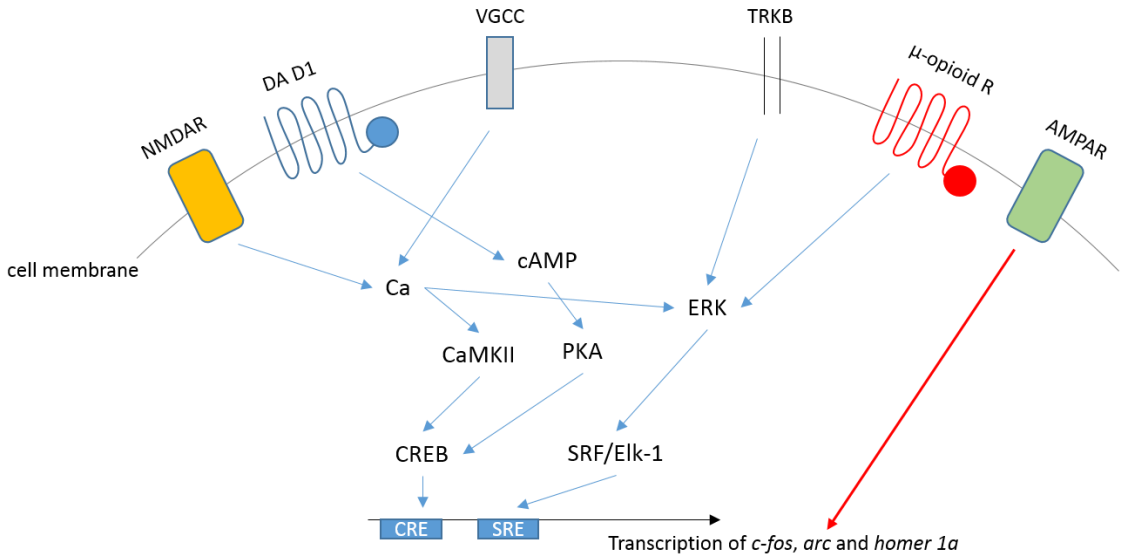

Fig. 1.4. A simplified representation of some receptors and intracellular signalling cascades involved in IEG transcription. Blue arrows represent direction of molecule interactions. Please note that some of the molecules in the signalling cascades are omitted for the sake of clarity. Following binding of ligands to receptors, second messengers (e.g. calcium, Ca, or cyclic adenosine monophosphate, cAMP) initiate signalling cascades which lead to phosphorylation of kinases (e.g. calcium-calmodulin kinase II, CaMKII, protein kinase A, PKA, extracellular-signal regulated kinase, ERK). These kinases in turn phosphorylate transcription factors such as cAMP-response element binding protein (CREB), serum response factor (SRF) or Elk-1. Transcription factors then bind to promoter regions of genes (e.g. cAMP response element, CRE, serum response element, SRE) to initiate transcription. The red line from the AMPA receptor (AMPAR) to *arc* signifies inhibition of transcription. Activation of μ-opioid receptors (R) on the other hand may activate the ERK pathway. This is to show that the relationship between excitatory neurotransmission and IEG transcription is not straight forward. DA D1: dopamine D1 receptor; VGCC – voltage-gated calcium channel; TRKB: growth factor receptor.

Neuronal ensembles and the c-fos IEG

The idea of neuronal ensembles can be traced back to the work of Charles Sherrington, who proposed that the central nervous system is composed of a number of so-called

"reflex arcs" that can influence each other and thus integrate information from the environment to produce movement (Sherrington, 1906). These arcs, he proposed, consisted of three key prototypical components – neurons which receive information from the environment, neurons which control muscles to produce movement and neurons connecting the two. Thus, Sherrington was one of the first proponents of the idea of specialised groups of neurons carrying information-integrating and sensorimotor functions: the basic principle of neuronal ensembles. Later on, a similar idea was put forward by Donald Hebb, who proposed that repeated stimulation of the nervous system gives rise to the so-called "cell assemblies": diffuse groups of neuronal cells capable of acting in a coordinated, independent manner, and capable of facilitating the activity of other such groups of cells. A chain of such "cell assemblies" bridging the gap between sensory input and motor output Hebb referred to as a "phase sequence" and it was suggested to underlie all thought processes (Hebb, 1949).

Nowadays, the term "neuronal ensembles" is being used widely, and generally refers to a population of neurons specialised in the processing of a given brain function. More specifically, a neuronal ensemble can be defined as a group of neurons with similar afferent and efferent connections, involved in the computation of similar behavioural functions, neuroendocrine regulation or sensorimotor gating (Pennartz, Groenewegen, & Lopes da Silva, 1994). Note that such a definition focuses on the neuroanatomical connections and functional properties of neuronal cells.

Electrophysiological research defines neuronal ensembles as groups of neurons that fire action potentials with the presentation of a given stimulus or during a particular behaviour. It is usually required that neurons pertaining to neuronal ensembles in this sense exhibit a synchronised (i.e. correlated) change in overall firing rate time-locked to the presentation of a stimulus, the execution of a behavioural response, or a change in a behavioural task parameter (Cohen & Kohn, 2011; Nicolelis, Baccala, Lin, & Chapin, 1995). For example, Hubel and Wiesel found that individual neurons in the cat visual cortex respond preferentially to lines of light presented in a particular orientation (Hubel & Wiesel, 1959). Their study was one of the first to provide direct evidence for preferential responding of neurons time-locked to the presentation of a particular stimulus. Such specialised neurons were found in other brain areas and it was believed

that they represent specialised “modules” for a given kind of information (Erickson, 2001). With time, it was accepted that a more accurate representation of the way specific functions in the brain are carried out is by population coding - orchestrated firing of motor neurons in the monkey cortex could be used to predict the direction of limb movements (Georgopoulos, Schwartz, & Kettner, 1986). These were seminal studies showing that neuronal activity can reflect (i.e. encode for) specific nervous system functions, not only in terms of sensation and perception, but also movement. Two features of neuronal ensembles (form an electrophysiological point of view) can be deducted from these two and other similar studies. First, the neurons forming a neuronal ensemble are expected to change their activity in a synchronised manner which is time-locked to the presentation of a stimulus or the expression of a particular behaviour; second, it should be possible to predict the behaviour of an animal from the activity of the neuronal ensemble encoding for said behaviour (T. J. Ebner, Hendrix, & Pasalar, 2009; Laubach, Wessberg, & Nicolelis, 2000).

Although electrophysiological approaches are indispensable for understanding the relationship between neuronal activity and behaviour, there is a limit to the number of neurons that can be recorded from simultaneously, especially in deep brain structures. Even with recent advances in large-scale recording using silicon electrode arrays (Buzsaki, 2004), recording from sparsely distributed neurons across multiple deep brain structures is still problematic. For this reason, the relationship between IEG expression and neuronal activity has been used to identify and manipulate neuronal ensembles across large deep brain areas through histochemical, and optogenetic techniques. A lot of focus in this area has been given to the IEG *c-fos*.

The insertion of transgenes downstream of the *c-fos* promoter has made possible tagging of strongly activated neurons with fluorescent markers such as GFP in living cells. This has allowed for electrophysiological measurements to be taken from neurons known to have been active during a specific event. For example, it has been recently shown that *c-fos*-expressing neurons in the nucleus accumbens activated by the presentation of sucrose-associated cue are more excitable relative to non-activated *c-fos*-negative neurons (Ziminski et al., 2017). Changes in excitability are a particular kind of neuronal plasticity which involves changes in receptor functioning (Zhang & Linden,

2003). This indicates that *c-fos* does not simply reflect any kind of activity, but activity in neurons that undergo plasticity.

Transgenes can also be used to express proteins that allow for manipulation and targeted inactivation of neurons active during a specific event in time. Such techniques have been indispensable in providing evidence for the causal role of neuronal ensemble activity in behaviour. For example, the Daun 02 technique uses a transgenic line of rats or mice where the *c-fos*-promoter is upstream of a *lacZ* promoter. In these rodents, the strongly activated neurons expressing *c-fos* also express the protein β-galactosidase. When the Daun02 drug is injected in a brain area of interest, the β-galactosidase produced by *c-fos* expressing neurons in the area interacts with Daun02 to produce daunorubicin and cause apoptosis in these cells. Using this method, it was shown for the first time that sparsely distributed neurons in nucleus accumbens (Nacc) are necessary for expression of context-specific sensitisation (Koya et al., 2009), and neuronal populations in the ventromedial prefrontal cortex (vmPFC) are necessary for context-induced relapse to heroin-seeking (Bossert et al., 2011). Thus, *c-fos* is expressed not only in neurons that undergo plasticity, but also in neurons that have a causal role in expression of behaviour. This causal role in behaviour is another defining feature of neuronal ensembles which is relevant to IEG expression as a marker of neuronal activity (Cruz et al., 2013; Koya, Margetts-Smith, & Hope, 2016).

To summarise, neuronal ensemble research has shown that there are distributed populations of neurons across the brain that act in concert to encode functions such as perception, movement and conditioned behaviour. A lot of neuronal ensemble research has been carried out within the field of electrophysiology, but the use of the *c-fos* IEG as a marker of activity has revealed a relationship of IEG expression with functional importance: *c-fos* is expressed not just in neurons that have been active, but also neurons that are crucial for expression of the behaviour for which they encode. In addition, they are often neurons that undergo certain types of plasticity. More recently, other IEGs have begun to gain popularity in ensemble research such as *arc* and *homer 1a*.

The arc gene

Characteristically of IEGs, *arc* is normally expressed at low levels, but is elevated following administration of electrical stimulation sufficient to produce convulsive seizures, and stimulation of the type that produces LTP. In addition, *arc* is expressed in response to natural neuronal activity: a monocular injection of tetrodotoxin (a sodium channel blocker) leads to reduced *arc* expression in the contralateral, but not ipsilateral, visual cortex (Link et al., 1995; Lyford et al., 1995).

The *arc* gene was first described as an IEG with a protein product that accumulates in the soma and dendrites of expressing cells. Thus, *arc* is different from *c-fos* in that it does not encode for a transcription factor, but an effector protein that directly affects the functioning of the cell.

The property of *arc* to be highly expressed under conditions of glutamatergic transmission makes it a useful marker of cellular activity as in the case of other IEGs. What makes *arc* different, however, is that once it is transcribed in response to cell-stimulating events, both its mRNA and protein products relocate to the dendrites which have received the stimulation (Moga et al., 2004). Although LTP-inducing type of stimulation leads to expression of *arc*, its protein is known to be involved in endocytosis of AMPA receptors (Chowdhury et al., 2006), and to be necessary for metabotropic glutamate receptor-dependent long-term depression (LTD) of synapses (Park et al., 2008). *Arc* transcription is also involved in long-term memory formation, but not short-term memory or learning (Plath et al., 2006). So, it seems that *arc* mRNA and protein are produced in response to events that reflect cellular activity and increased connectivity, while at the same time are involved in molecular processes which weaken synapses. To resolve this paradox, it has been suggested that *arc* is most likely involved in homeostatic plasticity whereby it weakens relatively inactive synapses onto dendrites that are receiving various inputs with different intensity (Minatohara, Akiyoshi, & Okuno, 2016).

Although *arc* mRNA levels are elevated similarly to *c-fos* and *zif268* following learning tasks such as the Morris water maze, the levels of *arc* and *c-fos* do not always correlate within relevant brain areas such as the hippocampus. In addition, *arc* levels are more

strongly correlated with task performance than *c-fos* (Guzowski, Setlow, Wagner, & McGaugh, 2001).

In summary, *arc* is similar to other well-known IEGs such as *c-fos* in that it is expressed under conditions of strong neuronal activation, and is involved in synaptic plasticity. However, *arc* seems to be involved in plasticity more directly by restructuring synapses through AMPA receptor trafficking. Finally, although correlated, *c-fos* and *arc* patterns of expression are not identical following learning tasks.

The homer 1a gene

The mRNA of the whole *homer 1* gene has four splice variants in total, two of which are constitutive (*homer 1b* and *1c*) and two are IEGs (*homer 1a* and *ania 3*). The *homer 1* gene spans ~100 kbp, and its constitutive forms are longer, encoded by exons 1-10, while the IEG forms are shorter spanning about half that length: exons 1-5 and parts of intron 5. The unique sequence that distinguishes the two IEG forms is found in the 3' UTR. The *homer 1* gene is constitutively expressed in its *1b/c* forms, but under conditions of neuronal activity transcription is terminated early and intronic sequence is converted to exonic, which is a unique way of adapting constitutive form of a gene to an IEG (Bottai et al., 2002).

Similar to *arc* and other IEGs, the transcription of *homer 1a* is rapidly induced in response to neuronal activation, is dependent on calcium influx through NMDA receptors, and the MAPK/ERK pathway is responsible for its transcriptional up-regulation (Sato, Suzuki, & Nakanishi, 2001).

Similarly to *arc,* the protein product of *homer 1a* has effects on receptor function and synaptic transmission. For example, *homer 1a* modulates endocannabinoid-mediated retrograde synaptic inhibition. It potentiates depolarization-induced suppression of excitation, and inhibits metabotropic suppression of excitation (Roloff, Anderson, Martemyanov, & Thayer, 2010). In addition, the Homer 1a protein reduces the coupling of the metabotropic glutamate receptor mGluR5 to postsynaptic effectors, reducing the inhibitory effects of the receptor (Kammermeier & Worley, 2007).

Homer 1a is also involved in some forms of learning. *Homer 1a* mRNA is expressed in the striatum and prefrontal cortex (PFC) during early instrumental training with food, with

patterns of expression changing over time: striatum levels are maintained, while PFC and cingulate cortex expression is reduced (Hernandez, Schiltz, & Kelley, 2006). Other effects on learning are possible through effects on glutamatergic neurotransmission, especially effects on the NMDA and metabotropic glutamate receptors: the Homer 1a protein disrupts the physical link between group 1 mGluRs and NMDA receptors by disrupting the Homer-Shank protein scaffold that links the two receptors. This results in inhibition of NMDA current by group 1 mGluR agonists (Bertaso et al., 2010).

Thus, *homer 1a* shares the characteristics of *arc* and *c-fos* in that it can be used as a marker of neuronal activity, because its expression is under the control of the MAPK/ERK pathway and the transcriptional factor CREB. It is also involved in learning, at least as far as classical conditioning goes, and has an effect on synaptic transmission by interacting with metabotropic glutamatergic receptors, the NMDA receptor and cannabinoid neurotransmission. However, the mechanisms of *homer 1a* transcription is unique and differs from those of other IEGs, and so do its interactions with receptor and neurotransmitter systems.

Psychoactive drugs induce IEG expression in the brain

One of many stimuli that lead to IEG expression in the central nervous system are psychoactive drugs. This expression is observed in several brain areas following drug administration, and the exact pattern and the magnitude of expression can vary depending on many factors including dose, type of drug, route of administration, environmental factors and behavioural tasks associated with drug administration. In addition, different IEGs can exhibit different patterns of expression under the same circumstances (Harlan & Garcia, 1998).

The definition of psychoactive drugs covers a vast amount of substances. The focus of this thesis is on heroin and cocaine for reasons elaborated in previous sections. For the sake of conciseness, this section will provide examples for these two drugs and their effects on *c-fos*, *arc* and *homer 1a* expression.

Acute intraperitoneal administration of cocaine induces significant increase of *c-fos* mRNA in the Nacc, but the this effect disappears after chronic treatment with the drug (Hope, Kosofsky, Hyman, & Nestler, 1992). Cocaine also increases *c-fos* mRNA in the

dorsal striatum and medial PFC. There the amount of expression varies from rostral to caudal areas, and across all areas the magnitude of the effect varies as a function of environmental context: novel environments increase IEG expression in response to cocaine and amphetamine (Uslaner et al., 2001).

Subcutaneous administration of heroin leads to elevation of *c-fos* protein levels in the shell of the Nacc, and this effect disappears with chronic treatment. Conversely, the opposite is seen in the dorsal striatum where elevated *c-fos* protein levels are seen only after chronic treatment (D'Este, Scontrini, Casini, Pontieri, & Renda, 2002).

Intraperitoneal injections of cocaine elevate *arc* mRNA in the PFC, striatum, hypothalamus and hippocampus of rats, and, importantly, this effect differs in magnitude from that of *zif268* expression as a function of brain area (Caffino, Racagni, & Fumagalli, 2011). While cocaine induces *arc* mRNA in the PFC and the magnitude of the effect depends on interactions with restraint stress, there was no elevation of *zif268* mRNA levels in this brain area regardless of stress treatment. In the striatum, expression of both IEGs was induced by cocaine, but the magnitude of the effect was higher for arc. There are no studies looking at the acute effects of heroin on *arc* mRNA expression, at least according to this author's knowledge.

Studies on the effect of cocaine on *homer 1a* expression are scarcer, but there is evidence that an intraperitoneal injection of cocaine elevates expression of the gene in the dorsal striatum, and the effect differs in magnitude from that seen with *zif268* (Unal, Beverley, Willuhn, & Steiner, 2009). Studies looking at the effect of heroin administration on *homer 1a* expression are also not known to this author.

In summary, it is evident that heroin and cocaine administration can induce the expression of IEGs in the brain, and the effect differs in magnitude depending on the brain area, IEG, and drug administered. There also appears to be a lack of studies on the effect of acute heroin administration on *arc* and *homer 1a* expression. Given that IEGs can serve as markers of activity to identify neuronal ensembles, and that they are involved in long-term effects of stimuli on cell functioning, it follows that IEG expression triggered by heroin and cocaine administration can be an informative area of research.

The relationship between these drugs and IEG expression is closely related to their pharmacology and their effects on neuronal systems and behaviour.

4. Using IEGs to identify neuronal ensembles activated by heroin and cocaine.

So far it was argued that heroin and cocaine act on brain areas including the striatum, amygdala, prefrontal cortex, where they interfere with activity of motivational systems and affect learning processes. Evidence was also presented that these two drugs induce IEG expression in these brain areas (among others) and these IEGs can be used as markers of neuronal activity and to identify specialised neuronal ensembles. There is also a substantial number of studies suggesting separate mechanisms of action of heroin and cocaine on the same neuronal circuits. One step closer towards clarifying the commonalities and differences between heroin and cocaine action in the brain would be identifying the neuronal ensembles responding to each drug in key brain areas where they act to produce their behavioural effects. One way to achieve this would be to take advantage of IEG expression and molecular techniques used to detect them in brain tissue at the cellular and subcellular resolution.

In situ hybridisation (ISH)

In situ hybridisation is a technique which allows for the detection and quantification of nucleic acids in intact tissues and cells. The techniques takes advantage of the natural property of DNA and RNA strands to bind to strands with complementary nucleotide bases (i.e. annealing or hybridisation). When a nucleotide sequence of interest has to be detected, and the code of this sequence is known, a "probe" can be synthesised using in vitro transcription of such that the probe is complementary to the RNA or DNA strand of interest (the "target"). Probes can be synthesised using nucleotides conjugated to fluorescent molecules which are then detectable using fluorescence microscopy (fluorescence ISH or FISH). More than one probe can be used simultaneously targeting several different sequences of interest within the same tissue.

Introducing the probes to the tissue where they bind their target happens after the tissue in question has been extracted from the organism that is subject of study. In the case of brain tissue the technique is therefore carried out post-mortem, as the sample

needs to be preserved and pre-treated chemically to provide optimal conditions for hybridisation.

CatFISH

Since FISH is a post-mortem analysis of gene expression, it usually allows for the quantification of gene expression resulting from a single event in time (usually up to a few hours before tissue extraction). This presents a limitation when the objective is the identification of gene expression within the same specimen resulting from two separate events. To overcome this limitation, Guzowski, McNaughton, Barnes, and Worley (1999) devised a variation of the standard FISH technique which takes advantage of the changes in mRNA location within cells as a function of time. This variation of the technique was named cellular compartment analysis of temporal activity by fluorescent ISH (catFISH). Immediately following gene expression, mRNA is mostly concentrated around the site of transcription within the cell nucleus, but then gradually diffuses to the cytoplasm where it takes part in other processes (e.g. translation). Since FISH can provide enough resolution to distinguish the two types of signal (i.e. nuclear vs. cytoplasmic), if the precise timing of the relocation of a given mRNA was determined empirically, it becomes possible to determine how long ago a cell has expressed a given gene. When this logic is applied to an IEG such as *arc*, which is expressed in response to cellular activation, conclusions can be made about whether a given neuron was activated by two separate events. *Arc* mRNA appears in the cell nucleus within 5 min following neuronal activation and relocates to the cytoplasm within ~30 min. Thus, if two discrete events are administered 30 and 5 min before brain tissue is collected for FISH, cells which are found to contain only nuclear *arc* mRNA would have been activated by the more recent event, while cells containing only cytoplasmic mRNA would have been activated by the more distant event, and cells expressing both nuclear and cytoplasmic signal would have been activated during both events. On theory, similar logic can be applied to any IEG. For example, *homer 1a* appears in the cell nucleus ~30 min following cell activation and moves to the cytoplasm within ~60 min. Therefore, using double-staining FISH for both *arc* and *homer 1a*, cells containing nuclear *arc* would represent activity 5 min before brain tissue has been collected, while cells containing nuclear *homer 1a* would represent activity 30 min before tissue collection, and the presence of both types of mRNA within

the nucleus would signify a cell was active during both events (Vazdarjanova, McNaughton, Barnes, Worley, & Guzowski, 2002). This double-staining variation of the catFISH technique makes it easier to detect cells activated twice on two separate occasions, as sometimes it might be difficult to distinguish between nuclear and cytoplasmic *arc* signal within the same cell. Nuclear signal appears as one or two bright dots at 20x to 60x magnification, while cytoplasmic signal is usually more diffuse and can occlude intranuclear signal especially given that the colour of the signal is the same. With two different types of mRNA being detected in two separate colour channels, such problems in detection are avoided.

Combining detection of *arc* and *homer 1a* with the logic behind catFISH allows to not only quantify the amount of activity associated with two separate events, but also to determine the overlap between the neuronal populations engaged by each event (as measured by amount of co-expressing cells). For example, when animals are exposed to the same novel environment twice, the two IEGs are expressed coincidentally in the same hippocampal neuronal population (Vazdarjanova et al., 2002), while exposing them to two distinct novel environments results in much less co-expression in hippocampal neurons (Vazdarjanova & Guzowski, 2004).

5. The aims of this thesis

A great deal of research on *c-fos* has shown that IEG expression is an important part of drug effects in the brain. Less attention has been paid to more recently discovered IEGs such as *arc* and *homer 1a.* Given that *arc* and, to some extent, *homer 1a* have proven to be involved in experience-dependent plasticity, it is worthwhile to characterise their expression in the brain following administration of heavily abused drugs such as heroin and cocaine. Some studies on the topic have already been undertaken, but most of them have studied heroin and cocaine separately, have used molecular techniques that have less than cellular resolution, and have mostly used the intraperitoneal route of administration.

The aims of this thesis were to expand on previous findings in three ways. First, we used fluorescent *in situ* hybridisation technique to quantify the expression of *arc* and *homer 1a* mRNA with a cellular (and even subcellular) resolution following non-contingent

intravenous injections of heroin and cocaine at doses self-administered by laboratory animals. Second, this allowed us to apply the logic of the catFISH technique to look for neuronal ensembles encoding for heroin and cocaine effects in the basolateral amygdala, striatum, or prefrontal cortex. Third, we applied this paradigm to a drug self-administration setup. Thus, there were three empirical questions to be answered.

First, can standard self-administration doses of heroin and cocaine elicit *arc* and *homer 1a* expression in the striatum and BLA if administered non-contingently? This empirical question is addressed in chapter 3. Cocaine self-administration doses have been shown to induce expression of *arc* following operant training with cocaine (Fumagalli et al., 2009). However, these findings cannot automatically be generalised to *homer 1a* and heroin, and it is not clear whether the effect is the result of a single dose within the self-administration session, or whether it is a cumulative effect. In addition, experimenter vs. self-administered morphine can cause different magnitude of plastic changes in the accumbens (Robinson, Gorny, Savage, & Kolb, 2002), and if IEG expression is linked to plastic changes, it is not unreasonable to expect differences there too.

Second, are there neuronal ensembles in the striatum encoding for non-contingently administered heroin and cocaine, can they be identified using *arc* and *homer* 1a as markers of activity, and are they overlapping or distinct? One Daun 02 study has shown that cocaine administration leads to IEG expression in specific accumbens neurons that encode for drug-context associations rather than random neuronal populations (Koya et al., 2009). Thus, it is reasonable to expect that there are neurons that will respond repeatedly to cocaine when the context is held constant, at least in the accumbens. Whether this is the case for heroin remains an open empirical question, and it is addressed in the study described in chapter 4.

Finally, can findings from non-contingent drug administration experiments be generalised to the case of self-administration. This question was addressed in the study described in chapter 5.

Chapter 2 – General methods

To avoid repetition, identical aspects of the methodology used for all experiments reported further on are presented here. Additional materials and procedures and/or alterations to procedures are described in the corresponding chapters wherever necessary.

Subjects

Male Sprague-Dawley rats were used in all of the reported experiments. The animals were supplied by ENVIGO (Harlan) breeding facilities in Italy and the Netherlands. Animals weighed 250-275g upon arrival and were tested at a weight of 300-375 g. The rats were housed and tested in a temperature- and humidity-controlled room (21±1°C, 50%) with a reversed 12 h light cycle (lights on at 7:00 pm). Food and water were provided *ad libitum* except during testing sessions and self-administration training. Initially, they were housed three per cage, then, after the implantation of intravenous catheters, the animals were housed individually. All regulated procedures were carried out in accordance with the A(SP)A 2013.

Materials

Housing cages. Following i.v. catheter surgery, rats were housed in cages measuring 40 x 22 x 22 cm in length, width and height, respectively. The cages had opaque plastic bottom and a removable metal cage top with slots for water and food. Sawdust bedding and shredded paper nesting were provided at all times.

Operant chambers. Most of the testing sessions were carried out in operant chambers (PRS Italia). These chambers measured 30 x 30 x 30 cm, had a wire mesh floor, two metal walls and a transparent Plexiglas front and back walls. Each chamber was placed in a sound-insulating wooden cabinet and was equipped with two retractable levers with white LED lights placed on the moving part of the levers. These were used only in self-administration sessions. A counterbalanced metal arm was attached to each chamber

which supported the spring and infusion line attached to the animals' catheter for drug delivery. The infusion line was connected to a remotely controlled infusion pump.

RNAscope®. All *in situ* hybridisation and catFISH procedures were carried out using commercially available kits from RNAscope (Advanced Cell Diagnostics, ACDbio). Currently, many probes targeting well-studied genes and mRNA sequences are commercially available, and the RNAscope brand has provided a significant improvement to the quality of the signal coming from fluorescent probes. A common issue with FISH is that probes are never 100% specific in binding to their target and can often produce background signal. This is most often the result of binding to partially complementary sequences. RNAscope overcomes this issue by using several separate stages of target detection and signal amplification (fig. 2.1., p. 39).

First of all, each RNAscope probes consist of 20 separate Z-shaped nucleic acid sequences. The lower part of those Z-shaped elements is complementary to a part of the target sequence such that there will always be two Z-probes that can bind next to each other (a Z-pair). The intermediate part is a spacer sequence and the top part of the Z-probe serves as one half of a binding site which will be bound by a pre-amplifier molecule via a hybridization process. The pre-amplifier molecule can only bind a site formed by a complete Z-pair. If only one Z-probe has bound non-specifically, amplification will not occur. Pre-amplifier molecules bound to a Z-pair are then bound by an amplifier molecule which in turn is bound by a fluorescent label. The Z-pair binding required for amplification provides very high specificity, while the amplification steps provide signal strength such that a single mRNA molecule can be visualised using standard fluorescence microscopy.

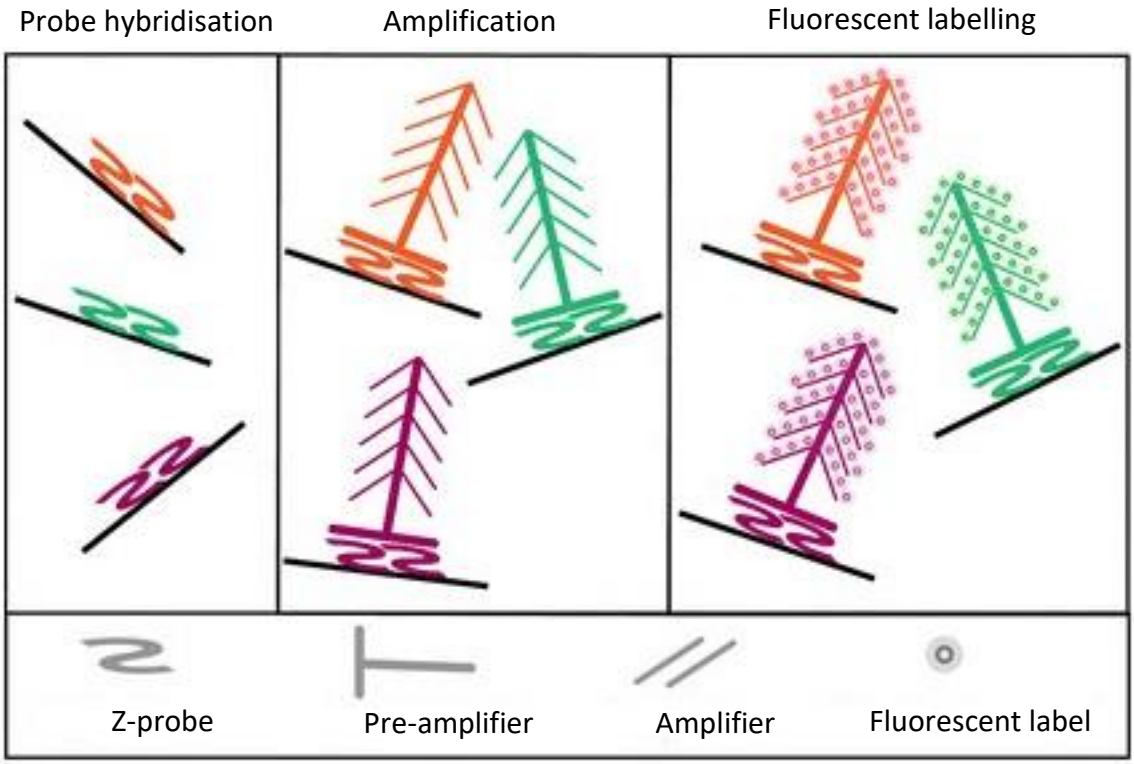

Fig. 2.1. RNAscope probe binding and signal amplification. Black lines on schematic represent target mRNA.

Arc and *h1a* hybridization probes (ACDbio, cat. No. 317071-C2 & 433261, respectively) were hybridized to fresh frozen brain coronal sections sliced on a Leica CM1900 cryostat. The signal was amplified with an RNAscope® Multiplex Fluorescent Reagent Kit (ACDbio, cat. No. 320850). Fluorescent signal was detected using a Zeiss Axioskop 2 plus epifluorescent microscope, and images were acquired using an Axiovision software (Zeiss).

The *arc* probe targeted the region spanning 1519-2621 base pairs of the *arc* gene mRNA, accession No. NM_019361.1.

The *h1a* probe targeted the 3' untranslated region of the *homer 1a* (*h1a*) gene mRNA, spanning 5001-5625 base pairs, accession No. U92079.1.

Procedures

Intravenous catheter surgery. All rats were acclimatized to the housing facilities for a period of 1 week, during which they were handled gently every other day. Following

acclimatization, all rats underwent intravenous catheter surgery. They were anesthetised with 110 mg/kg ketamine (Anesketin) and 12 mg/kg xylazine (Rompun). A small incision was made on the top of the head, the skull exposed, and four surgical screws were positioned around bregma. Another small incision was made just above the right clavicle to expose the jugular vein. The vein was separated from surrounding tissue and a small incision was made into it to allow for insertion of the catheter. Catheters consisted of a 10.5 cm piece of silastic tubing with a silicon bead at 3.2 cm from the end entering the right jugular vein. The catheter was secured to the vein with surgical thread on both sides of the bead. Then, the free end of the catheter was passed subcutaneously, exiting through the skin at the nape of the neck, and was attached to an L-shaped cannula, and secured to the animals' head via the surgical screws and dental cement. Following surgery, animals were left to recover for 7 days. Analgesic in the form of Metacam oral suspension (2 mg/kg) was given immediately after, and on the day after the surgery. Catheters were flushed daily with saline.

In situ hybridization. Coronal sections of brain tissue were mounted on Superfrost Plus slides, the coordinates and thickness of the slices is reported separately for each experiment in the corresponding chapters. On the first day of staining, the slides with sections were incubated in 10% neutral buffered formalin (Sigma, cat. No. HT501128-4L) for 20 min at 4°C, followed by 2x1 min washes in 1xPBS, and then serial dehydration in ascending concentrations of ethanol – 50%, 70%, and 2x 100%, 5 min incubation in each. Following this, tissue was stored in 100% ethanol overnight. On day 2, the tissue was air dried, and then incubated with protease for 20 minutes, followed by 2x1 min washes in dH20. Protease, probe and amplifier solutions were supplied by ACDbio as part of a commercially available RNAscope assay kit. The *arc* and *h1a* probes were then applied (50 µl per section), and the sections were incubated for 2h at 40°C in a humidity-controlled oven. After incubation with the probes, the signal was amplified at 4 separate stages with 15 min, 30 min, 15 min and 30 min of incubation in between (respectively) at 40°C in the hybridization oven. The probe and amplifier solutions were applied to the sections with the help of a hydrophobic pen barrier. There were 2x2 min washes in wash buffer after each incubation (including after probe hybridization). Finally, sections were

coverslipped and counterstained with DAPI mounting medium (Vector Laboratories, cat. No. H-1500) and left at 4°C overnight.

Image acquisition and analysis. Regardless of brain area studied, greyscale images were taken from both hemispheres of 2 adjacent sections for each animal at 20x magnification. This yielded 4 images per animal per brain area. Final counts of DAPI-, *arc*-, and *h1a*-positive nuclei were averaged from these 4 images for each animal and each brain area.

The resulting eight-bit images measured 1300 x 1030 pixels and represented a region of interest (ROI) of 700 x 550 µm. These images were analysed using the RIO Montpelier extension of the ImageJ software (Baecker & Travo, 2006). Greyscale images were analysed separately for each channel: DAPI, Alexa 488 (H1a) and Cy3 (Arc).

First, each DAPI image was analysed by applying a Gaussian blur filter (sigma = 2), then a "rolling ball" background subtraction algorithm (ball radius = 20), followed by the application of the default automatic global thresholding algorithm. This yielded a binary image which was then used to count objects selected on the basis of their size and circularity using the "analyse particles" function of ImageJ. The size criterion was set to 0.0045-0.045 square inches, and the circularity - to 0.7-1.00. This analysis resulted in a mask image containing only object fulfilling the aforementioned criteria. This image was used to estimate the total number of DAPI-positive nuclei, and was merged with the Alexa 488 and Cy3 images for counting of nuclear mRNA signal.

The images from the Alexa 488 and Cy3 channels were first adjusted for brightness so that the most visible signal was that coming from nuclear staining for *arc* and *h1a*. This was defined as any signal representing one or two bright dots close to each other, as opposed to cytoplasmic signal which is less bright and more diffused (Guzowski et al., 1999). A global threshold was then applied to the images (default algorithm), and the "analyse particles" function was used again to select only objects of 4-90 square pixels, and to create a mask of the images showing only the defined particles. These masks of the *h1a* and *arc* images were then merged with the DAPI mask image, and only those particles which coincided with DAPI-stained nuclei were counted.

Blinding procedures. All rats were assigned a number upon arrival to the animal housing/testing facilities. Following intravenous catheter surgery, rats were assigned to experimental treatment groups randomly, and the treatment they received was associated to their number by recording it on an Excel spreadsheet. This sheet was stored separately from spreadsheets containing behavioural and *in situ* data associated with the number of each rat. Thus, the video recordings of behaviour and microscopy image data analysis was carried out by experimenters blind to the experimental treatment of the animals, and the group membership of each animal was revealed only for the sake of statistical analyses.

Statistical analyses. All statistical analyses were carried out using the SPSS software. In all cases where between-group comparisons were made, the following procedure was used. First, a Shapiro-Wilk test was used to assess normal distribution of scores within each group. If the assumption for normal distribution of scores was met, a Levene's test was run to check for homogeneity of variance across groups. If this assumption was met as well, a one-way ANOVA was run to test for a significant main effect of treatment. Where there was more than one dependent variable (e.g. *arc*- and *h1a*-positive nuclei, single labelling analyses, chapters 3, 4 and 5), multiple one-way ANOVAs were run rather than factorial ANOVAs. This was done because the comparisons of interest in those cases were between treatment groups for each IEG in isolation, rather than main effects of treatment on average IEG expression levels (averaging *arc* and *h1a*), which a factorial ANOVA provides. Although in theory it is possible to obtain the former kind of information from a factorial ANOVA, the SPSS set-up does not allow it without the use of syntax. It was decided that such analysis does not provide a substantial advantage over a one-way ANOVA.

Provided that a one-way ANOVA revealed a significant difference between treatment groups, either Gabriel *post-hoc* tests, or Dunnett's tests were run to look for specific between-group differences. Gabriel's test is adequate when the assumption for homogeneity of variance is met, but group sizes differ (Field, 2017). However, the SPSS module for this test does not allow specific comparisons, but rather all possible group comparisons are made which results in loss of power. Thus, the test was used when all comparisons were of interest (e.g. in ch. 3, when the timing profile of IEG expression

was being established, and it was necessary to compare all time groups; also ch. 4, comparison of overlap measures across treatment groups). When only specific comparison was necessary, Dunnett's tests were chosen because SPSS allows for comparisons to be made only relative to a single reference group which serves as a control. This was suitable for single-labelling analyses in chapter 4, where it was sufficient to show that *arc* and *homer 1a* expression increase following drug treatment relative to saline.

For between-group comparisons where scores were not normally distributed in at least one group, Kruskal-Wallis tests were run instead of one-way ANOVAs. This was done because ANOVA is particularly sensitive to normality violations (Field, 2017). Provided that the Kruskal-Wallis test yielded a significant result, differences between particular groups were assessed using the Dunn-Bonferroni tests or Mann-Whitney tests, applying a Bonferroni correction. Similarly to the case of ANOVA, the Dunn-Bonferroni tests are included in the SPSS Kruskal-Wallis module, and are run for all possible comparisons. When only specific comparisons were necessary, the Mann-Whitney tests were used to avoid loss of statistical power.

If the normality assumption was met, but homogeneity of variance was violated, a Welch test was carried out in conjunction with a one-way ANOVA. If the Welch tests yielded significant result, it was followed up by Games-Howell post-hoc tests, if all between-group comparisons were of interest, or by Mann-Whitney tests (Bonferroni correction), if only some comparisons were made (following the logic explained above). The Games-Howell *post-hoc* tests are appropriate in cases where heterogeneity of variance across conditions is present (Field, 2017).

For within-group comparisons (e.g. self-administration data, ch. 5), the same logic was followed, using Friedman's ANOVA as the equivalent of the Kruskal-Wallis tests for within-group comparisons. Specific group comparisons were done using Wilcoxon's tests. None of the data for within-subject comparisons met the assumptions for a parametric ANOVA.

Where only two groups were compared (e.g. latency to lever press, ch.5), a *t*-test was used provided that the normality assumption was met, and the value of the *t*-test was

reported taking into account the result from Levene's test (SPSS includes it in the t-test analysis automatically and provides a *p*-value for each possible outcome).

Finally, categorical data was assessed using Fisher's exact tests due to small group sizes which make the use of chi-square tests problematic (Field, 2017). A Bonferroni correction was applied to correct for multiple comparisons.

Chapter 3 – Temporal profile of *homer 1a* and *arc* expression following a single intravenous injection of heroin or cocaine

Introduction

The aim of the experiment described in this chapter was to address several methodological issues surrounding the use of catFISH to study acute effects of heroin and cocaine. Previous studies have used the technique to identify ensembles activated after exposure to an environment or a discrete cue, where stimulus presentation is easily controlled by the experimenter (Grosso et al., 2015; Vazdarjanova & Guzowski, 2004). Drug effects are not so easily controlled in terms of either duration or magnitude. Thus, the main issue arising from using catFISH with drug stimuli is the issue of stimulus onset and offset. Additionally, drug-induced IEG expression patterns may differ between IEGs: for example, cocaine increases *zif268* but not *homer 1a* expression in the cortex (Unal et al., 2009). Such differences would inevitably affect measures of overlap with catFISH. These issues were addressed by choosing an appropriate dose, route of drug administration, and by measuring IEG expression over time and in several brain areas.

To reiterate briefly, catFISH is used to assess whether the same population of neurons respond to two separate stimuli using IEGs as markers of activity. The logic behind the technique requires that, timewise, the peak of nuclear expression of the IEGs used coincides with the exposure to the two stimuli of interest. Considering that *arc* and *homer 1a* were used as markers of activity, and *arc* peaks at 5 min following cell activation, while *homer 1a* peaks 30 min after activation, the stimuli of interest must be presented around 30 and 5 min before brain tissue collection, respectively. Then, cells activated *only* by the first stimulus would express *homer 1a*, but not *arc*. On the other hand, cells activated by both stimuli would express both *arc* and *homer 1a*.

However, if the first stimulus were to influence cell activity for too long (e.g. >20 min), then there is a chance that *arc* would be expressed in cells activated by that stimulus as

well. In that case, it would be impossible to distinguish cells activated by the first stimulus only, or by both stimuli. This constitutes the importance of properly controlled stimulus offset.

Alternatively, the first stimulus may have its effect on cellular activity with a delay. Then, there will not be enough time for *homer 1a* to be expressed before tissue is collected for analysis. In that case, cells activated by the first stimulus will not be detectable. This constitutes the importance of properly controlled stimulus onset.

When the stimuli of interest are exposure to a particular context or a discrete cue, the definitions of stimulus on- and offset is arguably straightforward, and control over them is easier. The experimenter can place an animal in a novel environment and remove it at a precise moment in time. Light cues and tones can be switched on and off at a precise time. However, when the stimuli of interest are systemic administrations of a drug, the issues of on- and offset become more apparent. The *onset* of the acute drug effect in this study was defined as the moment when the drug is binding to its target in the brain and causing changes in neuronal activity. Thus, the *onset* is not necessarily the moment in which the drug is injected (which is what the experimenter has direct control over), but when it reaches the brain. Even more so, the time it takes for the drug to stop acting at its target in the brain, the *offset*, is also out of the direct control of the experimenter. For example, there is always the possibility that if heroin was injected first, and cocaine was injected 25 min later, then cells responding to heroin might still be doing so at the time of the cocaine injection.

Finally, there is the issue of whether both heroin and cocaine can trigger the expression of both IEGs, *arc* and *homer 1a,* within the same brain area. Although many IEGs are expressed in response to cellular activity, they do not necessarily overlap completely in their pattern of expression between brain areas, or between tasks and stimuli that trigger their expression (Guzowski et al., 2001). If heroin or cocaine injections trigger the expression of only *arc* or *homer 1a* but not both, then it would be impossible to measure the degree of overlap between neuronal populations engaged by the two drugs: one of the populations will be technically undetectable.

There is some empirical evidence to suggest that cocaine administration can induce *arc* and *homer 1a* expression across the striatum. Fosnaugh et al. have shown that a single cocaine i.p. injection induces *arc* expression in the striatum, an effect mediated through dopaminergic receptors (Fosnaugh, Bhat, Yamagata, Worley, & Baraban, 1995). A brief history of i.v. self-administration (a single 2h session) can also elevate *arc* levels in the striatum (Fumagalli et al., 2009). Finally, it has been shown that cocaine-induced *arc* expression is dose-dependent, with higher doses leading to higher expression in the striatum, PFC and hippocampus, but the effect is dependent on drug-treatment history (Fumagalli et al., 2006). Single and repeated i.p. injections of cocaine are also capable of inducing *homer 1a* expression in the striatum, and the magnitude of the effect decreases with repeated treatment (Unal et al., 2009).

In contrast to cocaine, the literature on the acute effects of heroin administration on *arc* and *homer 1a* is scarce. In fact, there are no known studies to date that directly address the topic. The only studies pointing towards the possibility of opiate effects on *arc* and *homer 1a* expression have used morphine. One of them has shown elevated *arc* mRNA and protein levels in the rat striatum after repeated exposure to relatively high morphine doses (Marie-Claire, Courtin, Roques, & Noble, 2004). Another study has shown *arc* expression in mouse striatum following single morphine injections, an effect dependent on direct action on the μ-receptor (Ziolkowska, Urbanski, Wawrzczak-Bargiela, Bilecki, & Przewlocki, 2005). These findings suggest that *arc* expression in response to morphine action on the μ-receptor might reflect cellular processes that are not a direct consequence of cellular activity. However, it must be noted that the effect was evident 3 hours after morphine treatment, so any immediate *arc* response is still likely to be indicative of cellular activity. Such immediate response to opiates has not yet been demonstrated. Whether this is because of a lack of such a response or because studies up to date were not designed to look for it, remains an open empirical question.

In summary, existing literature suggests that cocaine treatment induces *arc* and *homer 1a* expression in the striatum, and an opiate drug (morphine) acting through the μ-receptor can also induce *arc* expression. However, one common feature between all studies cited is that they used almost exclusively the intraperitoneal route of administration, and molecular techniques that do not allow for high temporal and

spatial resolution of mRNA expression such as real-time PCR. Thus, although these studies suggests that *homer 1a* and *arc* can be used to identify neurons activated by cocaine and morphine, they do not resolve all the methodological issues associated with catFISH.

To address the issues of stimulus onset, offset and drug-class differences in IEG expression, the study presented in this chapter exposed rats to a single intravenous injection of either heroin or cocaine. Then, the amount of *arc*- and *homer 1a*-positive nuclei in the striatum and basolateral amygdala of these animal was quantified at several different time points following injection via standard fluorescence in situ hybridisation.

The intravenous route of administration was chosen because it allows for a very quick entry of the drug into the brain. As a consequence, the onset of the drug effect becomes almost synonymous with the onset of the drug injection. The clearance rate is also relatively quick. For cocaine, the peak levels of drug concertation in the brain are reached within 2.5 min after injection and decrease steadily afterwards. Even after a dose of 7.5 mg/kg i.v. cocaine is cleared out of the brain within 1 hour. The alternative, intraperitoneal injections, have a slower onset, reach a lower and delayed peak concentration in the brain (about 30 min after injection), and have a slower rate of clearance (Pan, Menacherry, & Justice, 1991). Thus, i.p. injections would have presented with both stimulus onset and offset issues. The picture gets more complicated with heroin due to it having several metabolites active at the μ-receptor. Nevertheless, it is known that even at i.v. doses as high as 1.28 mg/kg, heroin and 6-MAM are cleared form the brain within 1 hour (Gottas et al., 2013). In addition, it was intended to eventually incorporate a catFISH protocol into the rat self-administration paradigm, which also uses the i.v. route, so using the same route in this preliminary study was preferable for that reason as well. The intention to use catFISH with self-administration was also how the testing doses were chosen. Previous studies by Badiani et al. have shown that doses of 400 μg/kg/infusion cocaine and 50 μg/kg/infusion heroin maintain self-administration and have behavioural effects that differ between residents and non-residents (Avvisati et al., 2016; Caprioli et al., 2009; Caprioli et al., 2008; Caprioli, Paolone, et al., 2007; Montanari et al., 2015). It was of interest whether a single treatment with these doses

would be able to induce IEG expression acutely, and, if so, whether the neuronal populations expressing IEGs would differ between heroin and cocaine (a question to be directly addressed in follow-up experiments). It was expected that these doses would be low enough so that the drugs would be cleared quickly, but high enough to induce IEG expression.

The Nacc core and dorsomedial striatum (DMS) were chosen as two parts of the striatum known to be involved in reward and learning processes relevant to drugs of abuse (Di Ciano & Everitt, 2004b; Eagle et al., 2011). It is also known that opiates and cocaine elicit IEG expression in these areas (Harlan & Garcia, 1998). The BLA was studied since it is a key structure in the processing of associative learning, but has different roles in heroin and cocaine second-order reinforcement (Alderson, Robbins, & Everitt, 2000), and it is anatomically connected to the Nacc core and DMS (as discussed in ch.1, see fig. 1.1, p. 10). In addition, preliminary analysis of imaging data revealed that the central amygdala and Nacc shell did not exhibit levels of IEG expression sufficiently high to be analysed quantitatively.

By measuring both *arc* and *homer 1a* nuclear expression at different time points after an i.v. injection, it was possible to empirically determine their temporal profile of expression following drug administration. Based on previous findings on heroin and cocaine pharmacokinetics, and their ability to induce IEG expression in the brain, it was expected that heroin and cocaine administration under these conditions will produce a brief, temporary increase in IEG expression. This brief increase was expected to peak around 8 min following drug administration for *arc*, while for *homer 1a (h1a)* the peak was expected 25-35 min following injection. These predictions were based on previous observations with novel environment and cue-induced IEG expression in catFISH studies.

Methods

Animals

The rats used in this experiment, *n* = 40, were tested at a weight of 350-375g. Supplier, weight upon arrival, and housing conditions were as described in general methods.

Test chambers and drugs

The test chambers were the same as the housing cages described in general methods (i.e. the animals were housed and tested in the same cages).

Cocaine and heroin HCL were dissolved in sterile saline at a concentration of 800 μg/ml and 100 μg/ml, respectively. Injections (i.v.) were made at doses of 400 μg/kg for cocaine and 50 μg/kg for heroin, so that 0.5 ml of drug solution corresponded to the dose for 1kg. Thus, the volume of each injection was between 0.15 and 0.19 ml. Saline treated animals received the same volume of saline without any heroin or cocaine dissolved in it. Drug infusions were delivered by hand using a 0.5 ml syringe attached to the catheter cannulas via a piece of silicone tubing. Note that due to this method of drug delivery, the speed of drug infusions was not strictly controlled in this experiment. However, it is unlikely that the duration of each infusion exceeded 3 sec, because the total volume infused was not larger than 0.2 ml.

Design

The design was mixed, with drug treatment (heroin vs. cocaine) and time of sacrifice (0, 8, 16, 25, and 35 min after injection) as between-subject independent variables (IVs). Staining was a within-subject IV, so that each rat was used to measure both *h1a* and *arc* signal at the time point at which it was sacrificed. The DV was the percentage of DAPI-stained nuclei co-expressing *arc* or *h1a* intranuclear signal.

For the comparison of drug effects with saline, the within-subject IV was brain area, the between-subject IV was drug treatment (heroin, cocaine and saline), and the DV was only the percentage of DAPI-positive nuclei co-expressing *arc*.

Procedure

Intravenous catheter surgery and drug treatment. All rats underwent intravenous catheter surgery as described in general methods. Following the recovery period, each rat was assigned to one of 11 experimental conditions. Some rats received an i.v injection of 400 μg/kg cocaine (n = 18) and some of them were euthanised a few seconds later (0 min, n = 3), 8 min (n = 3), 16 min (n = 4), 25 min (n = 4) or 35 min later (n = 4). Another group of rats received a 50 μg/kg injection of heroin (n = 19), and different

number of them were euthanised at equivalent time points (*n* = 3 for the 0 and 8 min groups, *n* = 4 for the 16 and 25 min groups, and *n* = 5 for the 35 min group). A final group was treated with saline and euthanised 8 min later (*n* = 3). Animals were euthanised using a 0.15 ml i.v. injection of pentobarbital. Immediately after the pentobarbital injection, rats were decapitated, their brains extracted, and fresh brain tissue was snap-frozen in a 400 ml of isopentane cooled to -50°C. The tissue was stored at -80°C for later processing.

RNAscope FISH. The RNAscope probes and reagents, the *in situ* hybridisation and image acquisition and analysis protocols were as described in general methods. Whole brains were sectioned at 16 or 20 µm thickness, with tissue collected from two main brain areas – striatum (+2.10 to +1.90 mm from bregma) and amygdala (-1.80 mm to -2.00 mm from bregma, Paxinos & Watson, 1984). These sections contained the Nacc core and DMS, and the basolateral amygdalar nucleus, respectively, where images were taken for analysis (see Appendix 1). To ensure consistency, tissue sampling was always conducted as follows. Sectioning started from the tip of the olfactory bulbs and brain sections were removed until their ventral part (the olfactory bulbs and the basal forebrain region) became attached to their dorsal part. At this point, the anterior forceps of the corpus callosum (fmi) was visible, and the lateral fissure reached halfway to the midline. This was defined as the +3.70 mm from bregma rostrocaudal level (fig. 3.1, next page). At this point, either a 100 or 80 sections were removed (when sectioning at 16 µm and 20 µm, respectively) to reach approx. +2.10 mm from bregma. Then a few sections were removed if necessary (no more than 5) to reach the rostrocaudal level where the rostral end of the lateral ventricles, the anterior commissure, the fmi and the striatum were all visible in the same coronal plane. The fmi formed an inverted C-shape. This was defined as +2.00 mm from bregma (fig. 3.2, next page). Here, images of the accumbens core were taken by positioning the microscope field of view (MFV) so that its centre was at equal distances from the ventral end of the lateral ventricle, and from the anterior commissure. If any sections were damaged during collection, the first two intact sections were collected. Images of the DMS were taken by positioning the MFV so that its left margin coincided with the dorsal part of the lateral ventricle and the medial side of the corpus callosum. Then, brain sections were removed until the anterior commissure

crossed the midline of the brain, and was seen as a continuous bundle of white fibres as opposed to two separate white matter fibre tracts in each hemisphere. This was defined as 0.00 mm from bregma. From there, a 110 or 85 sections were removed to reach approx. -1.76 from bregma. Then, a few sections were removed if necessary to reach the rostrocaudal level where the rostral tip of the hippocampal formation was visible, but the CA1-3 regions had just begun to become distinguishable. This was defined as -1.80 mm from bregma (fig. 3.3, next page). There, the white matter tract of the posterior corpus callosum was followed in ventrolateral direction to reach its tip. The centre of the MFV was positioned slightly below that tip, where images of BLA were taken.

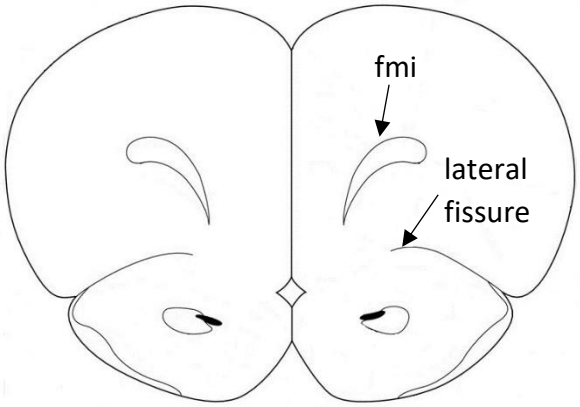

Fig. 3.1. Schematic representation of the rostrocaudal level defined as +3.70 mm from bregma during sectioning. The anterior forceps of the corpus callosum (fmi) is just visible, and the lateral fissure extends only halfway to the midline. The olfactory bulbs are attached to the ventral prefrontal cortex. This schematic is an approximation.

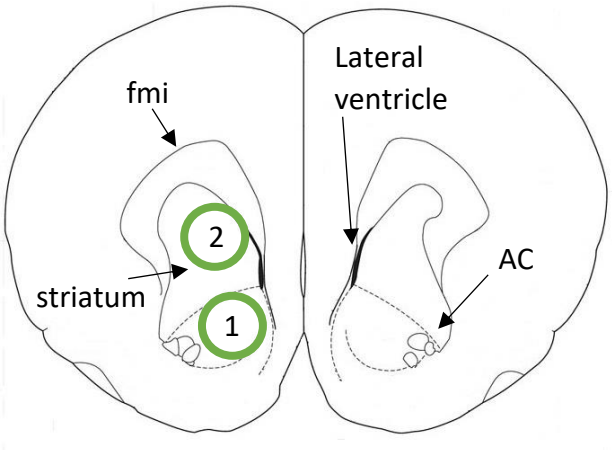

Fig. 3.2. Schematic representation of the rostrocaudal level defined as +2.00 mm from bregma. The Lateral ventricles, anterior commissure (AC), fmi and striatum are visible on one section. Green circles represent ROI where images of the Nacc core (1) and DMS (2) were taken. This schematic is an approximation.

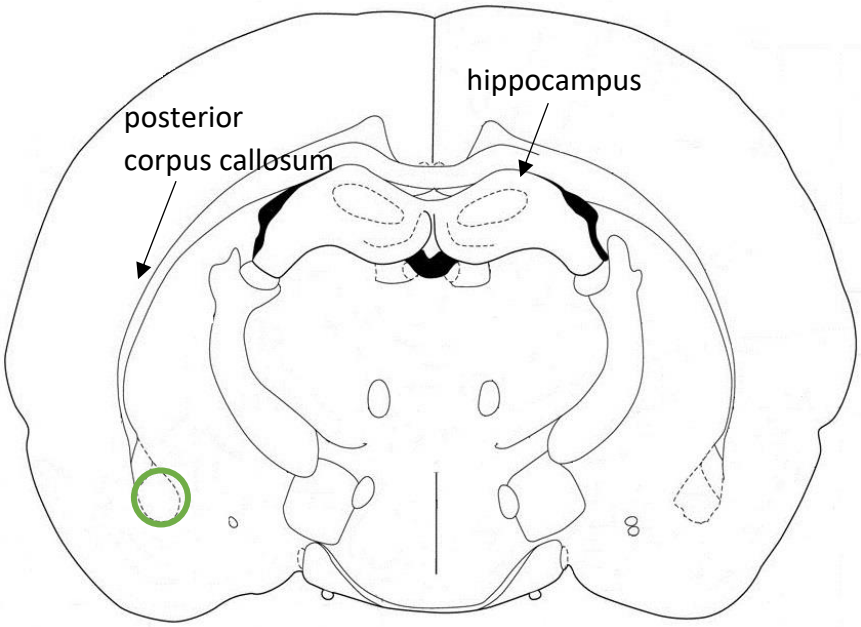

Fig. 3.3. Schematic representation of the rostrocaudal level defined as -1.80 mm from bregma during sectioning. The anterior tip of the hippocampus is visible, and the posterior corpus callosum is visible as an arc that extends ventrally towards the BLA (green circle), where images were taken, from both hemispheres.

Results

Nacc core – cocaine

Figure 3.4 (next page) shows the amount of *arc*- and *h1a*-positive nuclei in the Nacc core as a function of time following an i.v. injection of cocaine. The highest amount of *arc*-positive nuclei was found in the 8 min group (*M* = 3.55%, *SE* = 0.51), followed by the 0 min, 16 min, 35 min and the 25 min groups (*M* = 2.41%, *SE* = 0.13; *M* = 2.35%, *SE* = 0.34; *M* = 1.66%, *SE* = 0.23 and *M* = 1.45%, *SE* = 0.33, respectively). For *h1a*, the highest amount was found in the 35 min group (*M* = 3.98%, *SE* = 0.78), followed by the 25 min, 16 min, 0 min and 8 min groups (*M* = 2.75%, *SE* = 0.35; *M* = 2.20%, *SE* = 0.14; *M* = 1.50%, *SE* = 0.47 and *M* = 1.38%, *SE* = 0.27, respectively).

Shapiro-Wilk tests revealed normal distributions of the scores for *arc*-positive nuclei following a cocaine injection in all 5 time groups. Levene's statistic was also non-significant showing homogeneity of variance across groups, $F(4,13) = 1.02$, $p = .433$. A one-way ANOVA showed a significant effect of time, $F(4,13) = 6.09$, $p = .005$. Gabriel's *post hoc* tests revealed that the 8 min group had significantly higher amount of *arc*-positive nuclei relative to the 25 and 35 min groups.

Shapiro-Wilk tests for the scores of *h1a*-positive nuclei also revealed normal distributions in all conditions, and Levene's test showed homogeneity of variance, $F(4,13) = 2.27$, $p = .117$. A one-way ANOVA indicated a significant main effect of time, $F(4,13) = 4.87$, $p = .013$. According to Gabriel's post-hoc tests the 35 min group had significantly more *h1a*-positve nuclei than the 0 and 8 min groups.

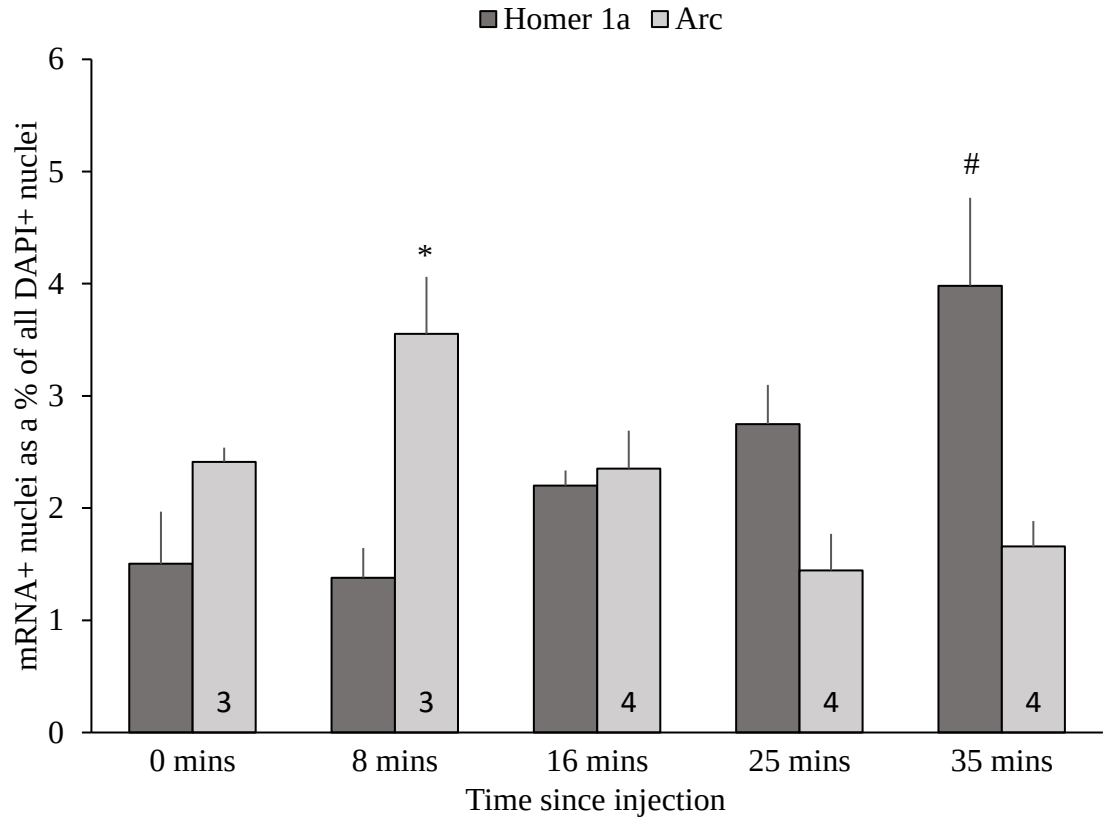

Fig. 3.4. mRNA expression in the **Nacc core** following an i.v. injection of **cocaine** as a function of time. Error bars represent ± 1SE. * significantly different from the 25 and 35 min groups. # significantly different from the 0 and 8 min groups. Numbers on bars refer to *n* for each time group.

Nacc core – heroin

The IEG expression in the Nacc core following an i.v. injection of heroin (fig. 3.5, next page) was similar to that following cocaine. On average, the highest amount of *arc*-positive nuclei was again found in the 8 min group ($M = 3.56\%$, $SE = 1.45$). This was followed by the 0 min, 25 min, 16 min and 35 min groups ($M = 2.55\%$, $SE = 0.72$; $M = 2.50\%$, $SE = 0.49$; $M = 2.18\%$, $SE = 0.58$ and $M = 1.22\%$, $SE = 0.34$, respectively). In the case of *h1a*, the 25 min group had the highest amount of mRNA-positive nuclei ($M =$

2.62%, *SE* = 0.64), followed by the 35 min, 0 min, 16 min and 8 min groups (*M* = 2.19%, *SE* = 0.58; *M* = 1.67%, *SE* = 0.33; *M* = 1.39%, *SE* = 0.5; and *M* = 1.25%, *SE* = 0.37, respectively).

Shapiro-Wilk tests revealed normal distributions of scores for *arc*-positive nuclei in all conditions except the 16 min group. A Kruskal-Wallis test revealed non-significant differences between time groups, $H(4) = 5.25$, $p = .262$.

The Shapiro-Wilk tests for *h1a*-positive nuclei scores revealed a non-normal distribution in the 35 min group. A Kruskal-Wallis test revealed no significant differences between groups $H(4) = 3.97$, $p = .41$.

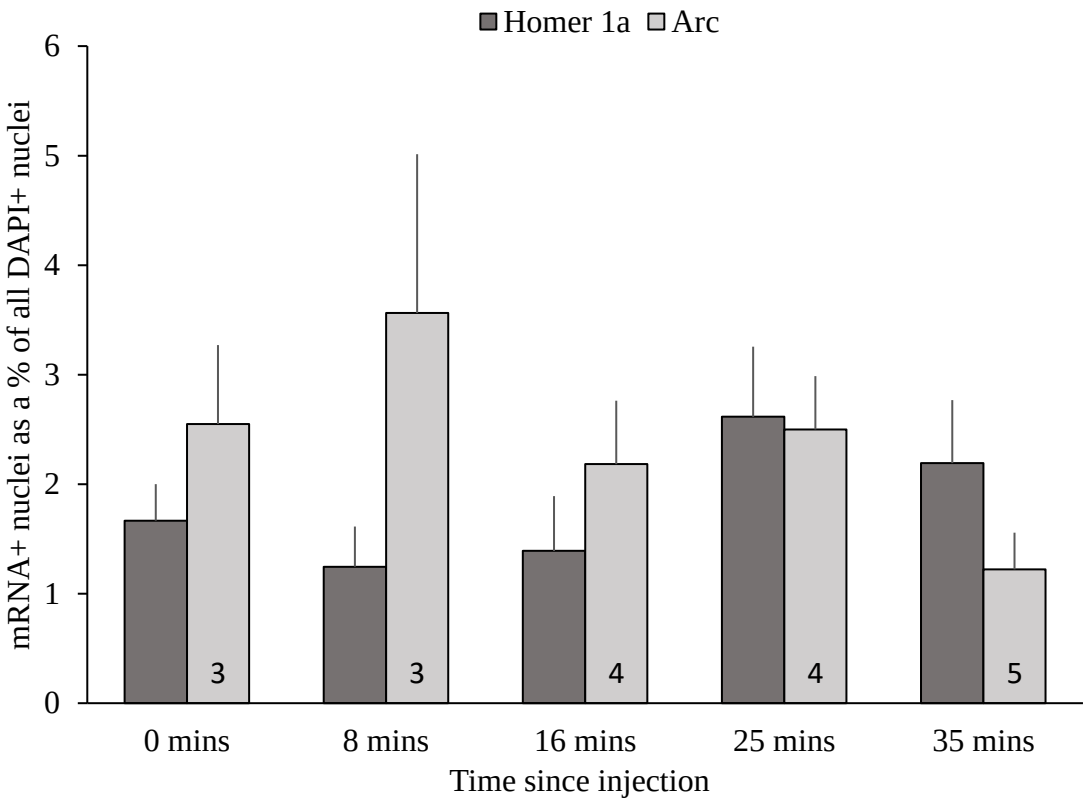

Fig. 3.5. mRNA expression in the **Nacc core** following an i.v. injection of **heroin** as a function of time. Error bars represent ± 1SE. Numbers on bars refer to *n* for each time group.

DMS – cocaine

The expression profile of *arc* an *h1a* in the DMS following an injection of cocaine was similar to that in the Nacc core, but the total amount of mRNA-positive nuclei was about twice as high (fig. 3.6, p. 57, note the range of values on the Y axis). Again, the highest

number of *arc*-positive nuclei was found in the 8 min group (*M* = 7.17%, *SE* = 0.97) followed by the 0, 16, 25 and 35 min groups (*M* = 4.26%, *SE* = 0.35; *M* = 3.52%, *SE* = 0.36; *M* = 2.39%, *SE* = 0.56 and *M* = 1.81%, *SE* = 0.25, respectively). On average, *h1a* levels were highest in the 35 min group (*M* = 9.39%, *SE* = 0.39), followed by the 25, 16, 0, and 8 min groups (*M* = 7.83%, *SE* = 0.61; *M* = 5.21%, *SE* = 0.4; *M* = 2.35%, *SE* = 0.86, and *M* = 1.9%, *SE* = 0.32).

Shapiro-Wilk tests revealed a non-normal distribution of the scores for *arc*-positive nuclei in the 25 min group. A Kruskal-Wallis test revealed a significant difference between time groups, $H(4) = 13.08$, $p = .011$. Dunn-Bonferroni tests revealed that the 8 min group had significantly more *arc*-positive nuclei than the 35 min group.

All distributions of scores for *h1a*-positive nuclei across the time groups were normal, as assessed by Shapiro-Wilk tests. Levene's statistic revealed homogenous variance across time groups $F(4,13) = 0.47$, $p = .76$. A one-way ANOVA indicated a significant effect of time, $F(4,13) = 35.97$, $p < .001$. Gabriel's *post-hoc* tests revealed that the 25 and 35 min groups had significantly more h1a-positive nuclei than all other groups, but did not differ significantly between each other. The 16 min group was significantly different from the rest of the time groups, and the 0 and 8 min groups did not differ from each other.

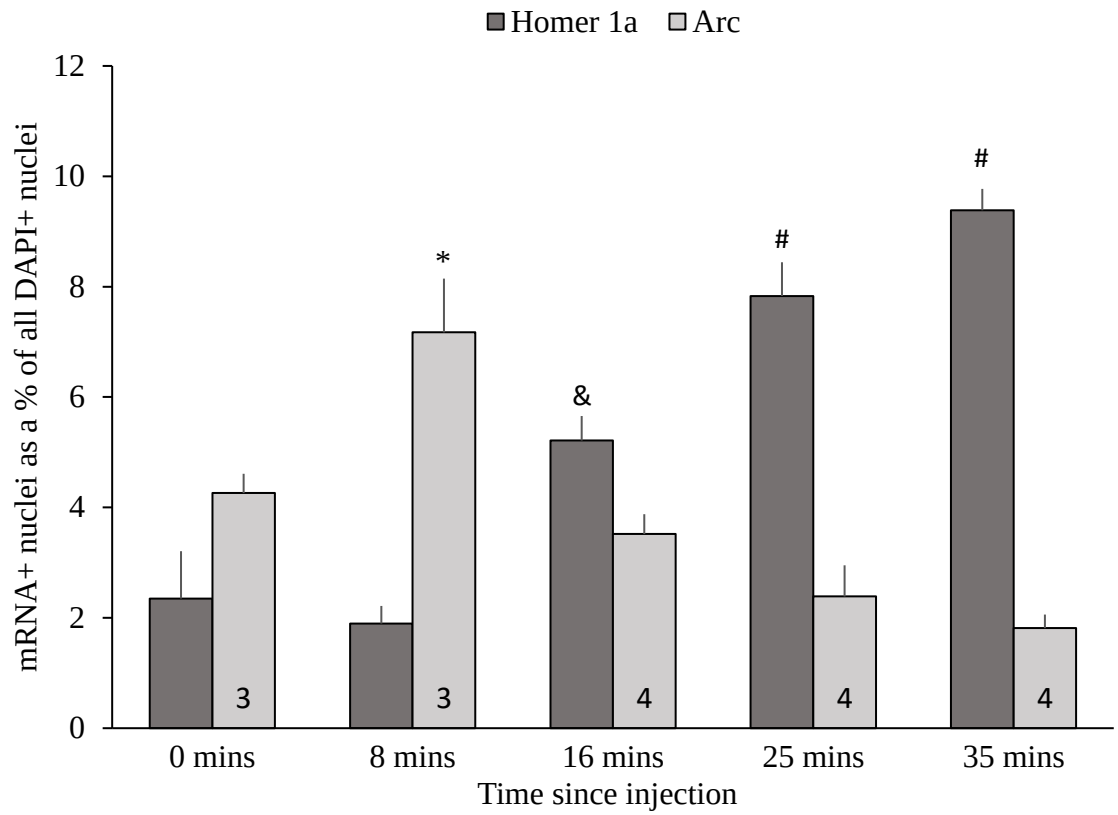

Fig. 3.6. mRNA expression in the **DMS** following an i.v. injection of **cocaine** as a function of time. Error bars represent ± 1SE. *significantly different from the 35 min group; #significantly different from the 0, 8, and 16 min groups. & - significantly different from all other time groups for *h1a*. Numbers on bars refer to *n* for each time group.

DMS – heroin

An injection of heroin seemed to produce much lower levels of IEG expression relative to cocaine (although no inferential statistics were carried out to compare the two). There was still a trend of higher amounts of *arc*-positive nuclei in the early time groups and higher amounts of *h1a*-positive nuclei in the later time groups (fig. 3.7, next page). On average, the highest amount of *arc*-positive nuclei was found in the 8 min group (*M* = 3.99%, *SE* = 0.89), followed by the 0, 25, 16, and 35 min groups (*M* = 3.39%, *SE* = 0.77; *M* = 2.37%, *SE* = 0.58; *M* = 2.10%, *SE* = 0.55, and *M* = 1.26%, *SE* = 0.42). The highest amount of *h1a*-positive nuclei was found in the 35 min group (*M* = 4.6%, *SE* = 0.63), followed by the 25, 16, 0 and 8 min groups (*M* = 4.46%, *SE* = 0.96; *M* = 3.65%, *SE* = 1.1; *M* = 2.47%, *SE* = 0.11, and *M* = 2.34%, *SE* = 0.88).

Shapiro-Wilk tests revealed a non-normal distribution of scores for *arc*-positive nuclei in the 16 min group. There was also one case of extreme score on this variable in the 35 min group as assessed by a box-plot. This case was excluded from the Kruskal-Wallis test analysis, which revealed a significant difference between groups, $H(4) = 11.17$, $p = .025$. Dunn-Bonferroni tests revealed that the 8 min group had significantly more *arc*-positive nuclei than the 35 min group.

Scores for *h1a*-positive nuclei were not normally distributed in the 25 min group. A Kruskal-Wallis test revealed no significant differences between groups for this variable, $H(4) = 4.49$, $p = .343$.

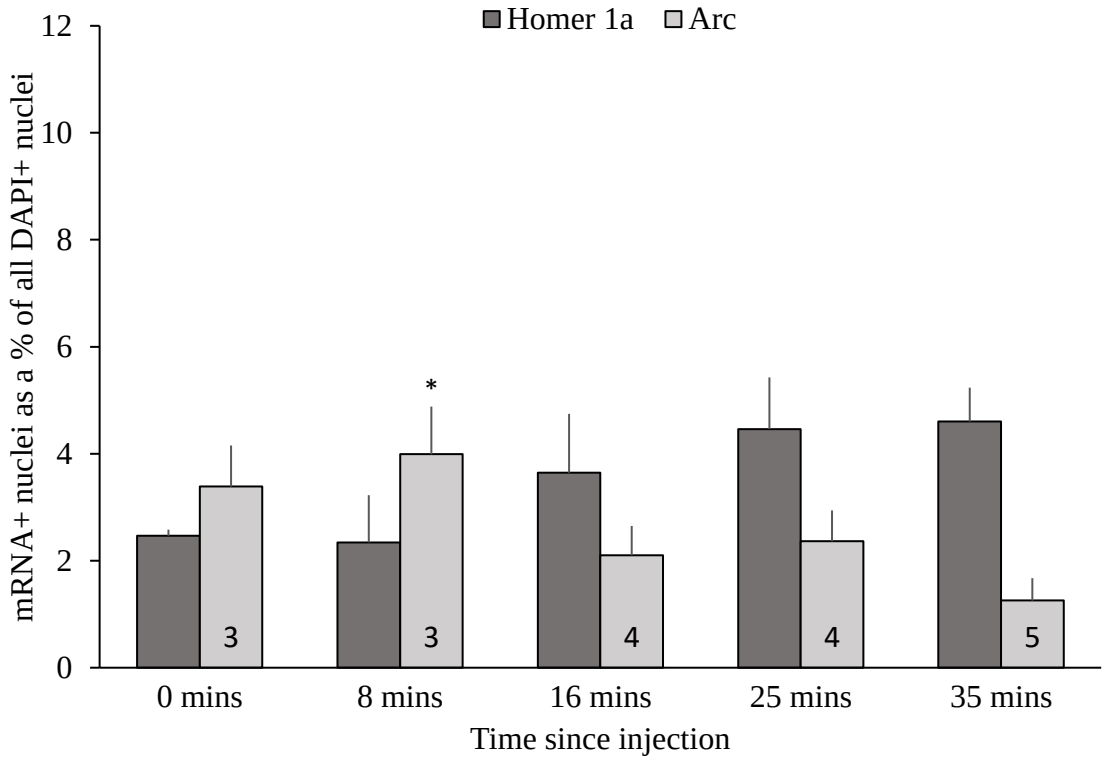

Fig. 3.7. mRNA expression in the **DMS** following an i.v. injection of **heroin** as a function of time. Error bars represent ± 1SE. *significantly different from the 35 min group. Numbers on bars refer to *n* for each time group.

BLA – cocaine

The IEG expression profile in the BLA following an injection of cocaine was somewhat different from the other two brain areas, mostly because of the high amount of *h1a*-positive nuclei in the 16 min group (fig. 3.8, next page). The highest amount of *arc*-positive nuclei was still found in the 8 min group ($M = 2.49\%$, $SE = 0.93$), followed by the

16, 0, 25 and 35 min groups (*M* = 2.14%, *SE* = 0.35; *M* = 1.75%, *SE* = 0.72; *M* = 1.64%, *SE* = 0.39, and *M* = 1.57%, *SE* = 0.53). For *h1a*, the highest amount of mRNA-expressing nuclei was found in the 16 min group (*M* = 4.21%, *SE* = 1.11), followed by the 35, 25, 8 and 0 min groups (*M* = 4.12%, *SE* = 1.45; *M* = 3.52%, *SE* = 0.32; *M* = 2.74%, *SE* = 0.97, and *M* = 2.45%, *SE* = 0.99).

Shapiro-Wilk tests revealed a normal distribution of scores for amount of *arc*-positive nuclei across time groups. Levene's statistic was also non-significant, $F(4,13) = 1.28$, $p = .327$, suggesting homogeneity of variance. A one-way ANOVA revealed no significant differences between groups, $F(4,13) = 0.45$, $p = .774$.

The distribution of scores for the amount of *h1a*-positve nuclei was also normal as assessed by Shapiro-Wilk tests. Variance was homogenous across groups, $F(4,13) = 1.19$, $p = .362$. A one-way ANOVA revealed no significant main effect of time, $F(4,13) = 0.53$, $p = .714$.

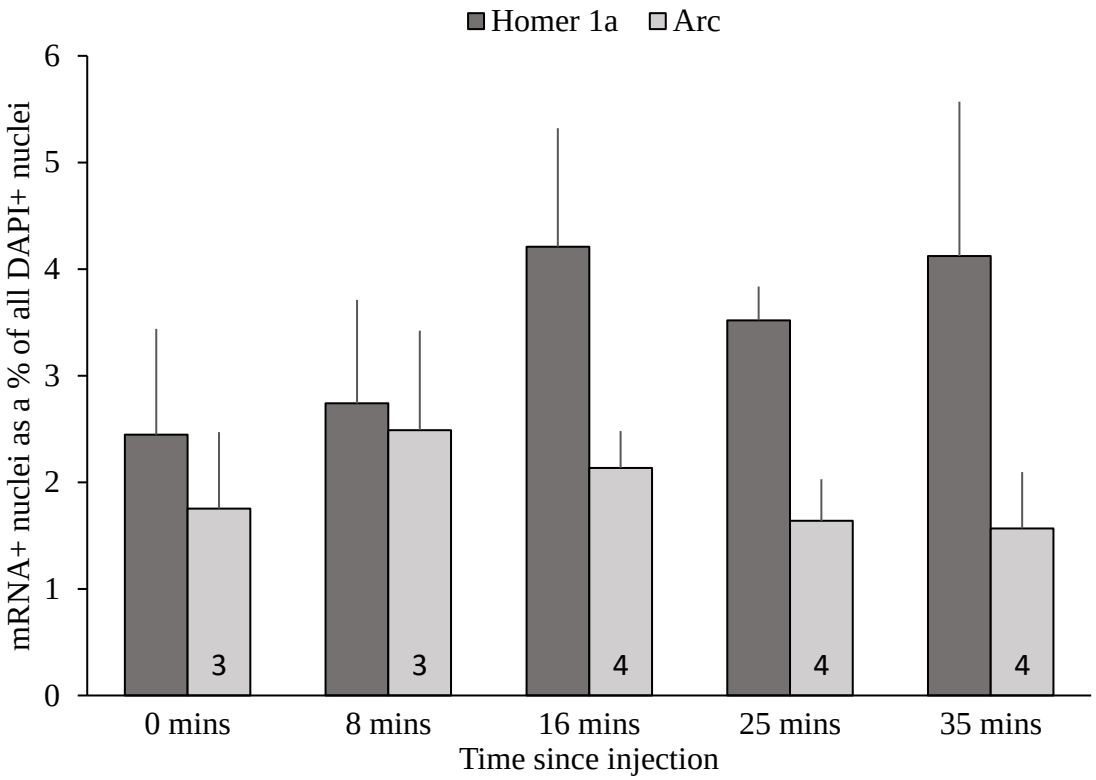

Fig. 3.8. mRNA expression in the **BLA** following an i.v. injection of **cocaine** as a function of time. Error bars represent ± 1SE. Numbers on bars refer to *n* for each time group.

BLA – heroin

Finally, following an i.v. injection of heroin, the IEG expression in the BLA was as follows (fig. 3.9, next page): the highest amount of *arc*-positive nuclei was found in the 25 min group (M = 1.76%, SE = 0.49), followed by the 16, 0, 35 and 8 min groups (M = 1.74%, SE = 0.44; M = 1.47%, SE = 0.21; M = 1.26%, SE = 0.41, and M = 1.16%, SE = 0.48). The highest amount of *h1a*-positive nuclei 16 min group (M = 4.05%, SE = 1.09), followed by the 35, 0, 25 and 8 min groups (M = 3.44%, SE = 0.46; M = 2.78%, SE = 0.28; M = 2.54%, SE = 0.26, and M = 2.47%, SE = 0.97).

Shapiro-Wilk tests revealed a normal distribution of scores for amount of *arc*-positive nuclei across time groups. Levene's statistic was also non-significant, $F(4,13) = 1.13$, $p = .381$, suggesting homogeneity of variance. A one-way ANOVA revealed no significant differences between groups, $F(4,13) = 0.38$, $p = .814$.

The distribution of scores for the amount of *h1a*-positve nuclei was also normal as assessed by Shapiro-Wilk tests. Variance was homogenous across groups, $F(4,13) = 1.44$, $p = .273$. A one-way ANOVA revealed no significant main effect of time, $F(4,13) = 0.96$, $p = .459$.

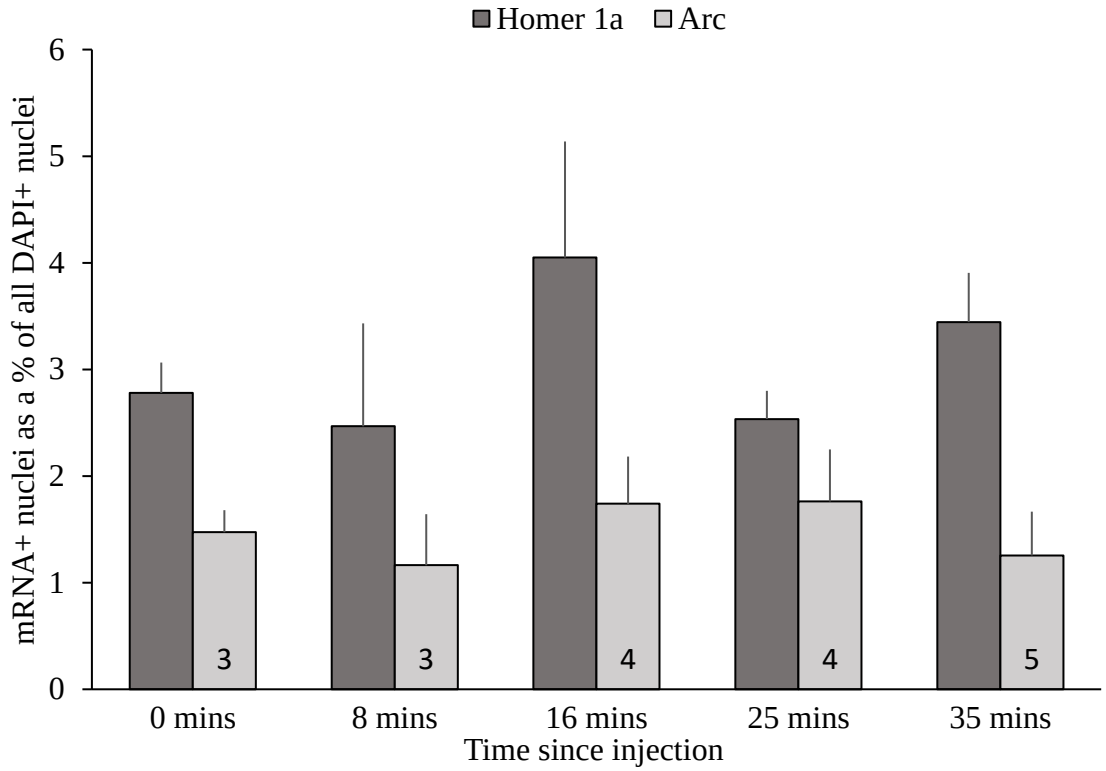

Fig. 3.9. mRNA expression in the **BLA** following an i.v. injection of **heroin** as a function of time. Error bars represent ± 1SE. Numbers on bars refer to *n* for each time group.

Arc expression relative to saline

Figure 3.10 (next page) shows the increase of *arc* expression 8 min following a drug injection relative to saline as a function of brain area. The percent of cells expressing *arc* following saline was highest in the DMS (*M* = 2.38%, *SE* = 0.18), followed by the Nacc core (*M* = 1.25%, *SE* = 0.11) and the BLA (*M* = 1.25%, *SE* = 0.19). *Arc* expression following heroin and cocaine is represented by the same data which were reported above.

Shapiro-Wilk tests revealed normal distribution of scores for *arc* expression in all conditions. Levene's statistics revealed homogenous variance in the DMS, $F(2,6) = 2.28$, $p = .184$, and a one-way ANOVA revealed significant differences between groups in this brain area, $F(2,6) = 10.07$, $p = .012$. Gabriel's *post-hoc* tests revealed that there was a difference between the cocaine and saline groups, p = .012. Levene's test was significant for the Nacc core, $F(2,6) = 7.42$, $p = .024$, indicating lack of homogeneity of variance. The one-way ANOVA was non-significant ($F(2,6) = 2.29$, $p = .187$), but the Welch tests approached significance, $F(2,2.78) = 8.98$, $p = .061$. Finally, Levene's test approached

significance in the BLA, $F(2,6) = 5.11$, $p = .051$, and the one-way ANOVA revealed no significant differences between groups for this brain area, $F(2,6) = 1.45$, $p = .307$.

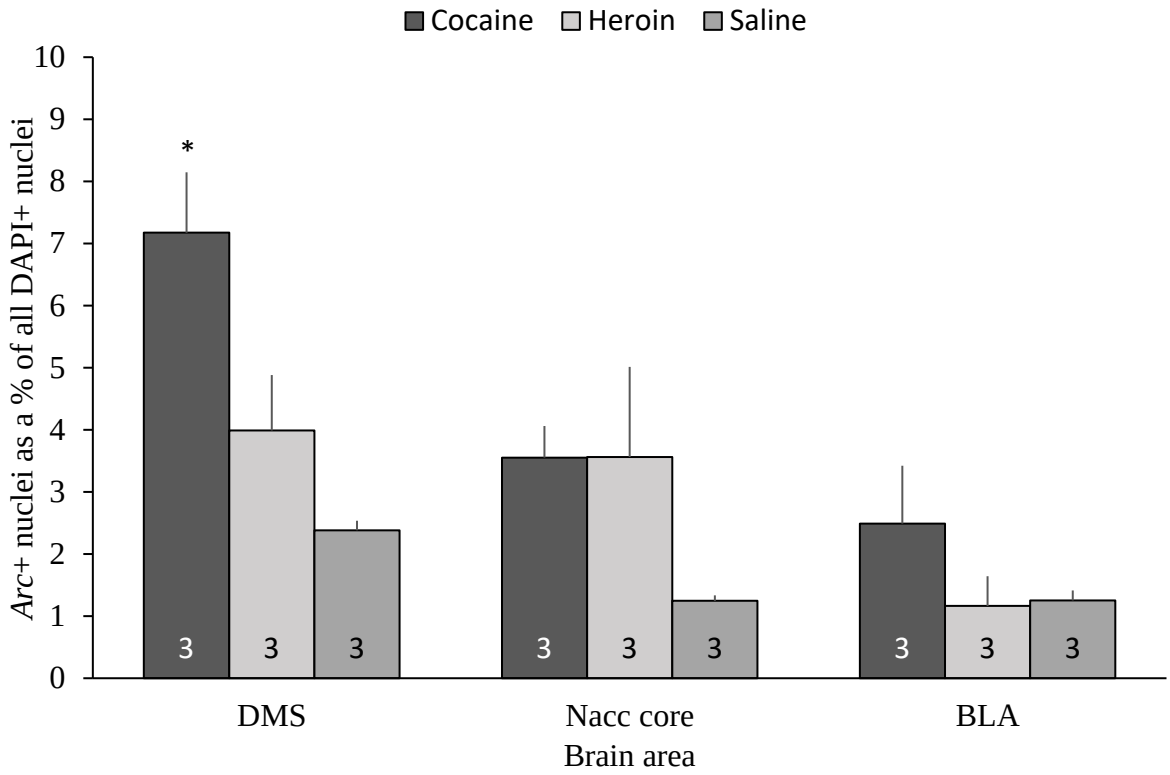

Fig. 3.10. Arc expression 8 min after a drug injection relative to saline as a function of brain area. Error bars represent ± 1SE. *Significantly different from saline. Numbers on bars refer to *n* for each drug group.

Discussion

The present study revealed that single intravenous injections of heroin or cocaine are followed by a quick and transient expression of *arc* and *h1a* in the striatum. This expression had a different temporal profile depending on the IEG in question, as well as a different magnitude depending on the drug administered and the brain area of interest.

Cocaine – Nacc core and DMS

The nuclear *arc* expression in the Nacc core and DMS following cocaine reached its highest levels 8 min after injection, and was lowest ~30 min after the injection. This temporal profile of expression mimics closely that of *arc* expression in response to conditioned cues or a novel environment (Grosso et al., 2015; Vazdarjanova et al., 2002). Similarly, *h1a* nuclear expression followed its predicted temporal profile – its levels were low soon after an injection of cocaine (0-16 min), but increased sharply after ~30 min.

However, there appeared to be subtle differences between the magnitudes of IEG expression between areas (i.e. the Nacc vs. the DMS), but these differences were not assessed using inferential statistics. However, it is notable that almost twice as large a proportion of observed neurons in the DMS were IEG positive compared to the Nacc core. This difference cannot stem from differences in total number of neurons observed, since IEG-positive nuclei were quantified as a percentage of all DAPI-positive nuclei. Similar findings have been reported before for *c-fos* (Steiner & Gerfen, 1993). In addition, *h1a* levels in the DMS were significantly elevated 16 min after cocaine, which is quicker from its previously reported speed of expression in the hippocampus (Vazdarjanova et al., 2002). In summary, the response to cocaine in the striatum met the expectations based on previous research with *arc* and *h1a*, and there was a slight tendency for a more pronounced response in the dorsal vs. ventral parts of this brain area. The magnitude and regional differences of mRNA expression reported here was similar to studies with *c-fos*. For example, it has previously been shown that only 2-3% within the NAcc express *c-fos* in response to cocaine (Koya et al., 2009).

Heroin – Nacc core and DMS

On average, the highest levels of *arc* nuclear expression in response to heroin occurred at about the same time as in the case for cocaine (8 min), although there was no statistically significant effect of heroin on *arc* expression in the Nacc core. Similarly, on average, *h1a* peaks of nuclear expression occurred at a delayed time after a heroin injection (~30 min), but no statistically significant main effect of time was found. There seemed to be a difference between heroin and cocaine in the magnitude of these expression levels in the DMS, where heroin seemed to have a weaker effect (however, this difference was not assessed using inferential statistics). Although heroin and cocaine have not been compared directly before, there is some evidence for lower *c-fos* levels in dorsal striatum following morphine vs cocaine (Harlan & Garcia, 1998). In addition, there was much higher variability in the levels of *arc* and *h1a* in the Nacc core and DMS after heroin, to the extent that a main effect of time was observed only in the DMS, and only for *arc* expression.

In summary, there was a trend for heroin to produce the expected temporal pattern of *arc* and *h1a* expression in the striatum, but the effect was much less evident in

comparison to cocaine. In fact, for heroin the only statistically significant change in expression over time was found in the DMS. Clearly, a direct comparison between the two drugs is impossible without a full dose-response analysis due to their differences in terms of pharmacology. Nevertheless, it is not unreasonable to aim for a similar effects on the temporal profile of IEG expression between the two drugs. One likely way of achieving that would be increasing the dose of heroin. Further arguments for this are presented in following sections with discussion of saline control results.

Cocaine and heroin - BLA

The expression of *arc* and *h1a* in the BLA following either heroin or cocaine did not produce similar temporal profile to the one seen in the striatum. Overall, there was a large amount of variability in the average levels of *arc* and *h1a* expression in this area for both drugs. Thus, any conclusions and generalisations about the results must be made with caution. Interestingly, *h1a* reached its peak levels 16 min following an injection of either heroin or cocaine, which was earlier than expected. This increase was maintained, on average up to 35 min following drug injections. Conversely, peak levels of *arc* were almost indistinguishable in the BLA for both drugs: *arc* expression was low across all time points with very little change between. Such inconclusive results are somewhat surprising given the previous findings of drug-induced *c-fos* expression in this brain area (Day et al., 2001), and the shared MAPK/ERK pathway of *arc*, *h1a* and *c-fos*. One possible explanation would be that *arc, h1a* and *c-fos* expression in this brain area arise after different type of electrophysiological activity. Only strongly activated neurons express *c-fos*, but no electrophysiological profile has been defined for *arc* and *h1a*.

Saline controls

A comparison was made between *arc* expression following drug and vehicle administration to examine the possibility that temporal changes in the level of IEG expression are the result of handling animals rather than a consequence of drug actions on neuronal activity. There was a clear effect of cocaine on *arc* expression in the DMS relative to saline. However, the effect of cocaine in the Nacc core and the effect of heroin across both areas of the striatum was more subtle and did not reach statistical significance. Given the small sample size, it is possible that the effect is genuine, but

small, or that the differences found are random variation. Although the latter possibility is less likely given that the effect was visible across several brain areas (suggesting it is in some way reliable), it is admittedly the case that higher doses should be used in the future to reach a more robust effect.

Implications for catFISH

The main goal of the present study was to examine the possibility of applying the catFISH method to the study of heroin and cocaine's acute effects. The main obstacles in such a task were the issues surrounding stimulus on- and off-set. The data presented here show that the use of the i.v. route of administration resolves these issues to a great extent. That is, the highest levels of *arc* expression were seen soon after drug injection, while the highest levels of *h1a* expression were seen about 25 min later. However, it must be noted that this was not true for all brain areas or both heroin and cocaine. There were no statistically significant effects of heroin on *h1a* expression in this study, which warrants the use of higher doses in following experiments. In addition, there was no statistically significant change in IEG expression in the BLA following any drug treatment.

When present, the IEG response was transient, so that if two injections were administered in succession, there wouldn't be substantial carry-over effects from the first to the second. Indeed, there are some notable differences in the magnitude of IEG expression between drugs and brain areas, and these can affect estimates of overlap measured by catFISH. Nevertheless, these differences can be controlled for statistically. Overall, given the data shown here, it should be possible to use catFISH to measure the overlap between neuronal populations activated by heroin and cocaine.

One obvious caveat is the small difference between drug-induced IEG expression and IEG expression in the saline-treated animals. These small differences raise the possibility that some of the IEG expression seen here is an artefact of the drug administration procedure, at least in the case of heroin. It is not possible to conclude whether the cells expressing IEG following heroin and saline are the same or different, so the possibility of a genuine heroin effect still remains. This issue will be addressed by increasing the doses of heroin and cocaine in following experiments. Regardless of this limitation, the

present study clearly showed that the IEG response to drug injections under these conditions is quick and transient, which was the main goal of this particular study.

In summary, intravenous injections of heroin and cocaine trigger transient expression of the *arc* and *h1a* in the striatum. The temporal profile of this expression is similar to the one seen in studies using conditioned stimuli and exposure to a novel environment. Although there are regional and drug-specific differences in the magnitude of this effect, it is an indication that it would be possible to incorporate the catFISH technique into the study of heroin and cocaine's acute effects in parts of the striatum. At the same time, the BLA is not a suitable target for such an investigation, because IEG expression there was markedly different from that of the striatum in terms of both temporal profile and magnitude.

Chapter 4 – Distinct neuronal populations respond to heroin and cocaine across the striatum

Introduction

In the previous chapter it was established that i.v. injections of heroin and cocaine at doses known to be self-administered by animals lead to a quick and transient IEG response in the Nacc core and DMS, albeit with some caveats regarding the effects of heroin (see discussion in previous chapter). It was concluded that the catFISH technique can be applied to the study of pharmacological stimuli. This chapter describes a follow-up experiment where catFISH was used to measure the overlap between neuronal populations active after two consecutive injections of either saline, cocaine or heroin (in different combinations). Here it was possible to find out if these neuronal populations are overlapping or distinct, and if there are neurons that reliably respond to heroin and cocaine on two separate occasions across the striatum (defined as neuronal ensembles).

Note that, in view of the various theoretical definitions of a neuronal ensemble reviewed in ch. 1, a distinction was made between *activated neuronal populations* and *neuronal ensembles*. The former was defined as a group of any neurons active at the time of a particular drug or saline injection, while the latter was defined as a group of neurons activated by the same type of injection on two consecutive occasions. This latter definition for a neuronal ensemble has previously been used by Xiu et al. (2014), and it implies that since neuronal ensembles are activated by a particular stimulus, then the presentation of that stimulus on two separate occasions should activate more or less the same neurons.

Using this definition, Xiu et al. have previously found neuronal ensembles activated by morphine in the NAcc shell (Xiu et al., 2014). The technique used in that study took advantage of the temporal difference between *c-fos* mRNA transcription and protein translation (TAI-FISH). Effectively, the logic behind the TAI-FISH technique is identical to catFISH: two stimuli are administered in succession, and the neuronal population

activated by the first one is labelled by the FOS protein, while for the second stimulus it is labelled by *c-fos* mRNA. The study found that two consecutive i.p. injections of morphine or two separate application of a foot shock activate almost exclusively the same populations of neurons in the Nacc shell and paraventricular nucleus of the thalamus, respectively. In addition, injections of morphine and then cocaine activated almost the same populations of neurons in the shell: there were significantly less neurons activated by only one stimulus or the other relative to neurons activated by both. Finally, neuronal populations in the shell activated by morphine and foot-shock were almost completely separated. Thus, there is evidence that pharmacological stimuli such as morphine injections reliably recruit more or less the same neurons in the shell, at least as long as *c-fos*-expressing neurons are concerned. The present study expands on these findings in several ways.

First, the present study used i.v. injections of heroin and cocaine instead of i.p. injections. The pharmacokinetic differences resulting from the two routes of administration and their methodological implications for catFISH were discussed in the previous chapter. In addition to those considerations, using one route vs. the other also has implications for drug effects on IEG expression. For example, a dose of cocaine administered over 5 or 25 sec elicits higher *arc* and *c-fos* mRNA expression in the Nacc core and shell compared to the same dose administered over 100 sec (Samaha, Mallet, Ferguson, Gonon, & Robinson, 2004). This indicates that the rate at which cocaine reaches the brain determines its effects on IEG expression. Potential reasons for it are that higher rate of administration leads to increased ability of cocaine to block dopamine (DA) re-uptake, and quicker rise in extracellular dopamine (Ferrario et al., 2008), because both *c-fos* and *arc* expression are to some extent dependent on DA transmission (Fosnaugh et al., 1995; Graybiel, Moratalla, & Robertson, 1990). Thus, it is reasonable to expect that using an i.v. route with a quicker rate of drug entry to the brain would result in more robust IEG response relative to an i.p. injection. That in turn means a different set of neurons being active, and, potentially, a different neuronal ensemble. Whether that would also mean a different degree of overlap between neuronal populations is a question addressed empirically in the present study.

A second difference from the Xiu et al. study is the use of heroin instead of morphine. As already mentioned, *arc* expression is dependent on DA, and so is in fact *homer 1a* (Yamada et al., 2007). There are big differences in DA release following heroin vs. morphine injections, heroin producing much higher levels of DA release, most likely because of the actions of 6-MAM (Gottas, Boix, Oiestad, Vindenes, & Morland, 2014). Thus, heroin is likely to recruit additional if not entirely different neurons across the striatum. Again, whether this would affect estimates of overlap between drug-activated neuronal populations is an open question.

Finally, the present study looked at several different parts of the striatum in addition to the Nacc shell, and identified the neuronal populations activated following different order and combinations of drug administration. More specifically, this study looked at the Nacc core, dorsomedial and dorsolateral striatum (DMS and DLS) in addition to the shell. All these areas are targets of dopaminergic innervation and are believed to undergo functional changes following drug administration (Belin & Everitt, 2008; Volkow et al., 2006). Also, each of these four subdivisions of the striatum receive different glutamatergic and dopaminergic projections, as discussed in ch.1 (e.g. see fig. 1.1, p.10). Additional combinations of drugs administered included consecutive injections of heroin as well as consecutive injections of cocaine. In this way it was possible to address the question of whether there are neurons that are reliably activated, and across different areas, following consecutive administrations of each drug. The order of drug administration (heroin-cocaine and cocaine-heroin) was also studied as a possible factor in IEG expression and neuronal population overlap measures. Due to the design implied by the catFISH technique, injections were administered with a relatively short amount of time in between. Thus, it was possible that some changes in the intrinsic properties of the cell following the first drug injection could have affected the effect of the second one.

The doses used for this study were increased to 800 µg/kg of cocaine and 100 or 200 µg/kg for heroin. This is a two-fold increase relative to the doses used in the study reported in chapter 3, where the temporal profile of mRNA expression was examined. The reason for this change was aiming at a more pronounced IEG response, especially in the case of heroin. In the previous chapter it was shown that, although heroin

injections produced the same quick and transient IEG response as cocaine, the response was much more variable and of a lower magnitude. In addition, elevation of IEG expression after cocaine vs. after saline treatment was somewhat subtle at lower doses. Making inferences about overlap between neuronal populations activated by heroin and cocaine could have been problematic if the number of active cells after each injection was too low. To make sure there was to be a reliable increase of IEG expression from baseline following heroin, the dose was increased two- and four-fold. Having two different doses also helped reveal any expression pattern differences for lower and higher doses of heroin. Saline control groups were also added to account for any changes in temporal profile of IEG expression due to the increased doses.

It was expected that the neuronal populations activated by two consecutive injections of cocaine would exhibit a high degree of overlap due to having the same stimulus administered twice. Considering the evidence that self-administration behaviour is mediated by different type of signalling in the NAcc, it was also predicted that an injection of cocaine and an injection of heroin would activate populations that overlap to a significantly lesser extent.

Methods

Animals, housing and testing cages

The rats used in this experiment (*n* = 50) were tested at a weight of 300-310g. Supplier, weight upon arrival, and housing conditions were as described in general methods.

Following acclimatization and catheter surgery, until the day before testing, rats were placed in the housing cages and were moved to the operant chambers for the night before and during the day of testing (details in procedure section).

Drugs

Heroin HCL was dissolved in sterile saline at concentrations of either 825 µg/ml or 1.65 mg/ml. Cocaine HCL was dissolved at a concentration of 6.6 mg/ml. All drug infusions had a volume of 40 µl, which corresponded to doses of either 100 µg/kg or 200 µg/kg for heroin, and a dose of 800 µg/kg for cocaine. As discussed above, the doses used in this study were increased in order to achieve a more robust IEG response. Note that in

this case drug infusions were delivered remotely, using a computer-controlled infusion pump (more details in procedure section).

Design

The study had a between-subject design. The independent variable was the combination of drugs administered. For the first two waves of rats (*n* = 35) these were: saline - saline, saline – cocaine, cocaine – saline, cocaine – cocaine, cocaine-heroin 100 µg/kg, cocaine – heroin 200 µg/kg. For a separate wave (*n* = 15) the groups were: heroin – heroin, heroin – cocaine, and heroin – saline (heroin given at 200 µg/kg in each case). Dependent variables were number of DAPI-positive cellular nuclei, cell nuclei positive for *h1a* mRNA, cell nuclei positive for *arc* mRNA, and nuclei positive for both types of mRNA.

Procedure

Acclimatization and catheter surgery were carried out as described in general methods.

Testing. All rats were moved to the testing chambers 18 hours before the testing session in order to habituate them to the novelty of the testing environment. This was done to avoid interaction effects between drugs and novelty, which could have influenced the final measures of IEG expression (Paolone et al., 2007; Uslaner et al., 2001). Following this habitation period animals were tethered to the infusion lines. All stimuli in the operant chamber such as levers and lights were inactive. The infusion pumps were programmed to begin the testing session automatically, one hour after the tethering. This was done to ensure minimum effect of the handling of animals on mRNA expression. Thus, in the absence of the experimenter, each animal received one of the following combinations of drug infusions, separated by 25 minutes: saline – saline (*n* = 4), saline – cocaine (*n* = 6), cocaine – saline (*n* = 6), cocaine – cocaine (*n* = 6), cocaine – heroin 100 µg/kg (*n* = 6), cocaine – heroin (*n* = 7), and heroin – cocaine (*n* = 6), heroin – heroin (*n* = 6), heroin - saline (*n* = 3). For the last 4 experimental groups heroin was given at a dose of 200 µg/kg. Five minutes after the second injection, rats were given an overdose of pentobarbital i.v., decapitated, brains were snap-frozen in isopentane at -80°C, and stored for cryosectioning.

To achieve the administration of two separate injections through the same catheter, the infusion lines were back-filled with the appropriate drug solutions for each condition, just before tethering of the animals. The volume of drug solutions in each line was 40 µl for both heroin and cocaine, with the corresponding drug concentration. The rest of the lines were filled with saline. Each drug solution was separated from the rest of the saline in the infusion line by a small air bubble. The infusion pumps operated at a default infusion rate of 10 µl/sec (±1.5 µl/sec). Thus, each drug infusion was delivered over 4 sec. The two drug solutions were separated by 90 µl of saline, and the pumps were switched on for 10 sec during each of the two infusions. This way two potential issues were avoided: first, the possible mixing of the first and second drug infusions due to slight infusion rate variability between infusions and between pumps. Second, the issue of a small amount of drug solution remaining in the catheter itself. For the saline-saline group, the whole set-up was kept the same except no drugs were dissolved in the saline. Thus, in this experiment the infusion rate was controlled more strictly than in the study described in chapter 3, where duration of infusions might have varied by up to 2 sec. It was not suggested that this might have had a substantial effect on overall levels of IEG expression in response to heroin and cocaine. Effects of infusion rate on IEG expression have been shown for much greater differences between infusion rates such as 5 vs. 100 sec, but not 5 vs 25 sec (Samaha et al., 2004). In addition, there is no reason to expect systematic variability between groups as a result of the drug administration procedure. Nevertheless, it was expected that better control over infusion rates might reduce within-group variability of IEG expression scores.

CatFISH, image acquisition and analysis. Whole brains were sectioned at 16 µm thickness, at the level of the anterior striatum containing the Nacc core and shell, DMS, and DLS, where images were taken for analysis (+1.70 mm from bregma (Paxinos & Watson, 1986), fig. 4.1 on next page, see also Appendix 2). As described in chapter 3, sectioning started from the tip of the olfactory bulbs and brain sections were removed until their ventral and dorsal parts became attached (defined as +3.70 mm from bregma). From this point, 120 sections (16 µm thick) were removed to reach approx. + 1.70 from bregma. If necessary, a few (no more than 5) additional sections were removed to reach the point where the dorsal tip of the corpus callosum formed a pointed angle. Then,

images of the accumbens core were taken by positioning the microscope field of view (MFV) so that its centre was at equal distances from the ventral end of the lateral ventricle, and from the anterior commissure. Images of the DMS were taken by positioning the MFV so that its left margin coincided with the dorsal part of the lateral ventricle and the medial side of the corpus callosum. Then, images of the DSL were taken by moving the MFV laterally so that its right margin coincided with the lateral part of the corpus callosum. For the Nacc shell, the centre of the MFV was positioned over the anterior commissure and moved in a medial direction until the left margin of the MFV reached the medial border of the Nacc shell, recognised as a less dense staining for cell nuclei by DAPI. The RNAscope probes and reagents, the *in situ* hybridisation and image acquisition and analysis protocols were as described in general methods.

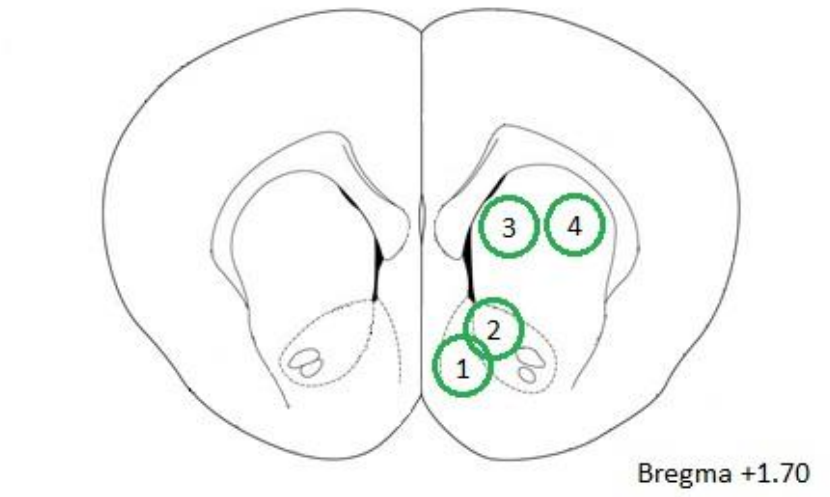

Fig. 4.1. Schematic representation of regions of interest (ROI) where microscopic images were taken: Nacc shell (1), Nacc core (2), DMS (3) and DLS (4).

Results

Homer 1a and arc expression – saline controls

The total amount of *h1a* expression in each condition was measured by adding up the number of nuclei expressing only *h1*a and the number of nuclei expressing both *arc* and *h1a* mRNA. Similarly, the total amount of *arc* expression was measured by adding up only *arc*-expressing nuclei and co-expressing nuclei. For simplicity, mRNA expression in

the cocaine-saline, saline-cocaine and heroin-saline control conditions is presented separately, before all other conditions.

Nacc core and shell

Figure 4.2 (next page) shows the average number of *h1a*- or *arc*-positive nuclei in the Nacc core and shell as a function of drug treatment. The punctuated lines represent mRNA levels in the saline-saline group for each brain area.

In the Nacc core, *h1a* expression was high in the cocaine-saline (*M* = 22.92, *SE* = 4.59) and heroin-saline (*M* = 20.67, *SE* = 2.48) groups, and much lower in the saline-cocaine group (*M* = 4.54, *SE* = 0.81). In the shell, on the other hand, *h1a* expression remained somewhat low in the cocaine-saline condition (*M* = 10.33, *SE* = 2.59), but was higher in the heroin-saline condition (*M* = 26, *SE* = 2.04). The saline-cocaine condition had low levels of *h1a* in the shell as well (*M* = 7.75, *SE* = 1.37).

Arc expression was relatively high only in the saline-cocaine conditions in both the core and shell (*M* = 37.17, *SE* = 9.21, and *M* = 21.88, *SE* = 3.49, respectively). The cocaine-saline and heroin-saline conditions had low levels of *arc* both in the core (*M* = 5.71, *SE* = 0.75, and *M* = 3.92, *SE* = 0.85, respectively) and shell (*M* = 4.79, *SE* = 0.92, and *M* = 4.58, *SE* = 1.08, respectively).

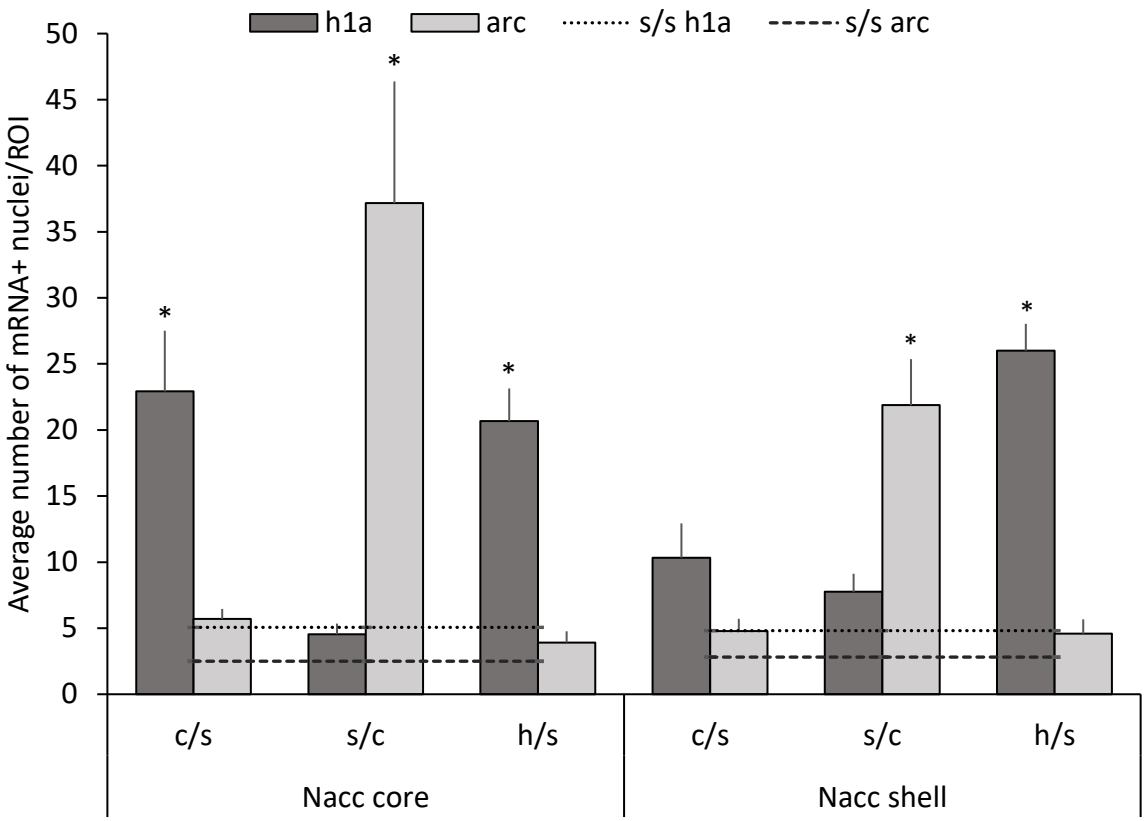

Fig. 4.2. Average number of *h1a*- or *arc*-positive nuclei in the Nacc core and shell as a function of drug treatment. s - saline, c - cocaine, h - heroin. Error bars: ±1 SE. *significantly different from saline-saline. *N* for each group: s/s = 4, c/s = 6, s/c = 6, h/s = 3.

Shapiro-Wilk tests revealed normal distributions of *h1a* and *arc* expression scores in the core. *H1a* score distribution was normal in the shell as well, but there was deviation from normality of the *arc* scores in the cocaine-saline condition. Levene's tests revealed heterogeneity of variance across groups for both *h1a* and *arc* scores in the core, $F(3,15) = 4.57$, $p = .018$, and $F(3,15) = 19.93$, $p < .001$, respectively. In the shell, the variance of *h1a* scores was homogenous $F(3,15) = 2.56$, $p = .094$, but the variance of *arc* scores was not $F(3,15) = 10.04$, $p = .001$.

A Welch test revealed significant differences between groups for *h1a* expression in the core, $F(3,6.29) = 14.68$, $p = .003$, as well as for *arc* expression, $F(3,7.36) = 5.59$, $p = .026$. Mann-Whitney tests with a Bonferroni correction ($\alpha = .017$) showed that the cocaine-saline and heroin-saline groups had significantly higher levels of *h1a* expression than the saline-saline group. Also, the saline-cocaine group had significantly higher levels of *arc* expression than the saline-saline group.

A one-way ANOVA revealed significant differences in *h1a* levels in the shell, $F(3,15) = 14.07$, $p < .001$. Dunnett's *t* tests revealed that there was significantly more *h1a*-positive nuclei in the heroin-saline condition relative to saline-saline. A Kruskal-Wallis test revealed significant differences in *arc* scores, $H(3) = 12.9$, $p = .005$, where the saline-cocaine condition was significantly different from saline-saline, $U = 24$, $p = .01$.

Dorsal striatum

Figure 4.3 (next page) shows the average number of *h1a*- or *arc*-positive nuclei in the DMS and DLS as a function of drug treatment. The punctuated lines represent mRNA levels in the saline-saline group for each brain area.

In the DMS, *h1a* expression was highest in the cocaine-saline group ($M = 54.71$, $SE = 8.31$), followed by heroin-saline ($M = 29.50$, $SE = 4.78$), and lowest in the saline-cocaine group ($M = 16.92$, $SE = 2.57$). In the DLS, *h1a* expression remained high in the cocaine-saline condition ($M = 69.54$, $SE = 8.59$), but was relatively low in the heroin-saline ($M = 21.42$, $SE = 2.04$) and the saline-cocaine conditions ($M = 11.54$, $SE = 2.58$).

Arc expression was relatively high only in the saline-cocaine conditions in both the DMS and DLS ($M = 76.33$, $SE = 15.56$, and $M = 85.38$, $SE = 12.58$, respectively). The cocaine-saline and heroin-saline conditions had low levels of *arc* both in the DMS ($M = 9.21$, $SE = 1.88$, and $M = 3.17$, $SE = 0.36$, respectively) and DLS ($M = 8.79$, $SE = 2.13$, and $M = 2.83$, $SE = 1.45$, respectively).

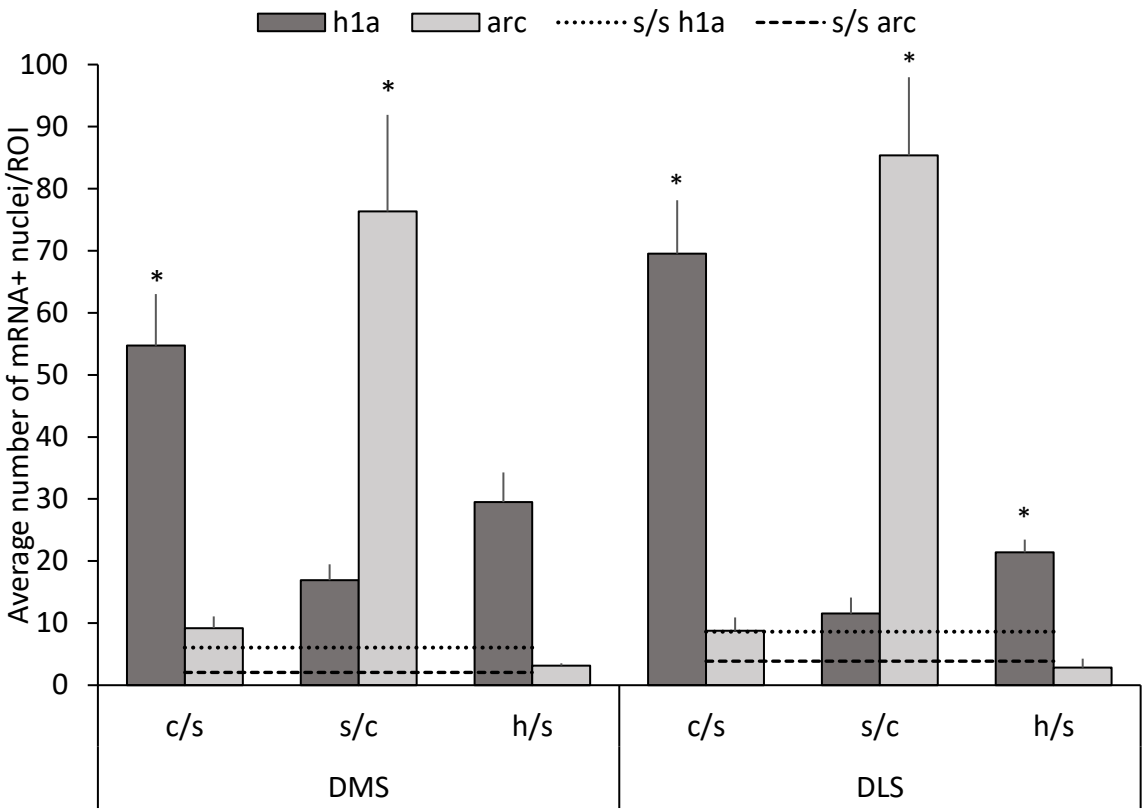

Fig. 4.3. Average number of *h1a*- or *arc*-positive nuclei in the DMS and DLS as a function of drug treatment. s - saline, c - cocaine, h - heroin. Error bars: ±1 SE. *significantly different from saline-saline. *N* for each group: s/s = 4, c/s = 6, s/c = 6, h/s = 3.

Shapiro-Wilk tests revealed normal distributions of *h1a* scores in the DMS, but not *arc* expression scores, where the deviation form normality was found in the cocaine-saline condition. Both *h1a* and *arc* score distribution was normal in the DLS. Levene's tests revealed homogeneity of variance across groups for *h1a* scores in the DMS, $F(3,15) = 1.18$, $p = .349$, while the tests was significant for *arc* scores $F(3,15) = 19.93$, $p < .001$. In the DLS, the variance of both *h1a* and *arc* scores was heterogeneous $F(3,15) = 23.09$, $p < .001$, and $F(3,15) = 5.77$, $p = .008$, respectively.

A one-way ANOVA revealed significant differences between groups for *h1a* expression in the DMS, $F(3,15) = 14.09$, $p < .001$. Dunnett's *post hoc* tests revealed that only the cocaine-saline condition differed significantly from the saline-saline condition. In addition, a Kruskal-Wallis test revealed significant differences in *arc* scores between groups in this brain area, where the saline-cocaine condition differed significantly from saline-saline, $U = 24$, $p = .01$.

Welch tests revealed significant differences in *h1a* and *arc* scores between groups in the DLS, $F(3,7.88) = 16.05$, $p = .001$, and $F(3,8.05) = 13.35$, $p = .002$, respectively. Mann-Whitney tests ($\alpha = .017$) revealed that the cocaine-saline and the heroin-saline conditions differed significantly from saline-saline in *h1a* levels, and the saline-cocaine condition differed significantly from saline-saline in *arc* levels.

Table 4.1. **Summary of *homer 1a* and *arc* expression in drug-saline conditions. The effect of heroin and cocaine on IEG expression is shown relative to saline in each brain area: (↑) significant increase over saline, (–) no sig. difference form saline, (×) no data.**

	Core		**Shell**		**DMS**		**DLS**	
	h1a	*arc*	*h1a*	*arc*	*h1a*	*arc*	*h1a*	*arc*
cocaine	↑	↑	–	↑	↑	↑	↑	↑
heroin	↑	×	↑	×	–	×	↑	×

Homer 1a and arc expression – experimental conditions

Nacc core

Figure 4.4 (next page) shows the amount of *h1a* and *arc* expression as a function of experimental condition in the Nacc core.

Animals in the saline-saline condition had the lowest amount of *h1a*-positive nuclei, $M = 5.06$, $SE = 0.73$. In all other conditions, the amount of *h1a*-positive nuclei was much higher: cocaine-cocaine $M = 26$, $SE = 6.15$, cocaine-heroin (100 µg/kg) $M = 25.33$, $SE = 5.21$, cocaine-heroin (200 µg/kg) $M = 22.11$, $SE = 4.7$, heroin-cocaine $M = 21.08$, $SE = 3.75$, and heroin-heroin $M = 16.17$, $SE = 2.59$. In the last two conditions heroin is at a dose of 200 µg/kg.

Shapiro-Wilk tests revealed a non-normal distribution of *h1a* scores in the cocaine-heroin (200 µg/kg) group, where $W(7) = 0.78$, $p = .027$. A Kruskal-Wallis tests was used to examine differences in number of *h1a*-positive nuclei between experimental conditions, and it yielded significant results, $H(5) = 11.11$, $p = .049$.

Multiple comparisons for *h1a* scores were made using Mann-Whitney tests and a Bonferroni correction was applied, so all effects are reported at .01 level of significance.

There was a significant difference between the saline-saline group and cocaine-cocaine (U = 24, p = .01), cocaine-heroin (100 µg/kg; U = 24, p = .01), cocaine-heroin (200 µg/kg; U = 28, p = .006), and heroin-cocaine (U = 24, p = .01). The difference from the heroin-heroin group was not significant at the .01 level, U = 22, p = .038.

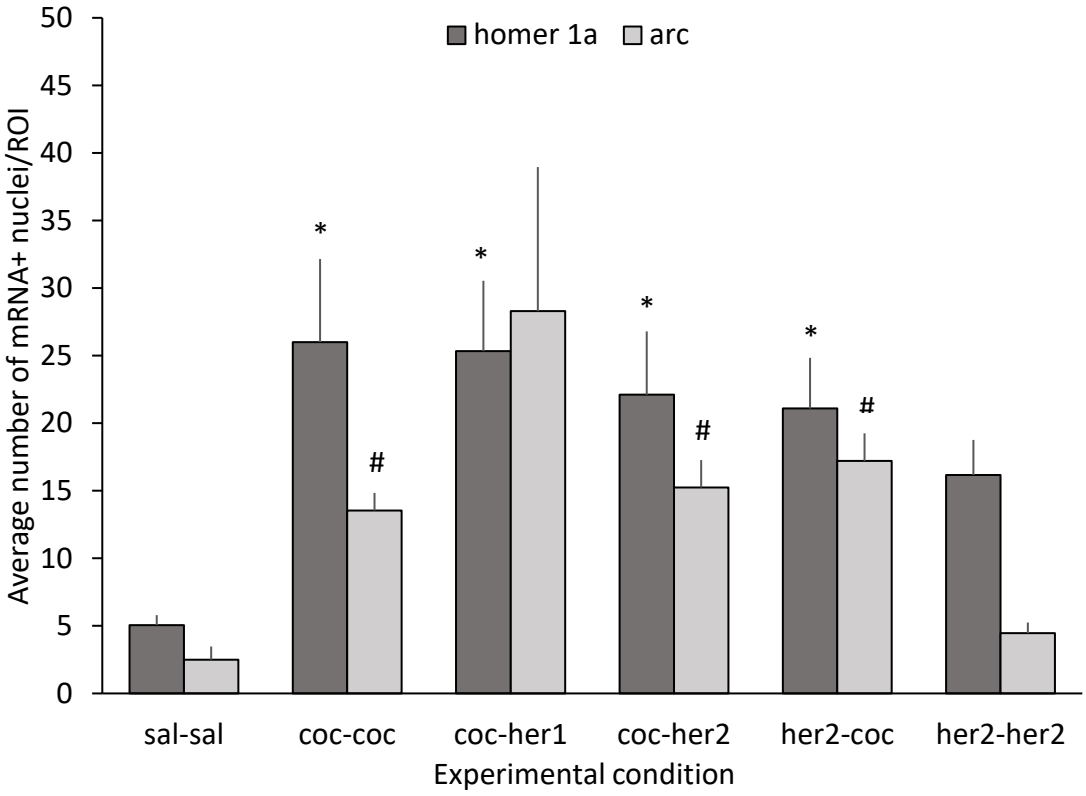

Fig. 4.4. Average number of *h1a*- or *arc*-positive nuclei in the **Nacc core** as a function of experimental condition. Error bars represent +1 SEM. */# - significantly different from saline-saline. *N* for each group: sal-sal (4), coc=coc (6), coc-her1 (6), coc-her2 (7), her2-coc (6), her-her (6).

The saline-saline group also had the lowest levels of *arc* expression: M = 2.5, SE = 0.97. Next lowest was the heroin-heroin group, M = 4.46, SE = 0.79. In the rest of the conditions, *arc* expression was relatively higher: cocaine-heroin (100 µg/kg), M = 28.29, SE = 10.66, cocaine-heroin (200 µg/kg), M = 15.25, SE = 2.02, cocaine-cocaine M = 13.54, SE = 1.3, and heroin-cocaine, M = 17.21, SE = 2.04. Shapiro-Wilk tests revealed normal distributions of *arc* scores in all conditions, but Levene's statistic was highly significant, $F(5,29) = 22.56$, $p < .001$.

A Welch test revealed significant differences between groups, $F(5,12.98) = 17.02$, $p < .001$. Mann-Whitney tests were used for multiple comparisons and a Bonferroni

correction was applied, so all comparisons are reported at α = .01. There were significantly less *arc*-positive nuclei in the saline-saline condition relative to all other conditions except the cocaine-heroin (100 µg/kg) and heroin-heroin conditions, where U = 22, p = .038, and U = 20, p = .114, respectively. The statistics for the other comparisons were as follows: cocaine-cocaine, U = 24, p = .01, cocaine-heroin (200 µg/kg), U = 28, p = .006, and heroin-cocaine U = 24, p = .01.

Nacc shell

Figure 4.5 (next page) shows the amount of *h1a* and *arc* expression in the Nacc shell as a function of drug treatment.

Overall, there was noticeably less *h1a* expression in this brain area relative to the rest of the striatum. The average number of *h1a+* nuclei in each condition were as follows: saline-saline, M = 4.81, SE = 1.68, cocaine-heroin (200 µg/kg), M = 9.32, SE = 2.1, cocaine-heroin (100 µg/kg), M = 10.5, SE = 2.97, cocaine-cocaine, M = 14.33, SE = 3.28, heroin-heroin, M = 19.17, SE = 2.22, and heroin-cocaine, M = 20.88, SE = 4.27. Shapiro-Wilk tests of normality revealed normal distributions in all conditions.

Levene's test revealed homogenous variance across all groups $F(5,29) = 0.91$, p = .488. A one-way ANOVA revealed significant differences between conditions, $F(5,29) = 3.86$, p = .008. Dunnett's two-sided tests revealed that the heroin-cocaine and heroin-heroin groups had significantly higher amounts of *h1a*-positive nuclei than the saline-saline group. The rest of the groups did not differ significantly from saline-saline.

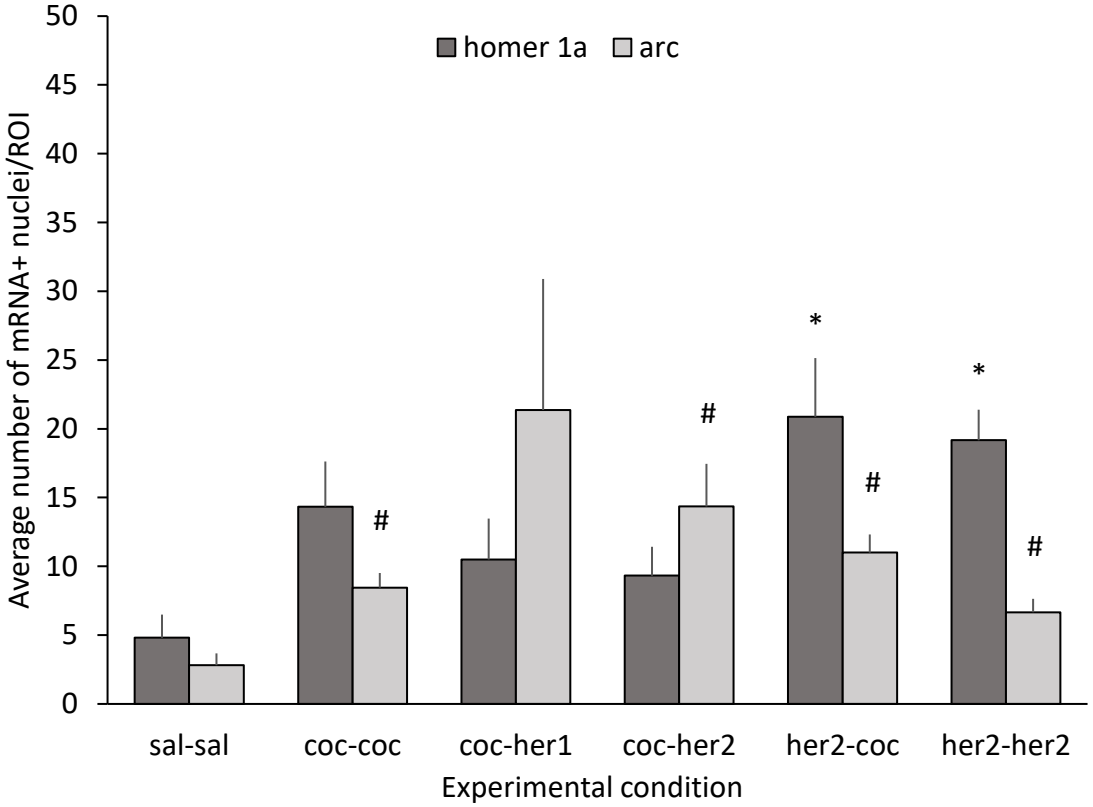

Fig. 4.5. Average number of *h1a*- or *arc*-positive nuclei in the **Nacc shell** as a function of experimental condition. Error bars represent +1 SEM. */# - significantly different from saline-saline. *N* for each group: sal-sal (4), coc=coc (6), coc-her1 (6), coc-her2 (7), her2-coc (6), her-her (6).

The average number of *arc*-positive nuclei in the Nacc shell was as follows: cocaine-heroin (100 µg/kg), M = 21.38, SE = 9.52, cocaine-heroin (200 µg/kg), M = 14.36, SE = 3.09, saline-saline, M = 2.81, SE = 0.86, cocaine-cocaine, M = 8.45, SE = 1.06, heroin-cocaine, M = 11, SE = 1.32, and heroin-heroin, M = 6.67, SE = 0.97. Shapiro-Wilk tests revealed normal distributions in all conditions.

Levene's test revealed a lack of homogeneity of variance, $F(5,29) = 8.76$, $p < .001$. A Welch test revealed significant differences between groups, $F(5,13.29) = 7.03$, $p = .002$. Multiple comparisons were carried out using Mann-Whitney tests at $\alpha = .01$. The cocaine-heroin (100 µg/kg) group did not differ significantly from saline-saline ($U = 21$, $p = .067$), but all other groups did: cocaine-cocaine, $U = 24$, $p = .01$, cocaine-heroin (200 µg/kg), $U = 28$, $p = .006$, heroin-cocaine, $U = 24$, $p = .01$, and heroin-heroin, $U = 24$, $p = .01$.

DMS

Figure 4.6 (next page) shows the amount of *h1a* and *arc* expression in the DMS as a function of drug treatment.

The lowest *h1a* expression was found in the saline-saline group, $M = 6.06$, $SE = 2.19$. The average amount of *h1a*-positive nuclei in the rest of the conditions were as follows: cocaine-heroin (200 μg/kg), $M = 53.79$, $SE = 7.69$, cocaine-cocaine, $M = 56.33$, $SE = 11.39$, cocaine-heroin (100 μg/kg), $M = 62.83$, $SE = 17.5$, heroin-cocaine, $M = 21.25$, $SE = 3.69$, and heroin-heroin, $M = 27.75$, $SE = 6.43$. Shapiro-Wilk tests revealed normal distributions in all conditions.

There was a violation of the homogeneity of variance assumption as indicated by Levene's test, $F(5,29) = 3.14$, $p = .022$. A Welch test revealed significant differences between conditions, $F(5,13.29) = 11.58$, $p < .001$.

Again, Mann-Whitney tests were used for multiple comparisons and are reported at a significance level of .01 following a Bonferroni correction. In all other conditions, there were significantly more *h1a*-positive nuclei than in the saline-saline condition: cocaine-heroin (200 μg/kg) $U = 28$, $p = .006$, cocaine-heroin (100 μg/kg), $U = 24$, $p = .01$, cocaine-cocaine, $U = 24$, $p = .01$, heroin-cocaine, $U = 24$, $p = .01$, and heroin-heroin, $U = 24$, $p = .01$.

The most *arc* expression was found in the heroin-cocaine group, $M = 27.95$, $SE = 5.26$, followed by cocaine-cocaine, $M = 26.71$, $SE = 2.96$. All other groups had relatively low levels of nuclear *arc*: saline-saline, $M = 2.06$, $SE = 1$, cocaine-heroin (100 μg/kg), $M = 7.79$, $SE = 1.11$, cocaine-heroin (200 μg/kg), $M = 8.29$, $SE = 0.72$, and heroin-heroin, $M = 3.54$, $SE = 0.68$. Shapiro-Wilk tests revealed normal distributions in all conditions.

Levene's statistic indicated heterogeneous variance across groups, $F(5,29) = 8.17$, $p < .001$. A Welch test indicated significant differences between conditions, $F(5,12.51) = 17.73$, $p < .001$. Multiple comparisons were carried out using Mann-Whitney tests at $\alpha = .01$. The saline-saline condition differed significantly from the cocaine-cocaine, $U = 24$, $p = .01$, cocaine-heroin (200 μg/kg), $U = 28$, $p = .006$, and the heroin-cocaine ($U = 24$, $p = .01$) conditions, but not from the cocaine-heroin (100 μg/kg), $U = 23$, $p = .019$, and the heroin-heroin ($U = 20$, $p = .114$) conditions.

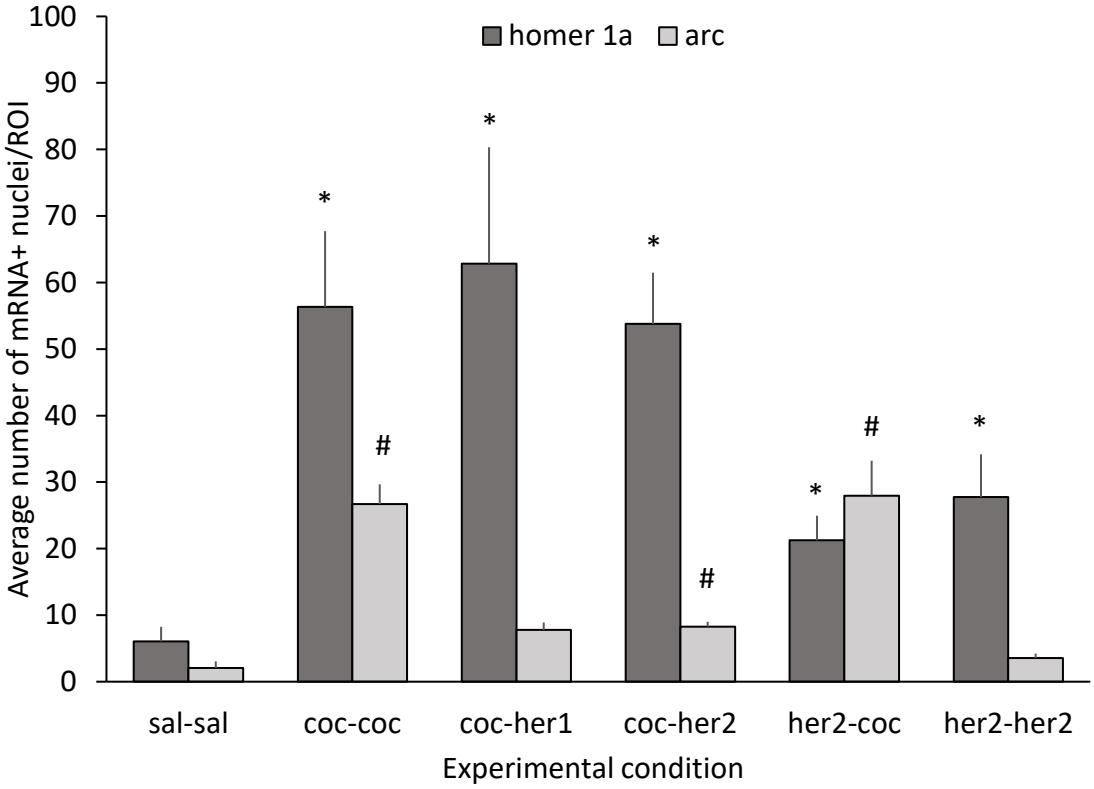

Fig. 4.6. Average number of *h1a*- or *arc*-positive nuclei in the **DMS** as a function of experimental condition. Error bars represent +1 SEM. */# - significantly different from saline-saline. *N* for each group: sal-sal (4), coc=coc (6), coc-her1 (6), coc-her2 (7), her2-coc (6), her-her (6).

DLS

Figure 4.7 (next page) shows the amount of *h1a* and *arc* expression in the DLS as a function of drug treatment.

The lowest *h1a* expression was found in the saline-saline group, *M* = 8.63, *SE* = 2.66. The average number of *h1a+* nuclei in the rest of the groups was: cocaine-cocaine, *M* = 71.04, *SE* = 7.66, cocaine-heroin (100 µg/kg), *M* = 69.67, *SE* = 11.61, cocaine-heroin (200 µg/kg), *M* = 60.39, *SE* = 8.13, heroin-cocaine, *M* = 12.67, *SE* = 2.86, heroin-heroin, *M* = 17.5, *SE* = 3.01. Shapiro-Wilk tests revealed normal distribution of scores in all conditions.

Levene's tests indicated homogenous variance across conditions, $F(5,29) = 1.78$, $p = .148$. A one-way ANOVA revealed significant difference between groups, $F(5,29) = 15.94$, $p < .001$. Dunnett's *post hoc* 2-sided tests revealed that all conditions where the first injection was cocaine had significantly higher levels of *h1a* expression relative to saline

(p < .001). The saline-saline, heroin-cocaine and heroin-heroin groups did not differ significantly from each other in terms of *h1a* expression.

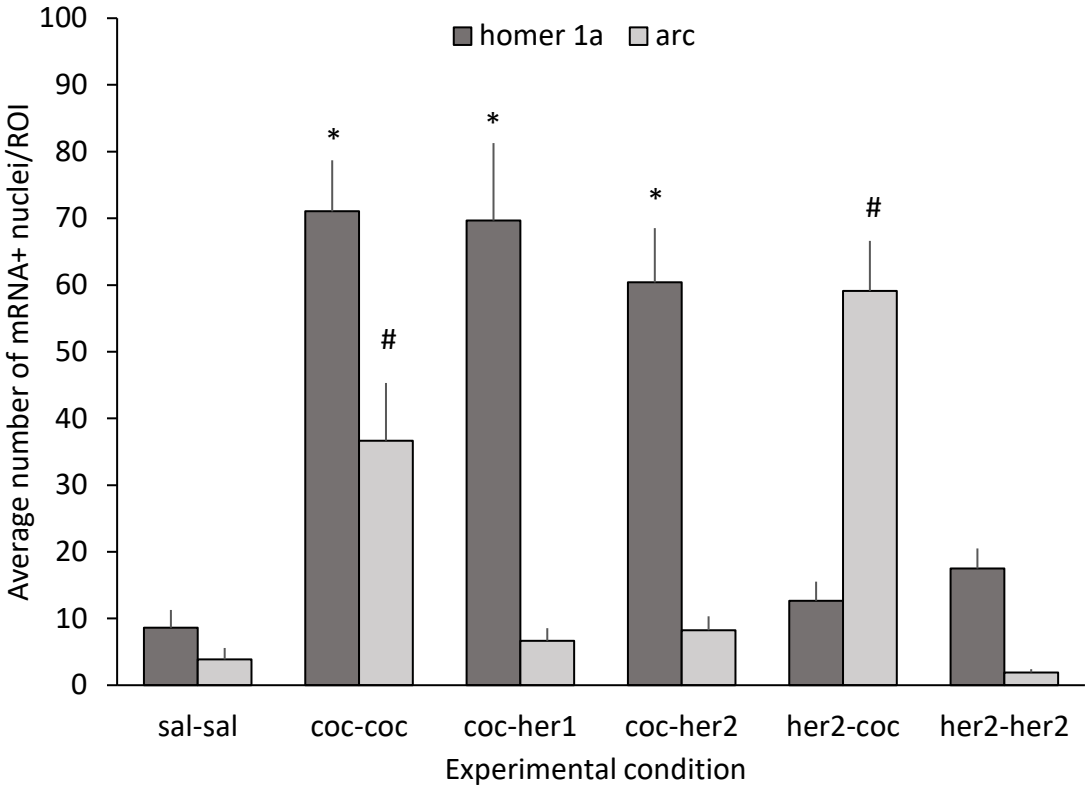

Fig. 4.7. Average number of *h1a*- or *arc*-positive nuclei in the **DLS** as a function of experimental condition. Error bars represent +1 SEM. *significantly different from saline-saline. *N* for each group: sal-sal (4), coc=coc (6), coc-her1 (6), coc-her2 (7), her2-coc (6), her-her (6).

The average amount of *arc*+ nuclei in the DLS depended on drug treatment as follows: cocaine-cocaine, M = 36.67, SE = 8.65, saline-saline, M = 3.88, SE = 1.71, cocaine-heroin (100 µg/kg), M = 6.67, SE = 1.88, cocaine-heroin (200 µg/kg) M = 8.25, SE = 2.08, heroin-cocaine M = 59.13, SE = 7.49, heroin-heroin M = 1.92, SE = 0.49. Shapiro-Wilk tests revealed normal distributions in all conditions.

Levene's statistic indicated heterogeneity of variance, $F(5,29) = 5.99$, p = .001. A Welch test revealed significant differences between conditions, $F(5,11.39) = 14.03$, p < .001, and was followed by multiple comparisons using Mann-Whitney tests at α = .01 (Bonferroni correction). There were significantly less *arc+* nuclei in the saline-saline condition relative to the cocaine-cocaine, U = 24, p = .01, and heroin-cocaine, U = 24, p = .01, groups. The saline-saline group did not differ significantly from the cocaine-heroin

(100 µg/kg), $U = 18$, $p = .257$, cocaine-heroin (200 µg/kg), $U = 21$, $p = .230$, and the heroin-heroin groups, $U = 28$, $p = .476$.

Table 4.2. **Summary of *homer 1a* and *arc* expression after administration of two drug injections. The effect of each drug combination on IEG expression is shown relative to saline-saline in each brain area: (↑) significant increase over saline, (–) no sig. difference form saline.**

	Core		**Shell**		**DMS**		**DLS**	
	h1a	*arc*	*h1a*	*arc*	*h1a*	*arc*	*h1a*	*arc*
c/c	↑	↑	–	↑	↑	↑	↑	↑
c/h1	↑	–	–	–	↑	–	↑	–
c/h2	↑	↑	–	↑	↑	↑	↑	–
h2/c	↑	↑	↑	↑	↑	↑	–	↑
h2/h2	–	–	↑	↑	↑	–	–	–

Co-expression (overlap) - saline controls

Figure 4.8 (next page) shows the average percent co-expressing nuclei (overlap) as a function of drug treatment and striatal area. The punctuated line represents the amount of co-expressing nuclei in the saline-saline group for each group and area.

The average percent co-expressing nuclei in each striatal area were as follows: Nacc core, cocaine-saline ($M = 5.58$, $SE = 0.75$), heroin-saline ($M = 4.57$, $SE = 1.18$), and saline-cocaine ($M = 1.79$, $SE = 0.42$). Nacc shell, cocaine-saline ($M = 13.29$, $SE = 2.03$), heroin-saline ($M = 5.84$, $SE = 1.25$), and saline-cocaine ($M = 4.14$, $SE = 0.99$). DMS, saline-cocaine ($M = 7.62$, $SE = 1.02$), cocaine-saline ($M = 6.06$, $SE = 0.39$), and heroin-saline ($M = 1.82$, $SE = 0.06$). Finally, DLS, saline-cocaine ($M = 5.96$, $SE = 0.92$), cocaine-saline ($M = 5.76$, $SE = 1.38$) and heroin-saline ($M = 3.32$, $SE = 1.68$).

Shapiro-Wilk tests revealed normal distributions of scores in all conditions across all areas. Levene's tests revealed homogenous variance across groups for all striatal areas: Nacc core, $F(3,15) = 2.76$, $p = .079$, shell, $F(3,15) = 1.41$, $p = .278$, DMS, $F(3,15) = 2.42$, $p = 106$, and DLS $F(3,15) = 1.97$, $p = .162$.

One-way ANOVAs revealed significant differences between drug treatment groups in terms of amount of co-expressing nuclei: Nacc core, $F(3,15) = 8.18$, $p = .002$, Nacc shell, $F(3,15) = 7.32$, $p = .003$, DMS, $F(3,15) = 11.02$, $p < .001$, and DLS, $F(3,15) = 4.36$, $p = .021$. Gabriel's *post hoc* tests revealed that the cocaine-saline condition differed significantly from the saline-saline and saline-cocaine conditions in the core and shell. In the DMS, the saline-cocaine and cocaine saline conditions differed significantly from the saline-saline and heroin-saline conditions. Finally, in the DLS, saline-cocaine and cocaine-saline differed significantly only from the saline-saline condition.

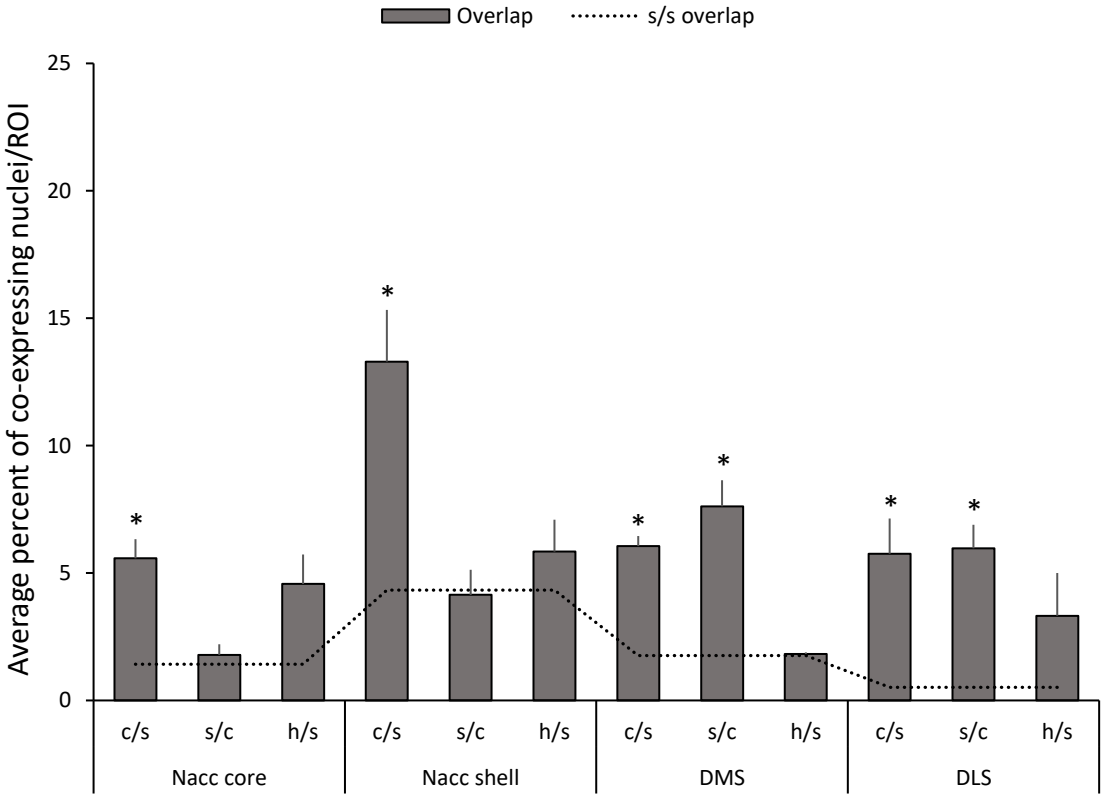

Fig. 4.8. Average percent of **co-expressing nuclei** as a function of drug treatment and striatal area. s - saline, c - cocaine, h - heroin. Error bars: ±1 SE. *significantly different from saline-saline. *N* for each group: s/s = 4, c/s = 6, s/c = 6, h/s = 3.

The co-expression of *arc* and *h1a* was calculated by dividing the number of nuclei positive for both Arc and H1a in each condition by the sum of *arc*-positive, *h1a*-positive and *arc*-and-*h1a*-positive nuclei in each condition. This ratio was then converted to percentage. Due to this choice of data representation, the number of *h1a*- and *arc*-positive nuclei in each brain area shown so far was represented in absolute numbers

rather than percentage of DAPI-positive nuclei. This way, it was avoided that the overlap was represented as percentage of a percentage which makes data interpretation somewhat convoluted.

Co-expression (overlap) - experimental conditions

Nacc core

Figure 4.9 (next page) shows the percentage of all mRNA-positive nuclei in each condition which were positive for both *arc* and *h1a*. The highest percentage of co-expressing nuclei were found in the cocaine-cocaine group, *M* = 16.44, *SE* = 1.98, followed by cocaine-heroin (200 µg/kg) M = 9.02, *SE* = 1.13, and cocaine-heroin (100 µg/kg) *M* = 8.27, *SE* = 2.53, heroin-cocaine, *M* = 12.71, *SE* = 0.79, heroin-heroin, *M* = 6.93, *SE* = 1.98, saline-saline, *M* = 1.42, *SE* = 0.86. Shapiro-Wilk tests revealed normal distributions of scores in all conditions.

Levene's tests revealed that the variance across groups was homogenous, $F(5,29) = 1.33$, $p = .278$. A one-way ANOVA revealed significant differences between conditions, $F(5,29) = 7.56$, $p < .001$. Gabriel's *post hoc* tests revealed that there was significantly higher percentage co-expressing nuclei in the cocaine-cocaine condition relative to all other conditions except heroin-cocaine. Only the cocaine-cocaine and heroin-cocaine groups were significantly different from saline-saline. All other comparisons were non-significant.

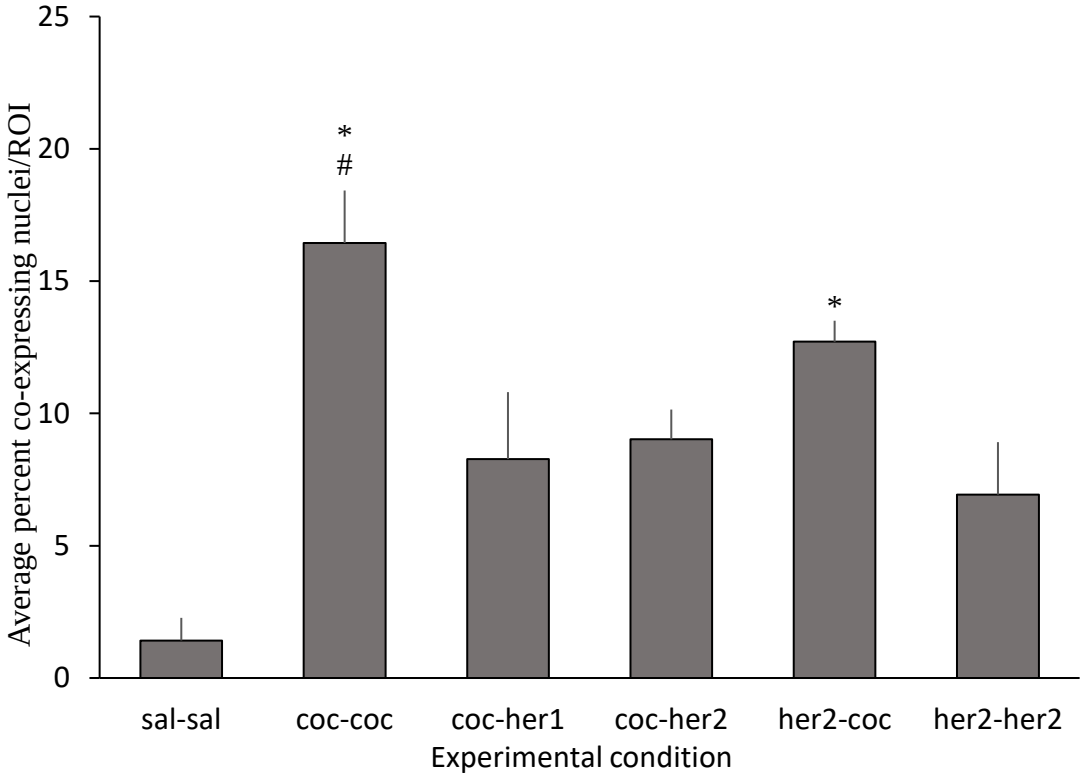

Fig. 4.9. **Co-expressing** nuclei as % of all mRNA+ nuclei in the **Nacc core**. Error bars represent +1 SEM. *significantly different from saline-saline. #significantly different from all other groups except heroin-cocaine. *N* for each group: sal-sal (4), coc=coc (6), coc-her1 (6),

Nacc shell

Overall, the pattern of co-expression in the shell was somewhat similar to that of the core (Fig. 4.10, next page). The highest levels of co-expression were found in the heroin-heroin, *M* = 17.92, *SE* = 3.69, heroin-cocaine, *M* = 17.79, *SE* = 2.69, and cocaine-cocaine conditions, *M* = 17.01, *SE* = 2.75. Co-expression was much lower in the rest of the conditions: saline-saline, *M* = 4.33, *SE* = 2.14, cocaine-heroin (100 µg/kg), *M* = 7.53, *SE* = 2.54, and cocaine-heroin (200 µg/kg), *M* = 7.64, *SE* = 1.3. Shapiro-Wilk tests revealed normal distributions in all conditions.

Levene's tests was non-significant, $F(5,29) = 1.38$, $p = .261$. A one-way ANOVA revealed significant differences between groups, $F(5,29) = 5.17$, $p = .002$. Gabriel's *post-hoc* tests revealed that there was significantly more co-expression in the heroin-cocaine and heroin-heroin groups relative to saline-saline. The difference between cocaine-cocaine and saline-saline only approached significance, $p = .059$.

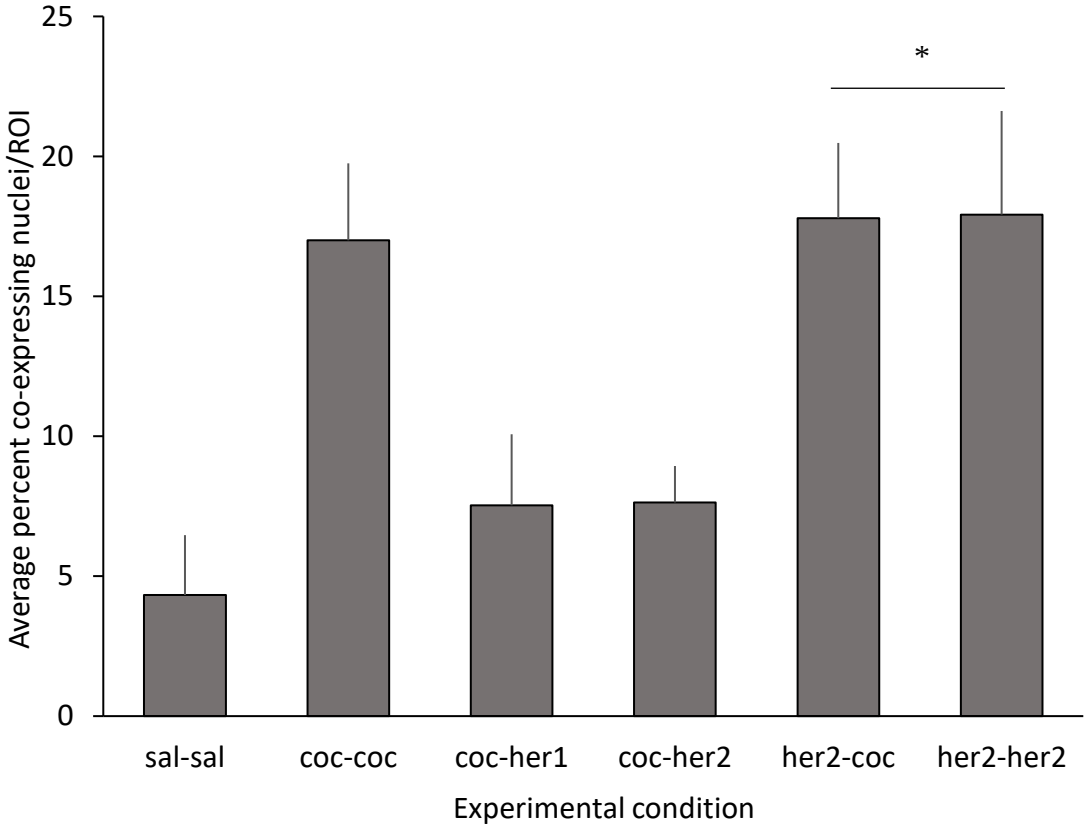

Fig. 4.10. **Co-expressing nuclei** as % of all mRNA+ nuclei in the **NAcc shell**. Error bars represent +1 SEM. *significantly different from saline-saline. *N* for each group: sal-sal (4), coc=coc (6), coc-her1 (6), coc-her2 (7), her2-coc (6), her2-her2 (6).

DMS

In this brain area, only the cocaine-cocaine condition had a noticeable amount of co-expressing nuclei relative to the overall level of mRNA expression, *M* = 19.43, *SE* = 2.57 (Fig. 4.11, next page). In all other conditions, the percentage of co-expressing nuclei was low: saline-saline (*M* = 1.76, *SE* = 1.24), heroin-cocaine (*M* = 7.69, *SE* = 1.44), heroin-heroin (*M* = 3.53, *SE* = 0.72), cocaine-heroin 100 µg/kg (*M* = 5.64, *SE* = 1.28) and cocaine-heroin 200 µg/kg (*M* = 5.31, *SE* = 0.48). Shapiro-Wilk tests revealed normal distributions in all conditions except heroin-heroin.

Levene's test did not reach significance, $F(5,29) = 2.05$, $p = .101$. A Kruskal-Wallis tests revealed significant differences between groups, $H(5) = 21.3$, $p = .001$, and was followed by multiple comparisons using Dunn-Bonferroni. The only significant differences found were between cocaine-cocaine and saline-saline, and cocaine-cocaine and heroin-heroin.

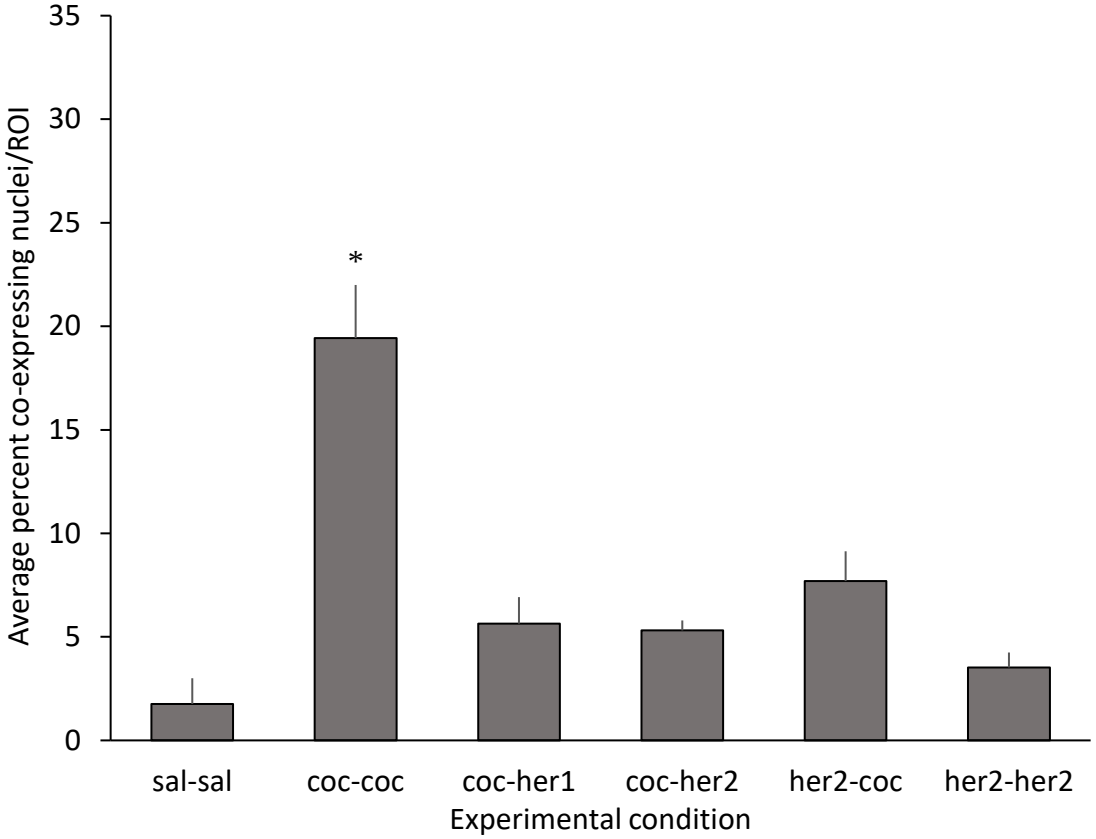

Fig. 4.11. **Co-expressing nuclei** as % of all mRNA+ nuclei in the **DMS**. Error bars represent +1 SEM. *significantly different from saline-saline and heroin-heroin. *N* for each group: sal-sal (4), coc-coc (6), coc-her1 (6), coc-her2 (7), her2-coc (6), her2-her2 (6).

DLS

The co-expression profile in this brain area was similar to that of the DMS (Fig. 4.12, next page). Again, the highest measure of co-expression was found in the cocaine-cocaine condition, *M* = 23.81, *SE* = 4.88. The percent co-expressing nuclei in the rest of the conditions was as follows: saline-saline (*M* = 0.52, *SE* = 0.52), heroin-cocaine (*M* = 6.97, *SE* = 1.26), heroin-heroin (*M* = 1.46, *SE* = 1.05), cocaine-heroin 100 µg/kg (*M* = 5.17, *SE* = 1.08) and cocaine-heroin 200 µg/kg (*M* = 5.87, *SE* = 1.07). Shapiro-Wilk tests revealed non-normal distributions in several conditions.

Levene's tests revealed lack of homogeneity of variance, $F(5,29) = 17.17$, $p < .001$. A Kruskal-Wallis test indicated a significant difference between groups, $H(5) = 24.82$, $p < .001$. Multiple comparisons were carried out using Dunn-Bonferroni. The only significant differences found were between cocaine-cocaine and saline-saline, and cocaine-cocaine and heroin-heroin.

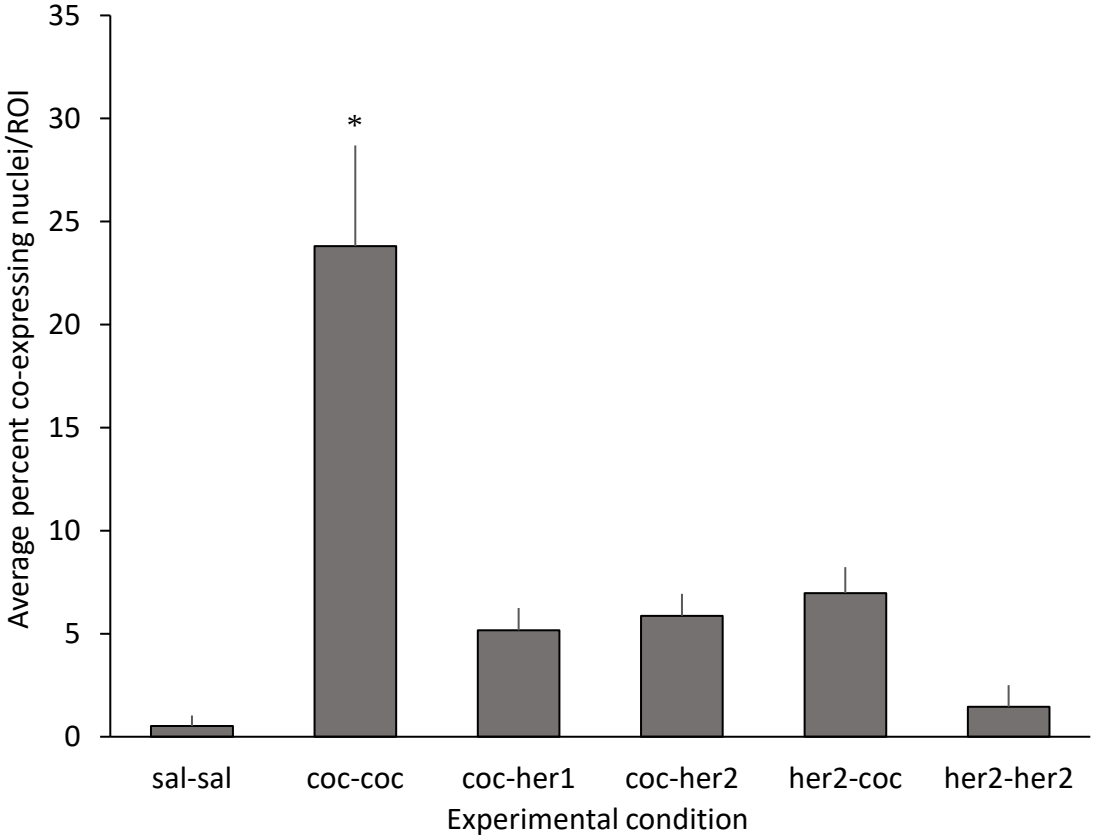

Fig. 4.12. **Co-expressing nuclei** as a % of all mRNA+ nuclei in the **DLS**. Error bars represent +1 SEM. *significantly different from saline-saline and heroin-heroin. *N* for each group: sal-sal (4), coc=coc (6), coc-her1 (6), coc-her2 (7), her2-coc (6), her2-her2 (6).

Table 4.3. **Summary of *homer 1a* and *arc* co-expression after administration of two drug injections. The effect of each drug combination on co-expression is shown relative to saline-saline and cocaine-cocaine in each brain area: (↑) significant increase, (–) no sig. difference, (↓) significant decrease.**

	Core		Shell		DMS		DLS	
	s/s	**c/c**	**s/s**	**c/c**	**s/s**	**c/c**	**s/s**	**c/c**
c/c	↑		–		↑		↑	
c/h1	–	↓	–	–	–	–	–	–
c/h2	–	↓	–	–	–	–	–	–
h2/c	↑	–	↑	–	–	–	–	–
h2/h2	–	↓	↑	–	–	↓	–	↓

Homer 1a and arc co-expression following two consecutive injection of the same drug

In all brain areas except the Nacc shell, two consecutive injections of cocaine elevated the proportion of co-expressing nuclei significantly above chance (i.e. relative to co-expression in the saline-saline condition). Conversely, two consecutive injections of heroin elevated co-expression levels only in the Nacc shell. As an additional measure of co-expression above chance, comparisons were made between heroin-heroin and heroin-saline in the shell, and cocaine-cocaine and cocaine-saline conditions in all other brain areas (fig. 4.13, next page). Where cocaine-cocaine and heroin-heroin did not differ from saline-saline, these comparisons were not made (see table 4.3 above).

Shapiro-Wilk tests revealed normal distributions in all conditions. One-tailed *t*-tests with a Bonferroni correction (α = .013) revealed significant differences between cocaine-cocaine groups and cocaine-saline in the Nacc core: $t(6.41) = 5.13$, $p = .001$, DMS: $t(10) = 5.15$, $p < .001$, and DLS: $t(5.79) = 5.13$, $p = .007$. The comparison between heroin groups in the Nacc shell was also significant, $t(6.02) = 3.09$, $p = .011$. Note that one-sided tests were used because these groups already showed increase above one measure of co-expression due to chance.

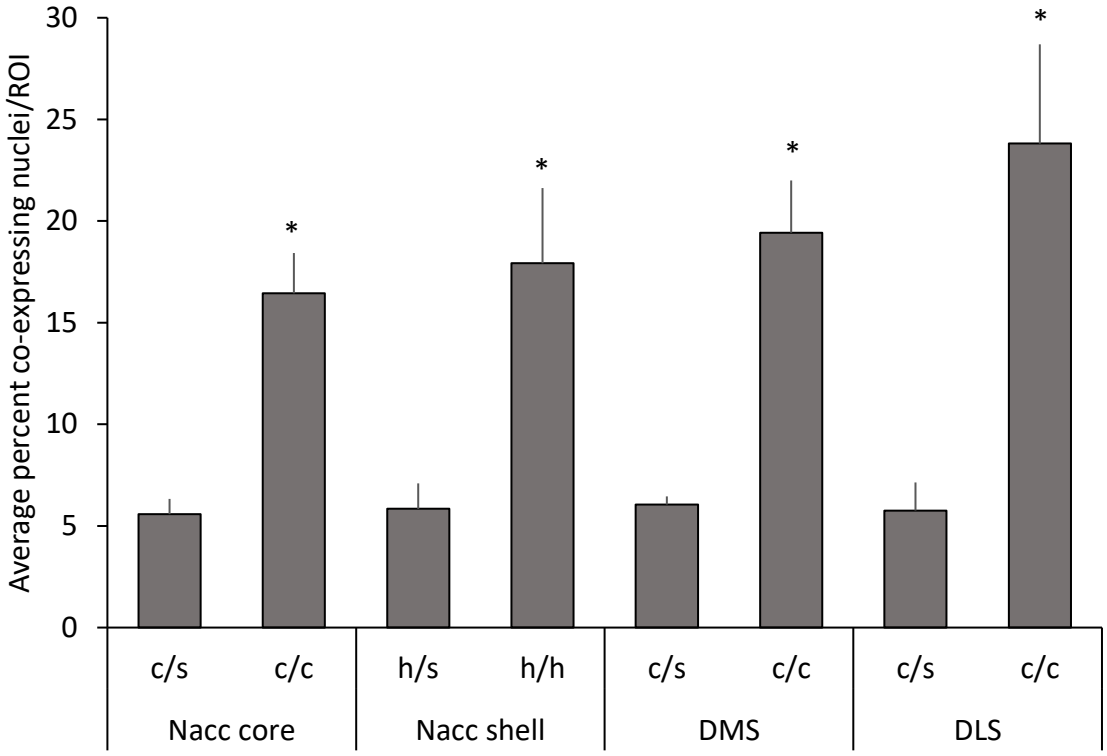

Fig. 4.13. **Co-expressing nuclei** as % of all mRNA+ cells following two consecutive injections of the same drug relative to one injection followed by saline. Error bars represent ± 1SE. s - saline, c - cocaine, h - heroin. *significantly different from the respective saline group. *N* for each group as follows: c/s (6), c/c (6), h/s (3), h/h (6).

Discussion

Main findings

The data presented here suggest that, when administered intravenously to the rat, both heroin and cocaine elevate levels of *h1a* and *arc* mRNA across the striatum. The magnitude of this effect varies depending on the particular gene and striatal area in question. Since both *arc* and *h1a* are markers of neuronal activity, it can be said that both heroin and cocaine activate neuronal populations across the striatum. By using the catFISH technique it was possible to determine if these neuronal populations represent neuronal ensembles, and whether these neuronal ensembles overlap between drugs. Indeed, across all examined areas of the striatum, there seems to be a group of neurons that are reliably activated by cocaine. Therefore, they likely represent a neuronal ensemble encoding for cocaine's effects. For heroin, this seems to be the case only in the Nacc shell, but not other areas.

When cocaine and heroin are administered to the same animal, the neuronal populations activated by each drug are more or less distinct, but this depends largely on the striatal area and the order in which the drugs were administered. In the dorsal striatum, the populations are largely distinct, regardless of order of administration. In the Nacc, heroin engages a different population of neurons than cocaine when cocaine is administered first. However, when cocaine is administered second, it engages many of the same neurons that were activated by heroin.

Cocaine effects on IEG expression

H1a expression was significantly elevated following cocaine administration in the Nacc core, DMS and DLS. On average, there was also more *h1a*-positive nuclei in the Nacc shell after cocaine relative to a saline injection, but this effect did not reach statistical significance. The magnitude of the effect was much more pronounced in the dorsal relative to ventral part of the striatum.

Cocaine also elevated *arc* expression, and this effect was statistically significant across all areas of the striatum, including the NAcc shell. Similarly to *h1a*, the effect was most pronounced in the dorsal relative to the ventral striatum. Note that *arc* expression reflects activity in response to the second injection, and the magnitude of the cocaine effect on *arc* expression depended on whether another drug was administered beforehand, and what drug that was. First and second injection interactions are discussed further on.

In summary, cocaine increased activity in all areas of the striatum that were studied, and this activity was reflected in elevated *h1a* and *arc* expression. Cocaine's effect was more pronounced in terms of *arc* expression relative to *h1a* expression, especially in the Nacc shell, where *h1a* mRNA was low after cocaine (figs. 4.2 and 4.3). The most activity (i.e. IEG mRNA) following cocaine was observed in dorsal parts of the striatum.

Heroin effects on IEG expression

Heroin injections increased *h1a* expression relative to saline injections in the Nacc core and shell. There was also an increase of *h1a* expression after heroin in the dorsal striatum, but it was of comparable magnitude to the accumbens, rather than more pronounced as was the case for cocaine.

The effect of heroin on *arc* expression was not assessed on its own, as there was no group of rats which received an injection of saline followed by and injection of heroin. However, the cocaine-heroin (200 µg/kg) group differed from saline-saline in levels of *arc* in the Nacc core, shell and DMS, while cocaine-saline did not differ from control in any of these areas. Thus, there was a difference between administering heroin vs. saline following an injection of cocaine. However, there remains the possibility that this increase in *arc* expression is (at least to some extent) the result of an interaction between heroin and cocaine. There was no effect of cocaine on *arc* expression at the time when heroin was administered (i.e. cocaine-saline and saline-saline groups did not differ in *arc* levels), so any such interaction would have resulted from an effect of cocaine which is not reflected in IEG expression per se.

The effect of heroin on *arc* varied not only as a function of brain area, but also depending on dose and the preceding drug infusion. Relative to the higher dose, the lower dose of heroin (100 µg/kg) produced higher *arc* expression in the Nacc core and shell, but not in the dorsal striatum. However, these effects were much more variable and did not reach statistical significance. There are no other studies that have studied the acute effects of heroin on *arc* expression, especially soon after drug administration. There are studies using other opiates. For example, it has previously been shown that *arc* protein levels are elevated in the core but not shell of the NAcc following 10 mg/kg i.p. injections of morphine in mice (Lv, Xu, Han, & Cui, 2011), and that *arc* protein levels are elevated in the DMS 1 day after a single i.v. injection of heroin (Li, Liu, Lu, Wang, & Liu, 2013). Taking into account both of these findings and the results presented here, it seems that opiates can have both short and long-term effects on *arc* expression in the striatum.

To summarise, heroin (200 µg/kg) produced a reliable increase in *h1a* and *arc* expression across the striatum, but, due to the lack of a saline-heroin group, it remains unclear whether the effect on *arc* is unique to heroin, or the result of a cocaine-heroin interaction. In contrast to cocaine, there were no stark differences in IEG expression (number of activated cells) between dorsal and ventral parts of the striatum. In fact, for the lower dose of heroin (100 µg/kg), the difference went in the opposite direction, with more active cells on average in the Nacc. There was also a lot of variability in the magnitude of *arc* expression depending on preceding drug or saline injections.

Interactions between first and second drug injections

When heroin (200 µg/kg) was preceded by cocaine, *arc* levels increased relative to saline-saline in the Nacc and DMS. However, this was not the case if two 200 µg/kg doses of heroin were administered in succession, suggesting a habituation to the IEG-inducing effects of heroin. *Arc* levels in the cocaine-cocaine group remained elevated above saline-saline controls. However, a *post-hoc* analysis revealed that they were lower relative to *arc* levels in the saline-cocaine group across all areas except the Nacc core (*t*-tests with a Bonferroni correction, all *p'*s < .025, except Nacc core – $p = .05$). Habituation effects to repeated injections of cocaine have been reported previously for the protein product of *c-fos* (Hope et al., 1992).

There are three possible explanations for the reduced IEG expression following repeated drug injections. First, there could have been an adaptive change in the receptors involved in *arc* transcription. One candidate for such change could be the AMPA glutamate receptor, activity at which is known to inhibit *arc* transcription (Rao et al., 2006), because levels of the receptor are up-regulated in the accumbens following a single injection of cocaine. However, AMPA receptor up-regulation is not evident before 24h following a single cocaine injection, so it is unlikely to have had an effect when the two injections were separated by 25 min (Ferrario, Li, & Wolf, 2011). Also, it is not known if heroin affects AMPA receptor trafficking in a similar way. Second, there could have been negative feedback within the signalling cascade leading to IEG transcription. Both *arc* and *h1a* transcription are initiated through the MAPK/ERK pathway (Sato et al., 2001; Waltereit et al., 2001) which is inhibited by the MKP-1 phosphatase, a product of the IEG *mkp-1*, also transcribed as a result of neuronal activity through ERK, as shown on fig. 4.14 (Sgambato, Pages, Rogard, Besson, & Caboche, 1998). Thus, a negative-feedback loop within intracellular cascades could have resulted in the suppression of *arc* transcription in recently activated neurons. Finally, it should be considered that all animals in the present study were drug naïve. The higher levels of IEG expression in the case of the first drug injection might simply reflect arousal produced by the novelty of the stimulus. For example, the novelty aspect of an environment (i.e. even when physical characteristics are controlled for) increases *c-fos* expression in the striatum (Badiani et al., 1998). It is possible that a similar effect occurs with drug stimuli as well.

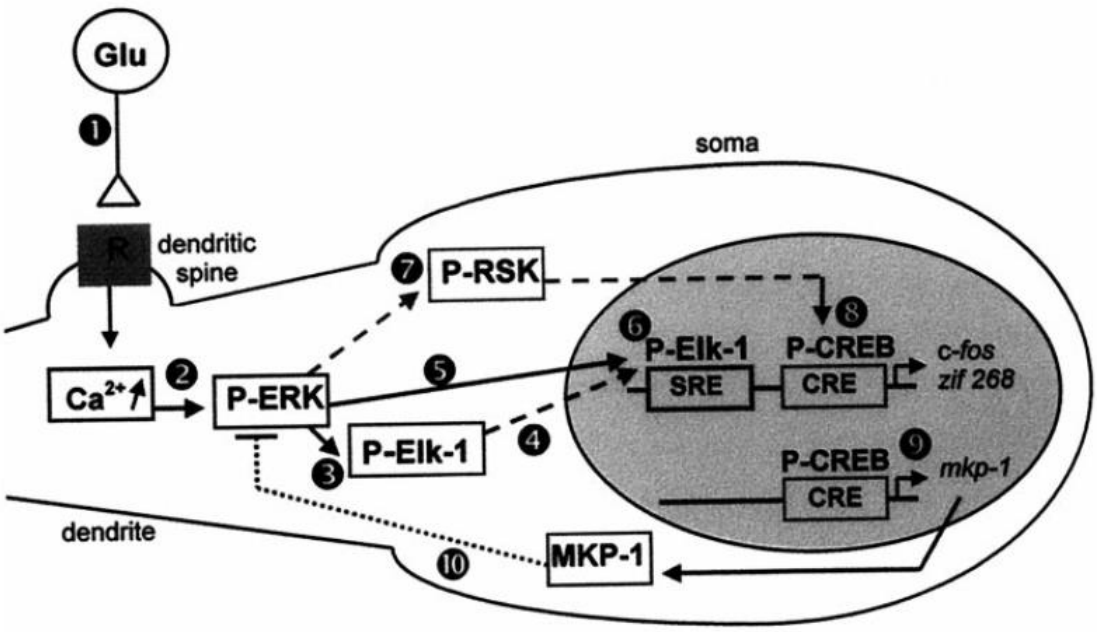

Fig 4.14. A schematic representing a possible negative feedback loop in the ERK intracellular signalling cascade. Following glutamatergic action at the NMDA receptor (1) calcium enters the postsynaptic cell (2) and activates the ERK pathway which ultimately promotes transcription of the gene *mkp-1* via CREB (9). This gene's protein product in turn deactivates ERK. Since the ERK pathway is one way in which *arc* transcription can be initiated, this negative feedback loop provides one explanation as to why *arc* expression is reduced with consecutive drug injections. Adapted from Sgambato et al. (1998).

Overlap between neuronal populations engaged by heroin and cocaine

Methodological considerations

Before discussing the overlap results reported here, it is necessary to take into account several methodological considerations. First, the measure of overlap employed here was somewhat different from those employed by other studies using catFISH or similar methods. For example, some studies report the amount of cells activated *only* by one stimulus (first *or* second), and then the amount of cells activated by *both* stimuli. In such cases, if the former type of cells are many and the latter few, it is concluded that the two stimuli activate different neuronal populations. Conversely, if there are many cells activated by both stimuli, but only a few activated by one stimulus only, then the stimuli activate the same neuronal population. This method is perhaps the most straight-forward, but the least flexible. It assumes that the first and second stimuli always activate roughly the same number of cells. It has been used in studies of the

hippocampus and exposure to a novel environment (Vazdarjanova & Guzowski, 2004; Vazdarjanova et al., 2002), and of the amygdala and fear conditioning (Zelikowsky, Hersman, Chawla, Barnes, & Fanselow, 2014).

Other studies report the amount of *all* cells active during the presentation of the first stimulus and then calculate the percentage of those cells that were also activated by the second stimulus – a "re-activation" measure in a sense. This method is useful in cases where activated neurons form a more variable pattern, and has been used for studies of the cortex (Grosso et al., 2015). Xiu et al. (2014) used a similar approach in their study, but instead calculated the percentage of cells activated by second stimulus that were also activated by the first. However, this method does not allow for a comparison between the degrees of overlap between two pairs of stimuli except under very specific circumstances. Namely, it is required that that there are no differences between the activity induced by each stimulus in each pair, or at least that the difference between the activity induced by each stimulus in each pair is the same.

In the study presented here there was a difference between the amount of cells activated by the first and second stimuli: cocaine-cocaine or cocaine-heroin. In addition, there were differences between the amounts of cells activated by each stimulus in different pairs of stimuli. Thus, neither of the methods described above was applicable. The solution was to calculate the overlap by representing the amount of cells activated by *both* stimuli as a percentage of all active cells (regardless if activated by first, second or both stimuli). In this way, any differences in magnitude of IEG response to each stimulus was controlled for. In addition, saline groups were added to measure overlap by chance. Thus all overlap measures must be considered in relation to saline-saline and drug-saline controls.

A second consideration should be that the reduced response to a second injection of cocaine might have led us to underestimate the amount of overlap in the cocaine-cocaine condition. Perhaps of this reason, the co-expressing cells in that condition did not exceed 25% of all mRNA+ cells. Since this experimental condition was used as point of comparison when it comes to estimating overlap between cocaine- and heroin-activated neuronal populations, it is likely that the reduction of overlap between heroin and cocaine populations is more substantial than it seems at first glance.

Finally, the *arc* response to a cocaine injection was consistently higher than the *h1a* response across all areas of the striatum. This means that the reduction in the response to a second injection of cocaine is quite substantial: *arc* expression in the saline-cocaine condition was almost three-fold higher than that in the cocaine-cocaine condition (e.g. figs. 4.3, p.77 and 4.6, p.83). In itself, this is an interesting finding suggesting that *arc* expression is switched on in an additional set of cells to those expressing *h1a*. Although the way overlap was calculated should have controlled for this difference, it is nevertheless relevant in interpreting high overlap in some cases where the response to the first injection of cocaine was low, such as in the Nacc shell.

In summary, the overlap measured by catFISH in this study was influenced by interaction between the first and second injections, differences between the amounts of cells activated by heroin and cocaine, and differences between the amounts of cells expressing *h1a* and *arc* in response to cocaine. To correct for these factors, overlap was calculated by controlling for overall amount of mRNA expression, and saline conditions controlled for overlap by chance.

Evidence for a neuronal ensemble encoding cocaine effects

Across all striatal areas except the Nacc shell, each of two consecutive injections of cocaine elevated mRNA expression levels more than vehicle. Thus, there was significantly more activity following a cocaine injection relative to a saline injection. Out of all these activated neurons, between 16 and 23% on average were activated by both cocaine injections, suggesting they were responding reliably to the drug. The saline-saline conditions revealed low levels of overlap by chance in these areas (Nacc core, DMS and DLS). Cocaine-saline conditions revealed low levels of overlap between drug-activated neurons and baseline activity (fig. 4.8, p.86 and fig. 4.13, p. 93). Therefore, the neurons activated by each of two consecutive injections of cocaine are likely to represent a part of a neuronal ensemble encoding for cocaine effects in the striatum.

The Nacc shell is an exception to this observation. The effect of cocaine on *h1a* expression was small, and did not reach significance. Conversely, there was an effect of cocaine on *arc* expression which was significantly increased (see fig. 4.2, p.75, saline-cocaine). Thus, cocaine does activate neurons in this area, but this activity is not

detected as an *increase* of *h1a* expression. This leaves two possibilities: (i) cocaine activates cells in the shell, but the activated cells do not express *h1a*; (ii) cocaine does not lead to a change in the *amount* of *h1a*-expressing cells relative to saline, but determines *which* cells will be expressing it. In the second case, the pattern of IEG expression produced by cocaine as a first injection is specific to the drug, but is undetectable as an increase relative to saline. This is possible considering the lower *h1a* response to cocaine relative to *arc* which is observed across all four areas of the striatum (figs. 4.2, p. 75 and 4.3, p. 77, saline-cocaine and cocaine-saline conditions). There is further support for this interpretation. Note that there was little activity and overlap in the saline-saline condition in the shell, suggesting that baseline activity and random overlap in this area are low. However, while there was no more overlap in the saline-cocaine condition relative to saline-saline, there was much higher degree of overlap in the cocaine-saline condition (fig. 4.8, p. 86). Thus, cocaine activates a particular set of neurons which do not overlap with baseline activity – hence no overlap in the saline-cocaine condition. A second injection of cocaine does very little in terms of IEG expression in the shell (fig. 4.5, p. 81), and the overlap in the cocaine-cocaine condition is not much higher than in the cocaine-saline condition. Therefore, the simplest explanation is that when cocaine is administered first, it produces activity that is sustained until the time of the second injection (i.e. 25 min later) – hence the overlap in the cocaine-saline condition. A more speculative explanation would be that the overlap in the cocaine-cocaine condition has been underestimated, while the overlap in the cocaine-saline condition has been overestimated due to the way overlap was measured (amount of co-expressing cells as a percentage of all mRNA+ cells). However, this is unlikely given the big difference between overlap in the cocaine-saline and saline-saline conditions in the shell (fig. 4.8, p. 86) which indicates that the overlap in the former condition was significantly above chance. In addition, the overall activation in the cocaine-cocaine and cocaine-saline conditions was similar (figs. 4.5. p. 81 and 4.2, p.75). Thus, the high overlap score in the cocaine-saline conditions is not simply the result of a small number of co-expressing cells forming a large percentage of all mRNA-expressing cells.

In summary, there is a population of neurons in the Nacc core, DMS, and DLS that respond reliably to consecutive injections of cocaine and are likely to represent a neuronal ensemble. In the Nacc shell, the existence of such a population of cells cannot be ascertained based on the data presented here, but it is safe to say that cocaine activates neurons in this striatal area as well. Some of these neurons remain active for at least 25 minutes.

Evidence for a neuronal ensemble encoding heroin effects

A dose of 200 µg/kg of heroin reliably elevated IEG expression in the Nacc core and shell. Heroin also reliably elevated *h1a* expression in the dorsal striatum, but *arc* expression in response to heroin was significantly elevated only in the DMS, but not DLS. In contrast to cocaine, two consecutive injections of heroin did not result in a large proportion of neurons activated by both injections in the core, DMS and DLS. The degree of overlap in these areas did not differ much from the overlap between neuronal populations active after heroin-saline injections or saline-saline injections. Thus, any overlap in these areas cannot be attributed to a group of cells that were reliably activated by heroin. However, there was relatively high level of overlap after consecutive injections of heroin in the Nacc shell. This was the case even though the overlap in the heroin-saline condition was low, and very similar to overlap in the saline-saline condition (figs. 4.10, p.89 and 4.8, p.86; see also fig. 4.13, p. 93). Thus, it seems there is a neuronal ensemble encoding the effects of heroin in the Nacc shell, but not the other areas of the striatum that were studied.

As already mentioned, *arc* expression was reduced following a second injection of heroin relative to an injection of heroin following cocaine. Thus, it is not possible to exclude the possibility that the low degree of overlap in the heroin-heroin condition is in part due to undetected active neurons. However, the *arc* expression in the shell following a second heroin injection was low as well, and the overlap score was high in this area. Thus, it is likely that if *arc* expression in the other areas was specific to heroin, it would have reflected in the overlap score there as well.

In summary, a 200 µg/kg dose of heroin activated more neurons across the striatum relative to saline, but only in the Nacc shell the same neurons responded reliably to the

drug. In contrast to the case of cocaine, the methods used here suggest that there are no neuronal ensembles encoding for heroin effects in other parts of the striatum.

Overlap between neuronal populations activated by heroin and cocaine

When an injection of cocaine was followed by an injection of heroin, the amount of neurons expressing both *arc* and *h1a* formed a small proportion of all mRNA-positive neurons. In fact, the cocaine-heroin conditions and the cocaine-saline conditions did not differ significantly in terms of the proportion of co-expressing neurons. Therefore, any overlap in the cocaine-heroin conditions can be attributed to the effect of cocaine alone. This was true regardless of heroin dose or striatal area. Thus, the neuronal ensembles responding to cocaine do so not just reliably, but also preferentially, at least under certain conditions. One of these conditions is the order in which heroin and cocaine were administered. When heroin is administered 25 min after cocaine, the populations of neurons activated by each drug are largely distinct. However, when heroin is administered first and cocaine second, there is an increase in the degree of overlap. Although this increase was not necessarily statistically significant, it was reliably observed across all striatal areas. The increase is most notable in the Nacc shell, where the overlap is similar to the overlap following two consecutive injections of heroin.

In summary, the overlap between the neuronal populations activated by heroin and cocaine in the striatum varies as a function of brain area and the order of drug administration. It can be said that the overlap is predominantly lower than the overlap between neuronal populations activated by consecutive injections of the same drug. The only exception is the Nacc shell, where heroin and cocaine activate largely the same neurons when cocaine is administered second.

Summary and conclusions

The study presented here was first of all an attempt at applying the catFISH technique to the study of pharmacological stimuli, and second – a characterisation of the neuronal populations activated by heroin and cocaine in different parts of the striatum. The results reported suggest that catFISH can indeed be used to study acute drug effects, but only with a number of methodological considerations in mind. Even with these considerations in mind, the results also point to the existence of a neuronal ensemble

encoding cocaine effects in both dorsal and ventral parts of the striatum. Heroin, on the other hand, activates a particular subset of neurons only in the Nacc shell, but not other part of the striatum.

There is also a significant separation between the neuronal populations activated by heroin and cocaine. These findings could explain why both heroin and cocaine produce activity in these areas as measured by IEG expression, while electrophysiological recordings from the Nacc core reveal different responses of the same neurons after administration of the two drugs (Chang et al., 1998). The order of drug administration matters for the amount of overlap between neuronal populations activated in the Nacc shell. As discussed earlier, previous studies have found high degree of overlap between neuronal populations activated by an injection of morphine and a following injection of cocaine in the Nacc shell of mice. Here, the same was found for heroin followed by cocaine, but not when cocaine was administered before heroin. Thus, future studies comparing the effects of heroin and cocaine in the same animal should take into account the order in which drugs are administered.

It is likely that neuronal activation and the corresponding IEG transcription that accompanies it are mostly the result of excitatory glutamatergic transmission. Cocaine-induced dopamine release potentiates the effects of glutamate on IEG expression, but is not sufficient for IEG expression in itself: chloral hydrate abolishes cocaine-induced c-fos expression by reducing glutamate but not dopamine release in the striatum (Kreuter, Mattson, Wang, You, & Hope, 2004). Thus, the separation of the neuronal populations found in this study could be the result of distinct glutamatergic afferent inputs to the Nacc being activated following cocaine and heroin injections. The three major sources of glutamatergic input to the Nacc shell are the hippocampus, BLA and PFC, with the hippocampus being the major one of the three. Optogenetic activation of all three inputs can sustain operant behaviour suggesting activity of neurons in the shell is sufficient for reinforcement (Britt et al., 2012). Both heroin and cocaine seem to engage a specific neuronal population in the shell. Thus, the activity elicited by heroin and cocaine in this brain area may reflect their reinforcing properties, regardless of whether this activity occurs in distinct neuronal populations.

In contrast, the separation of neuronal populations encoding for heroin and cocaine in the Nacc core could mean that these populations have non-equivalent functions. Transmission through the NMDA receptor in the Nacc core is necessary for food (Kelley, Smith-Roe, & Holahan, 1997) and cocaine self-administration, but not heroin self-administration (Pulvirenti, Maldonado-Lopez, & Koob, 1992). In addition, DA transmission in the accumbens plays a lesser role in heroin self-administration than in cocaine self-administration. The question remains open as to whether the neuronal activity in the Nacc core observed here is encoding the reinforcing properties of cocaine but not heroin. One way to test this hypothesis would be to selectively disrupt the cocaine-activated neuronal ensembles - e.g. using Daun02 (Koya et al., 2016) - and see if they would affect the reinforcing properties of heroin.

Chapter 5 – Applying catFISH to the context of drug self-administration

Introduction

The catFISH technique revealed that non-contingent administration of heroin and cocaine leads to the activation of neuronal ensembles that respond to cocaine across the whole striatum, while an ensemble for heroin is found only in the Nacc shell. Additionally, there was a significant separation of the neuronal populations responding to heroin and cocaine, at least under certain conditions. Thus, the striatum is encoding the effects of heroin and cocaine differently, at least in terms of neuronal activity marked by IEG expression. However, it remains unclear what is the functional significance of this activity. This chapter presents an attempt to apply the catFISH technique to a self-administration paradigm where rats get the opportunity to press a lever to obtain heroin and cocaine. In this way activity reflected in IEG expression could potentially be related to behaviours caused and driven by the two drugs.

Indeed, operant reinforcement has been shown to depend on neuronal activity in the striatum. For example, excitotoxic lesions of the Nacc can disrupt both heroin and cocaine self-administration (Zito, Vickers, & Roberts, 1985). In addition, optogenetic stimulation of dopamine D1 but not D2 receptor expressing medium spiny neurons (MSNs) of the dorsal striatum maintains operant responding in mice (Kravitz, Tye, & Kreitzer, 2012). Thus, activity across the striatum can be both necessary and sufficient to maintain operant conditioning, and this activity occurs in specific neurons. For this reason it is of special interest that heroin and cocaine activate largely distinct neuronal populations across the striatum: it is possible that dissociated circuitries underlie the operant reinforcing properties of the two drugs. However, it is also possible that the early encoding of heroin and cocaine effects does not reflect encoding following prolonged exposure to the two drugs. This is especially the case when activity is measured by IEG expression. The expression levels of *c-fos*, for example, can increase or

decrease (depending on brain area) from day 1 to day 6 of cocaine self-administration (Zahm et al., 2010). Thus, it is an empirical question whether *arc* and *homer 1a* will also change their patterns of expression following prolonged drug self-administration.

There are some studies that can inform a plausible hypothesis regarding the question. Fumagalli et al. have shown that a single 2h cocaine self-administration session has been found to elevate *arc* but not *zif268* mRNA levels in the mPFC of rats. In addition, the effect is not found in yoked controls receiving the same amount of drug. In the dorsal striatum, *arc* expression is significantly increased in both yoked and self-administering animals, while a non-significant increase is found only for the self-administering rats in the Nacc. For *zif268*, the increase in expression is found in both Nacc and dorsal striatum, regardless of whether cocaine is self-administered or received passively (Fumagalli et al., 2009). Following long-term cocaine self-administration (10 or 60 days), *arc* expression is slightly but significantly elevated in both the dorsal and ventral striatum. Increase in expression in these areas is also found for *c-fos*, with an even greater magnitude of the increase. However, *homer 1a* is not expressed above control levels in the striatum under the same conditions. In the mPFC, *c-fos* expression is also increased after long-term self-administration, while *homer 1a* and *arc* are only elevated following 60 but not 10 day self-administration (Gao, Limpens, Spijker, Vanderschuren, & Voorn, 2017). Thus, IEG expression following cocaine self-administration depends on many factors including length of self-administration training, type of IEG, and brain area of interest. It also noteworthy that passively received cocaine and self-administered cocaine can have different effects on IEG expression.

To date, there are no known studies that have examined the effects of heroin self-administration on *homer 1a* expression in the brain. Only one study has investigated the effect of heroin self-administration on *arc* protein expression, where rats were trained to self-administer heroin in a runway self-administration model (Li et al., 2013). After 1, 7, and 15 runway trials (1 trial per day), *arc* protein levels were significantly elevated in the mPFC, Nacc and DMS. *Arc* protein expression and run time were reduced to control levels after administration of NMDA and D1 receptor antagonists, suggesting that glutamatergic and dopaminergic neurotransmission are necessary for the behaviour and

protein expression. Passively received heroin also increased *arc* protein levels in all three brain areas.

In summary, there are only a few studies that have studied the effects of heroin and cocaine self-administration on *arc* and *homer 1a* expression, but they all point to increased expression of both genes in the mPFC. It also seems that, at least under certain conditions, *homer 1a* expression in the striatum is not increased above baseline after several days of cocaine self-administration. While *arc* is, there are regional differences between ventral and dorsal striatum, and the length of self-administration also matters, with longer training having a more pronounced affect.

The self-administration paradigm constitutes a model of motivated drug-taking behaviour where drugs act as reinforcers (Panlilio & Goldberg, 2007). Considering that drug self-administration can also induce IEG expression in some brain areas and under certain conditions, the model provides a good opportunity to study active neuronal populations using catFISH. It was already shown that non-contingent administration of heroin and cocaine activate largely different neuronal populations across the striatum. Applying the catFISH technique to a self-administration paradigm would provide an opportunity to associate the activity of these neuronal populations with the reinforcing properties of the two drugs. Provided that dissociation is found again, it would be direct evidence for distinct circuitry underlying heroin- and cocaine-taking.

The self-administration procedure also provides an opportunity to record and characterise other drug-induced behaviour such as locomotion and stereotypy. These behaviours are thought to be mediated in large part by dopaminergic neurotransmission and activity within the ventral and dorsal striatum. For example, microinjections of cocaine in the Nacc elicit locomotor behaviour in rats, and the effect is blocked by the DA receptor antagonist cis-flupenthixol (Delfs, Schreiber, & Kelley, 1990). Amphetamine (an indirect DA agonist) injected into the ventral lateral striatum elicits robust oral stereotypies such as gnawing, biting and licking (Kelley, Lang, & Gauthier, 1988). Thus, it can be expected that increases in IEG expression in the striatum will be correlated to locomotor behaviour and stereotypy, because IEG expression also reflects neuronal activity and DA neurotransmission to a great extent. In addition, drug-induced locomotor behaviour does not predict how well rats learn cocaine self-administration,

so it is likely that the two types of behaviour are mediated by separate neuronal systems within the striatum (Mandt, Johnston, Zahniser, & Allen, 2012; Mandt, Schenk, Zahniser, & Allen, 2008; Mantsch, Ho, Schlussman, & Kreek, 2001). Finally, operational definitions of locomotor behaviour can influence outcome measures of the behaviour, and stereotyped behaviour can be misrepresented as lack of drug effects on behaviour. For this reason several approaches to measuring drug-induced behaviour are preferred.

The study presented here describes a pilot experiment where the catFISH technique was applied to a self-administration paradigm. Rats were trained to self-administer heroin and cocaine at doses of 100 µg/kg and 800 µg/kg, respectively. These doses were chosen to correspond to the doses used in the experiment reported in chapter 4. The higher dose of heroin (200 µg/kg) was not used, because preliminary observations revealed that some rats became sedated and did not respond on the second lever during test sessions (test session described in procedure). After training, each rat was allowed to self-administer a combination of two drug injections 25 min apart, or was simply exposed to the self-administration environment. The aim of the study was to explore the possibility of measuring overlap between neuronal populations active during operant responding for drugs as opposed to passively received heroin and cocaine. Since *arc* and *homer 1a* differ in their expression patterns following drug self-administration, and their expression is not always elevated in the striatum, the PFC was also examined for changes in IEG expression. Finally, several measures of behaviour were taken immediately after self-administered heroin and cocaine infusions in order to find potential correlations between IEG expression and different aspects of behaviour.

Methods

Animals, housing and testing cages

The rats in this experiment (*n* = 20) weighed 300-340g at the time of testing. Two of them were supplied by Charles-River, UK. Otherwise, supplier, weight upon arrival, and housing conditions were as described in general methods.

The animals were kept in the housing cages described in general methods except during self-administration training and testing sessions, when they were moved to the operant chambers. These had two operating retractable levers positioned on opposite walls of

the chamber, with white LED lights positioned on the protruding part of each lever. Cameras were placed on top of each operant chamber to allow for recording of behaviour during testing sessions (details in procedure section).

Drugs

Heroin HCL was dissolved in sterile saline at a concentration of 0.6-0.68 mg/ml (depending on the weight of individual rats). Cocaine HCL was dissolved at a concentration of 4.8-5.5 mg/ml. All drug infusions had a volume of 50 µl, which corresponded to doses of 100 µg/kg for heroin, and a dose of 800 µg/kg for cocaine. The infusions were delivered over 5 sec using the same pumps as described in chapter 4, (infusion rate 10 µl/sec), but here these were activated contingently on the rats pressing a lever.

Procedure

Acclimatization and catheter surgery were done as described in general methods.

Self-administration training. After a recovery period of 7 days, rats were habituated to the self-administration chambers during two 2h periods on two separate days. During habituation, the rats were connected to the infusion lines via their implanted cannulas and the springs suspended on the counterbalanced arms of the self-administration chambers. The animals were left to explore freely and no drugs were available for self-administration.

Following habituation, each rat underwent several stages of self-administration training where intravenous infusions of heroin or cocaine (on alternate days) were contingent upon the pressing of a lever (FR1 schedule). Each drug was paired with a different lever.

There was one self-administration session a day, and each session lasted for a total of 6h, and there were no more than 50 infusions available. In cases where rats reached the maximum number of infusions early, they were left in the chamber for the whole duration of the session. The sessions began with the insertion of the lever with the LED lights on. It remained extracted until the rat pressed it which led to the lever being retracted, lights going off, and the delivery of a 50 µl infusion over 5 sec. This was followed by a timeout period (TO). The duration of the TO between infusions progressed

from one session to another in the following way: 1 min, 5 min, 10 min, 15 min and 25 min. This was done so that the rats got accustomed to the conditions of the final test session, described further on. The progression from one TO to the next for each rat was dependent on it reaching one of two criteria: self-administering 30% of the infusions available for the previous session or pressing the lever within 2 min after its first presentation for 4 consecutive sessions. Note that the total number of available infusions differed depending on the TO for the session (e.g. for 10 min TO, there were 36 infusions available, for a 15 min TO, there were a total of 24 infusions available).

Self-administration test session. After each rat had undergone at least two self-administration sessions with each of the 5 TOs, it was subjected to a test session on a separate day which lasted ~30 min. The session began with the insertion of one of the levers which remained extracted for a maximum of 2.5 min or until the rat pressed it, whichever came first. If the rat failed to press, the session ended, and testing was restarted. If the rat did press within the designated time, it received an infusion of the drug associated with the lever. Then, a 25 min TO period followed after which a lever was presented for a second time, again for a maximum of 2.5 min or until the rat pressed it. If the rat did not press, the session ended and the rat was tested again on the following day. If it did press, a second infusion was delivered, followed by a period of ~5 min. At the end of this period, the rat was euthanised with an intravenous injection of pentobarbital delivered manually through its catheter via a syringe. Then, the rat was decapitated, brain tissue was extracted, snap-frozen in isopentane and stored for catFISH analysis. There were 4 different drug combinations that the rats were allowed to self-administer: heroin-heroin, cocaine-cocaine, heroin-cocaine and cocaine-heroin. A final group of rats was exposed to the self-administration environment for the duration of the tests session, but no levers were presented and no drug infusions were available (exposure only, control group).

The reason for choosing this type of control was that some animals in the control group had blocked catheters at the time of the test session, so having animals press for saline infusions was not possible. Additional animals could not be tested due to time constraints. It was possible to expose animals in the control group to the drug-associated levers without any infusions available. However, preliminary observations revealed that

rats would rarely press a second time if pressing the first lever had no consequences. So, exposing control animals to the drug-associated levers would have resulted in differences between experimental and control conditions in terms of lever-pressing behaviour. Thus, choosing only exposure to the drug-associated environment as a control condition was the best compromise in light of practical issues (catheter patency issues and time constraints) rather than a choice made on theoretical grounds. It is noted that, as a result, some of the behavioural changes in the experimental groups can be attributed to the conditioned effects of a discrete cue - presentation of the drug-associated lever.

Precise administration of two separate doses of the same or different drugs was achieved through back-filling of the infusion line similarly to the experiment described in the previous chapter. Behaviour was recorded for the whole duration of the test session, but different behavioural measures were taken at different time points (detailed description available in continuation).

CatFISH. Whole brains were sectioned at 16 µm thickness, and coronal slices were taken at the level of the mPFC and the anterior striatum, (+3.70 mm, and +1.70 mm from bregma, respectively (Paxinos & Watson, 1986)). As in the procedures described in chapters 3 and 4, sections were removed starting from the tip of the olfactory bulbs until the ventral part of the brain sections (most caudal part of the olfactory bulbs) became attached to the dorsal part of the sections. At this point, the anterior forceps of the corpus callosum (fmi) was visible, and the lateral fissure reached halfway to the midline (+3.70 mm from bregma, fig. 5.1, next page). At this point, images of the PFC were taken from three locations. First, an imaginary line was followed from the tip of the lateral fissure to the midline. Where the two met was the point where the bottom left margin of the microscope field of view (MFV) was positioned. This was defined as the infralimbic cortex (IL). The MFV was then moved in a dorsal direction, until it was positioned between the midline and the medial side of the fmi. This was defined as the prelimbic cortex (PL). Finally, the MFV was moved further in the dorsal direction, along the midline, until the top margin of the MFV reached the angle between the midline and the most dorsal end of the section. This was defined as the anterior cingulate dorsal cortex (ACd). The sections of the striatum were delineated as described in chapter 4 (fig 5.2, next

page). The RNAscope probes and reagents, the *in situ* hybridisation, and the image acquisition and analysis protocols were as described in general methods.

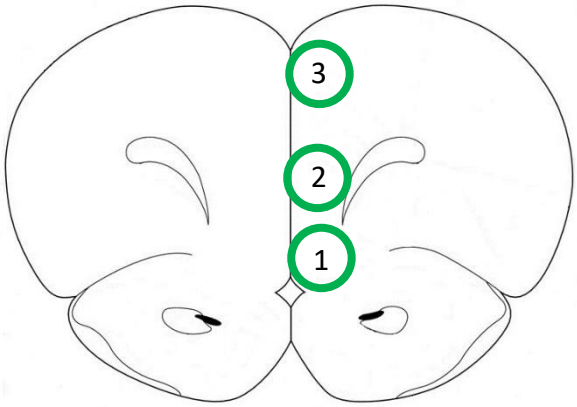

Fig. 5.1. Schematic representation of the rostrocaudal level defined as +3.70 mm from bregma during sectioning. Reference structures were as described in ch. 3. Green circles represent ROIs where images of the IL (1), PL (2), and ACd (3) were taken.

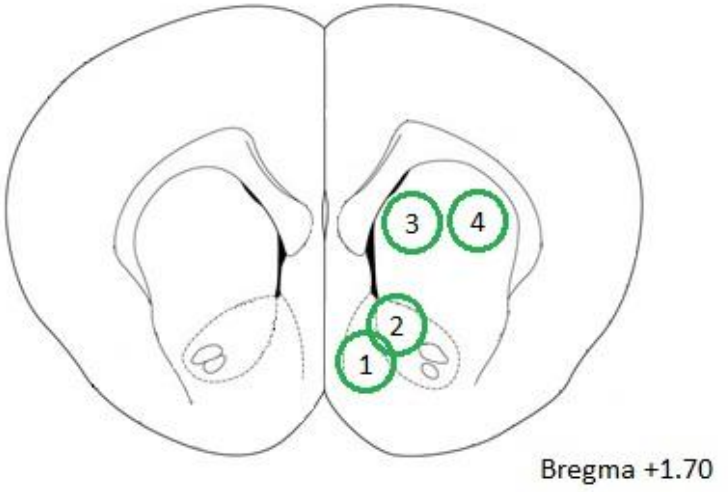

Fig. 5.2. Schematic representation of regions of interest (ROI) where microscopic images were taken across the striatum: Nacc shell (1), Nacc core (2), DMS (3) and DLS (4). Reference structures were the same as described in ch.4.

Design and behavioural measures

This study had a mixed design. The between-subject independent variable was the combination of drugs that each rat was allowed to self-administer during the final testing session (heroin-heroin, cocaine-cocaine, heroin-cocaine, cocaine-heroin or no drug, n = 4 in each group). For some of the statistical analyses rats were grouped based

on the first or second drug they self-administered. Within-subject independent variables were the different time periods during which behaviour was recorded within the testing session. Each rat was observed at several time points after the administration of the first drug and immediately after the administration of the second drug. The dependent variables were several behavioural measures. For the sake of analysis, the testing session was divided in 2 main periods – first 25 minutes reflecting the behavioural effects of the first drug, and last 5 min reflecting the effect of the second drug together with any potential carry-over effects of the first infusion. The first 25 min period was further divided in 5 min bins (labelled a-e, fig. 5.3, below).

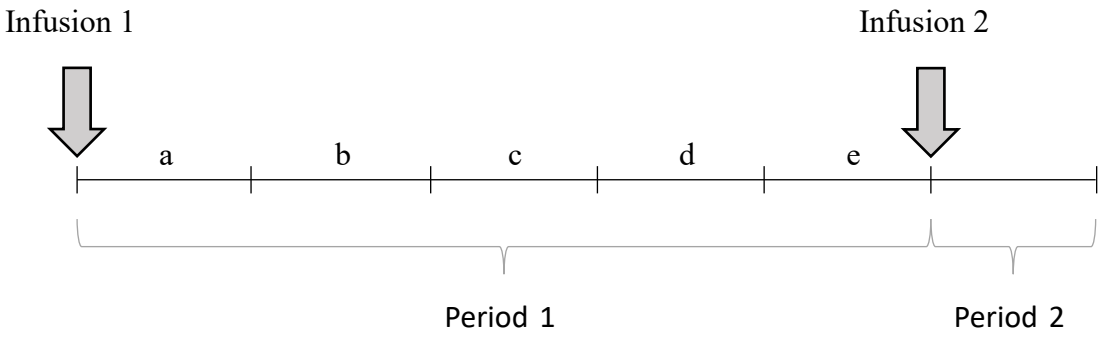

Fig. 5.3. Schematic representation of the final tests session timeline. Sections "a – e" represent 5 min bins of period 1.

First, locomotion was measured by dividing the bottom of the self-administration chamber in four quadrants of equal size and counting the number of crosses each rat made over the borders of these quadrants. A crossover was defined as when the shoulder blades of the animal cross from one quadrant to another. Crossovers were counted during the whole periods 1a, 1c, 1e and the whole duration of period 2, and scores were converted in crosses/min.

Second, the activity scale proposed by Ellinwood and Balster (1974) was used to provide an additional measure of drug-induced motor activity and stereotypy. Originally, the scale consists of 9 points corresponding to progressing levels of normal to drug-induced motor activity starting from none (0) and ending in stereotypical movements and dyskinesia (8, 9). A slight modification was made to the original order of the 5^{th} and 6^{th} points of the scale (the two were switched), because it was concluded that such order better reflects the progression from normal to drug-induced activity (table 5.1, next

page). Scoring using this scale was done by observing animals for the last 20 sec of periods 1a-e and the last 20 sec of the 5th min of period 2 and assigning the appropriate score.

***Table 5.1*. Scores, corresponding activity levels and operational definitions as adapted from the scale proposed by Ellinwood and Balster.**

Score	Activity level	Definition
1	**asleep**	lying down, eyes closed
2	**inactive**	lying down, eyes open
3	**in place activity (grooming)**	normal grooming (scratching, licking self, rubbing face with paws)
4	**normal/alert/active**	moving about cage, sniffing, rearing
5	**slow-patterned**	repetitive exploration of the cage at normal level of activity
6	**hyperactive**	running movement, rapid changes in position (jerky)
7	**fast patterned**	repetitive exploration of the cage with hyperactivity
8	**restricted**	remaining in the same place in cage with fast repetitive head/foreleg movement (licking, chewing, gnawing stereotypies)
9	**dyskinetic-reactive**	backing-up, jumping, seizures, abnormally maintained postures, dyskinetic movements

Third, the latency to press the lever after each of the two lever presentations during the test session was recorded in seconds. This was defined as the amount of time between the moment the lever was extracted and the moment the rat pressed it.

Finally, the occurrence of pre-defined categories of behaviour were counted according to methodology adopted from Fray, Sahakian, Robbins, Koob, and Iversen (1980). There were 10 behavioural categories in total (table 5.2, next page). Animals were observed during the last 10 sec of periods 1a-e as well as during the last 10 sec of the first 5 min of period 2 and the presence of any of the aforementioned behaviours was noted down.

***Table 5.2*. Categories and operational definitions for counting the occurrence of particular behaviours (adopted from Fray et al.).**

Category	Definition
still	Asleep or not moving, with an occasional sniff
locomotion	All four legs moving
rearing	Both front feet off the cage floor
sniffing	Sniffing for more than 3 s
licking	Licking for more than 3 s
gnawing	Gnawing for more than 3 s
head down	Animal standing, walking or running with its nose below horizontal for more than 5 s
swaying	Rhythmic swaying movements of the animal's head or body for more than 3 s
grooming	Grooming for more than 3 s
miscellaneous	Any category of behaviour not already listed that occurs for more than 3 s

Results

Self-administration training

A number of caveats must be taken into account when interpreting the self-administration data presented. These caveats arise from the differences in the speed of learning of individual rats. Every rat had to reach a criterion to progress from one stage of training to another, and different stages had different number of available infusions (due to different TO periods). In addition, the dose of cocaine was varied for some rats during the initial stages of training (to facilitate drug-taking in case the final testing dose is too high for acquisition). Finally, some rats required less training sessions than others overall. For all these reasons, the self-administration data are presented in several different ways, as seen in continuation.

Figure 5.4 (p. 117) shows the average number of infusions self-administered as a function of session and drug. Since individual rats were trained for varying number of sessions depending on how quickly they acquired the behaviour (min 5, max 10 sessions), the data in fig. 5.4 represents self-administration for the last 7 sessions of training only.

Where rats learned quicker than that (4 out of 20 cases in total), their score was not included in the calculation of the mean for sessions they did not participate in.

Rats pressed more (and more consistently) for heroin than for cocaine across all of the last 7 sessions of training. There was an increase in number of lever presses for heroin from the first to the last session (M = 11.75, SE = 1.38, and M = 15.63, SE = 0.93, respectively), while this was not the case for cocaine (first session: M = 8.4, SE = 3.19; last session: M = 4.25, SE = 1.3).

Shapiro-Wilk tests revealed non-normal distributions of the scores for several of the sessions. Friedman's ANOVAs revealed a significant difference between levels of heroin self-administration across sessions ($\chi^2(6) = 32.49$, $p < .001$), but there was no such difference in the case of cocaine ($\chi^2(6) = 4.02$, $p = .674$). Follow-up Wilcoxon's tests were run with a Bonferroni correction ($\alpha = .025$) to compare the amount of lever presses between the first and last sessions for both heroin and cocaine. Both tests were non-significant: z = -2.15, p = .031 (heroin) and z = -0.41, p = .682 (cocaine).

It must be noted here that the apparent decrease in self-administration over the last three sessions for both heroin and cocaine arises from the transition to sessions with longer TO periods and fewer available infusions.

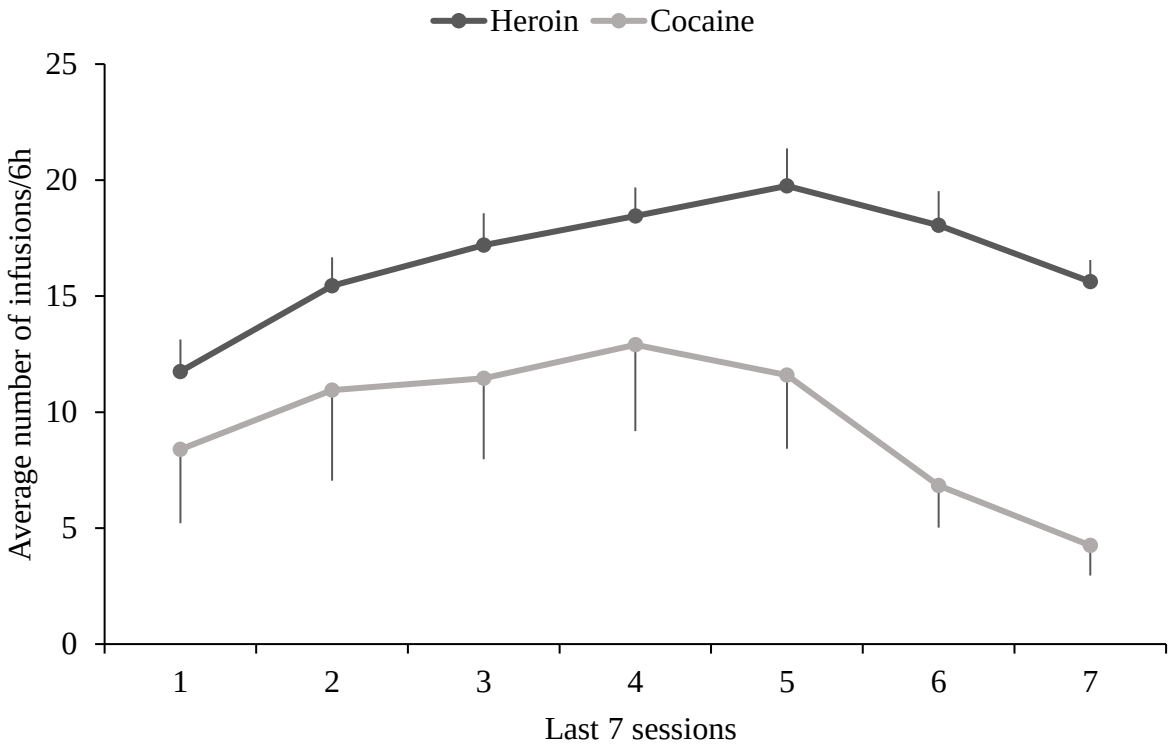

Fig. 5.4. Average number of infusions self-administered as a function of session and drug. Data is shown for last 7 sessions of training only. Error bars represent ± 1 SE. N = 20 for each drug.

To account for the change in TO and number of available infusions, the same data was also presented as percent of total available infusions self-administered for each session (fig. 5.5, next page). On average, rats increased heroin intake from the first session (M = 23.5%, SE = 2.76), approaching the maximum amount of available heroin infusions by the last session (M = 87.45%, SE = 4.51). In contrast, they were much less inclined to self-administer cocaine, seen as a lower amount of infusions self-administered on session 1 (M = 16.6%, SE = 6.4) and as reaching less than a third of the available infusions by the last session (M = 27.86%, SE = 8.71). Note that the variability in cocaine self-administration is also higher.

Shapiro-Wilk tests revealed a non-normal distribution of scores across sessions for both heroin and cocaine. Friedman's ANOVA tests were run and revealed significant changes between sessions for both heroin ($\chi^2(6) = 68.46$, $p < .001$) and cocaine ($\chi^2(6) = 15.54$, $p = .016$). Post-hoc Wilcoxon tests were run to compare self-administration scores between the first and last sessions for each drug. A Bonferroni correction was applied to correct for multiple comparisons, so $\alpha = .025$. Rats self-administered significantly

higher percentage of available infusions on the last session relative to the first session for both heroin (z = -3.52, p < .001) and cocaine (z = -2.27, p = .023).

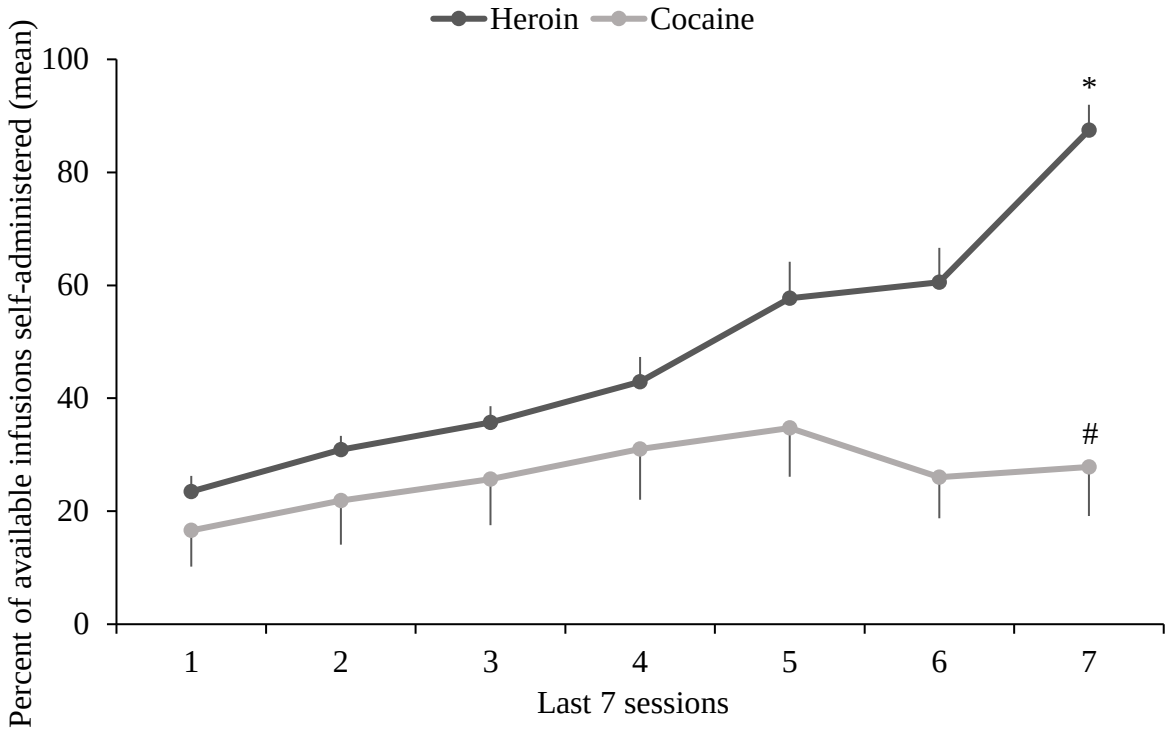

Fig. 5.5. Self-administration represented as percent of available infusions for each session, as a function of drug. Error bars represent ± 1 SE. */# - significantly different from the 1st session of the corresponding drug. *N* = 20 for each drug.

Figure 5.6 (next page) shows the cumulative amount of heroin and cocaine self-administered for each of the last 7 sessions in µg/kg. These data were presented to account for changes in the dose of cocaine across early training sessions for some rats that did not acquire the self-administration behaviour at the testing dose (800 µg/kg). For heroin, the amount of drug self-administered follows the lever-press data exactly, as the dose of 100 µg/kg did not change across sessions. On average, the highest amount of heroin and cocaine self-administered was found in session 5 (M = 1975, SE = 161.55 and M = 8400, SE = 2439.33, respectively).

Shapiro-Wilk tests revealed non-normal distributions of scores across several sessions. Friedman's ANOVAs revealed a significant change across sessions in the case of heroin ($\chi^2(6) = 32.49$, p < .001), but not cocaine ($\chi^2(6) = 9.99$, p = .125). A post-hoc Wilcoxon test revealed a significant difference between amounts of heroin taken in the first and last sessions (z = -2.15, p = .031).

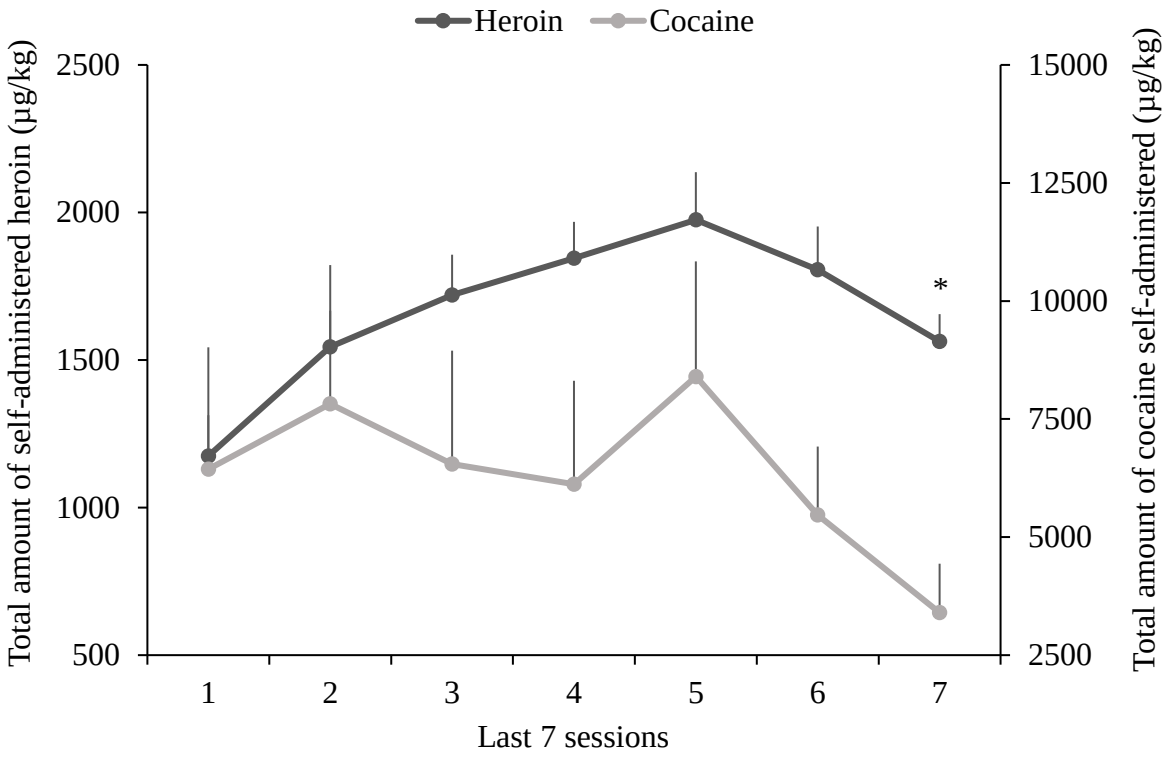

Fig. 5.6. Amount self-administered as a function of session and drug. Error bars represent ± 1 SE. * significantly different from session 1. *N* = 20 for each drug.

Finally, figure 5.7 (next page) shows the amount of heroin and cocaine taken during training by rats in each of the 4 experimental groups and the control group (testing day grouping). There was negligible difference in the amount of heroin taken by rats in all 5 groups (coc-coc: *M* = 12650, *SE* = 1657.56; coc-her: *M* = 10350, *SE* = 870.34; her-coc: *M* = 11650, *SE* = 629.15; her-her: *M* = 10900, *SE* = 1368.70; and control: *M* = 10125, *SE* = 2392.48). However, there was a more noticeable difference in the amount of cocaine taken by rats in each of the test day groups (coc-coc: *M* = 84050, *SE* = 43192.00; coc-her: *M* = 69200, *SE* = 24227.39; her-coc: *M* = 51750, *SE* = 13866.84; her-her: *M* = 6350, *SE* = 1206.58; and control: *M* = 3500, *SE* = 1173.31).

Shapiro-Wilk tests revealed normal distributions of the scores for amount of heroin taken across groups. The scores for amount of cocaine taken in the cocaine-cocaine group were not normally distributed. A one-way ANOVAs revealed that there was no significant difference between groups for amount of heroin taken ($F(4,15) = 0.46$, $p = .762$), while a Friedman's ANOVA revealed a significant difference in the case of amount of cocaine taken ($\chi^2(4) = 12.71$, $p = .013$).

Post-hoc Mann-Whitney tests were run to follow up on the Friedman's ANOVA, with a Bonferroni correction (α = .01). None of the comparisons were significant: heroin-heroin vs. control (U = 4, p = .343), heroin-heroin vs heroin-cocaine (U = 0, p = .029), heroin-heroin vs cocaine-heroin (U = 2, p = .114), heroin-heroin vs. cocaine-cocaine (U = 0, p = .029), and cocaine-cocaine vs. heroin-cocaine (U = 8, p = 1).

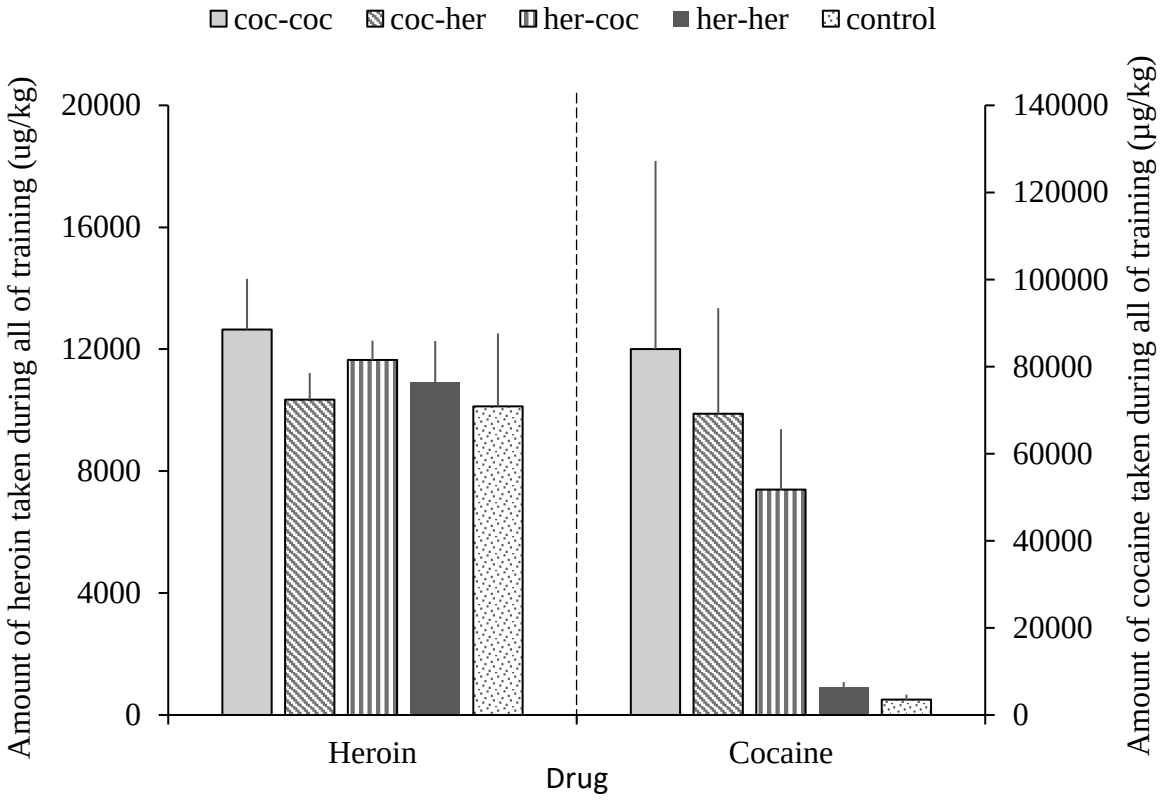

Fig. 5.7. Total amount of heroin and cocaine self-administered during training by rats in each of the test day groups. Error bars represent ± 1 SE. "exp only" - exposure only/control group. N = 4 for each group.

Measures of drug-induced behaviour

Measures of locomotion, activity as defined be the Ellinwood et al. (1984) scale, and categorical classification of behaviour are presented for the two periods following drug infusions on test day. The first period (period 1) spans 25 min following the first infusion, while the second period (period 2) consists of the 5 min following the second infusion.

Locomotion

Figure 5.8 (next page) shows the level of locomotor behaviour during the tests session as a function of first drug received and time. The control group was only exposed to the

self-administration environment. In the first 5 min following the drug infusion, rats that received heroin and cocaine moved the most (*M* = 8.3, *SE* = 3.5 and *M* = 8.85, *SE* = 1.1, respectively), relative to rats that did not receive a drug (*M* = 3.65, *SE* = 1.55). Ten minutes later, activity remained high in the heroin group (*M* = 7.44, *SE* = 1.61), while for cocaine and the control group locomotion diminished (*M* = 3.81, *SE* = 0.52 and *M* = 1.43, *SE* = 0.69, respectively). In the 20-25 min period post injection, locomotion in the heroin group was still relatively high (*M* = 6.36, *SE* = 1.69), while in the cocaine group it was closer to controls (*M* = 2.84, *SE* = 0.57 and *M* = 1.25, *SE* = 0.85, respectively).

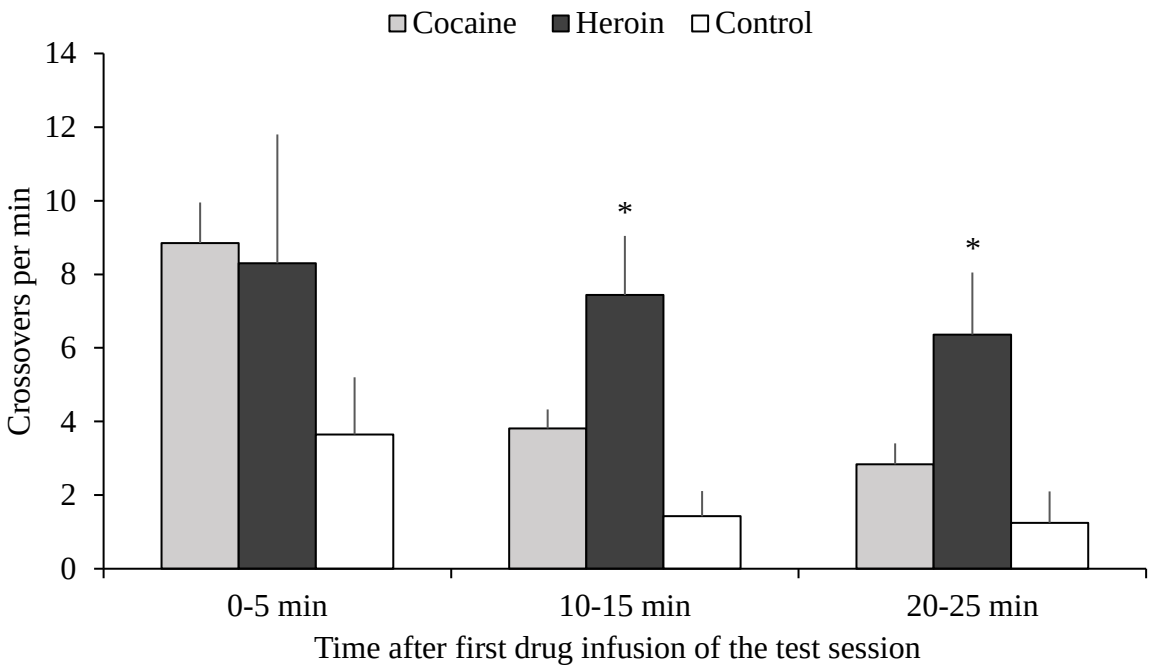

Fig. 5.8. Locomotion as a function of time after first drug infusion (period 1) and type of drug. Error bars represent ± 1 SE. *significantly different from control. *N* = 4 for control, 8 for drug groups.

Shapiro-Wilk tests revealed non-normal distributions of scores across some conditions. Therefore, drug groups in each 5 min bin were analysed separately using Kruskal-Wallis tests.

The differences between groups in the first 5 min post-injection only approached significance, $H(2) = 4.96$, $p = .084$.

In the 10-15 min period post-injection, there was a significant difference between drug treatments, $H(2) = 9.28$, $p = .01$. Mann-Whitney post-hoc tests (at $\alpha = .017$) revealed that there was significantly more locomotion elicited by heroin relative to control, $U = 1$, p

= .008, but not relative to cocaine, U = 14.5, p = .065. Cocaine and control conditions were also not significantly different from each other, U = 13.5, p = .028.

Finally, in the 20-25 min period post-injection, there was a significant difference between levels of locomotion across drug conditions, $H(2)$ = 8.1, p = .018. Again, this difference stemmed from significantly higher locomotion levels in the heroin condition relative to control, U = 2, p = .016 (at α = .017).

Figures 5.9 (next page) and 5.10 (p. 124) show the levels of locomotion in the first 5 min following the second drug infusion of the tests session. Figure 5.7 shows locomotion as a function of the second drug infusion only, while figure 5.8 shows locomotion as a function of the combination of drugs infused during the tests session (first and second).

Considering the second infusion only (fig. 5.9), on average, the highest level of locomotion was elicited by cocaine (M = 9.31, SE = 1.83), followed by heroin (M = 3.03, SE = 0.85) and then the control condition (M = 0.66, SE = 0.49). Shapiro-Wilk tests revealed lack of normality in the cocaine and control conditions.

A Kruskal-Wallis tests revealed a significant difference between conditions, $H(2)$ = 12.6, p = .002. Post-hoc Mann-Whitney tests (at α = .017) revealed that there was significantly more locomotion in the cocaine relative to both the heroin and the control conditions (U = 6, p = .005 and U = 0, p = .004, respectively). Heroin and control conditions were not significantly different from each other, U = 4, p = .048.

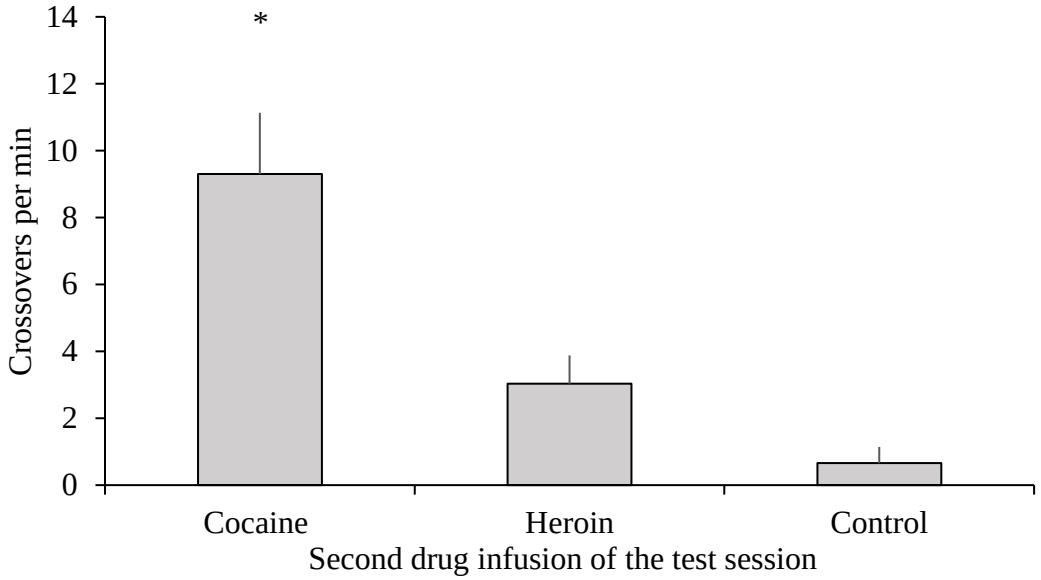

Fig. 5.9. Locomotion during the 5 min following the second drug infusion (period 2) as a function of second drug received. Error bars represent ± 1 SE. *significantly different from heroin and control conditions. *N* = 4 for control, 8 for drug groups.

When considering the combination of drugs received during the test session (fig. 5.10), the highest levels of locomotion were found in the heroin-cocaine group (*M* = 9.31, *SE* = 1.83), followed by the cocaine-cocaine (*M* = 9.31, *SE* = 1.83), heroin-heroin (*M* = 9.31, *SE* = 1.83), cocaine-heroin (*M* = 9.31, *SE* = 1.83) and control/exposure-only groups (*M* = 9.31, *SE* = 1.83). Shapiro-Wilk tests revealed that there was a non-normal distribution of scores in the control group.

A Kruskal-Wallis test revealed a significant difference between groups, $H(4) = 13.3$, $p = .01$, and post-hoc Mann-Whitney tests revealed that differences between the cocaine-cocaine group and control, and heroin-cocaine and control only approached significance after a Bonferroni correction ($U = 0$, $p = .029$ and $U = 0$, $p = .029$, respectively, if $\alpha = .025$).

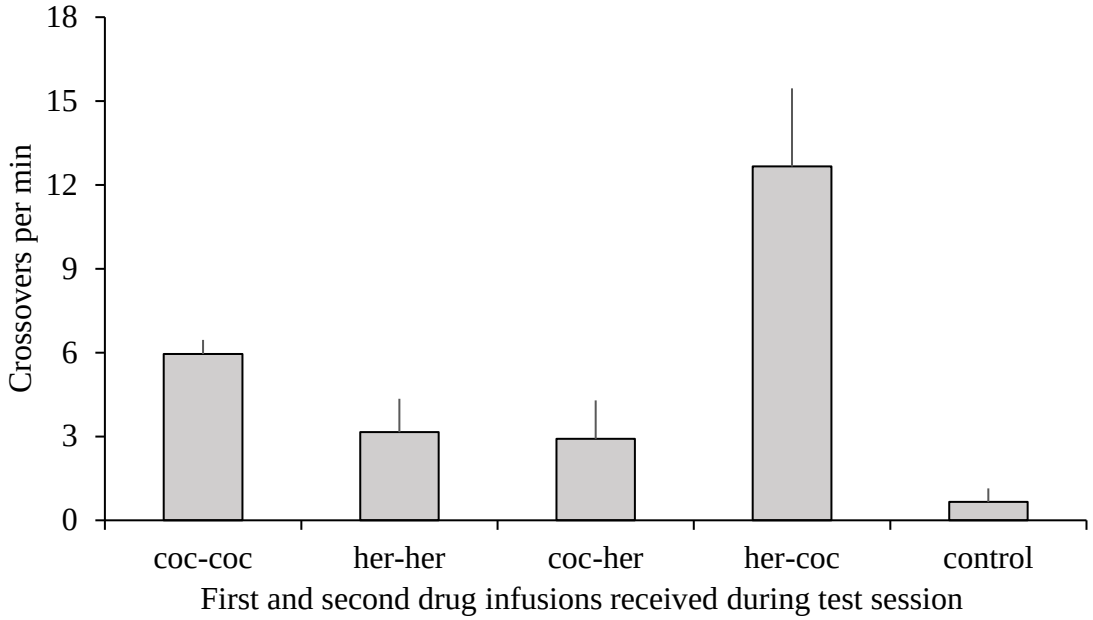

Fig. 5.10. Locomotion during the 5 min following the second drug infusion (period 2) as a function of combination of drugs received during the tests session. Error bars represent ± 1 SE. *N* = 4 for each group.

Latency to press

Figure 5.11 (next page) shows the latency to press a lever depending on the drug associated with it. On average, rats took longer to press a lever associated with cocaine (*M* = 52.46, *SE* = 11.39) than to press a lever associated with heroin (*M* = 31.10, *SE* = 7.13). Where rats had to press twice for the same drug (cocaine-cocaine and heroin-heroin groups), data were averaged from the two presses. Rats in the control condition were excluded from this analysis, as they were not given the opportunity to press.

Shapiro-Wilk tests revealed normal distributions of the latency scores for both the heroin and cocaine levers. A paired-samples *t*-test revealed a significant difference between conditions, $t(11) = 2.21$, $p = .049$.

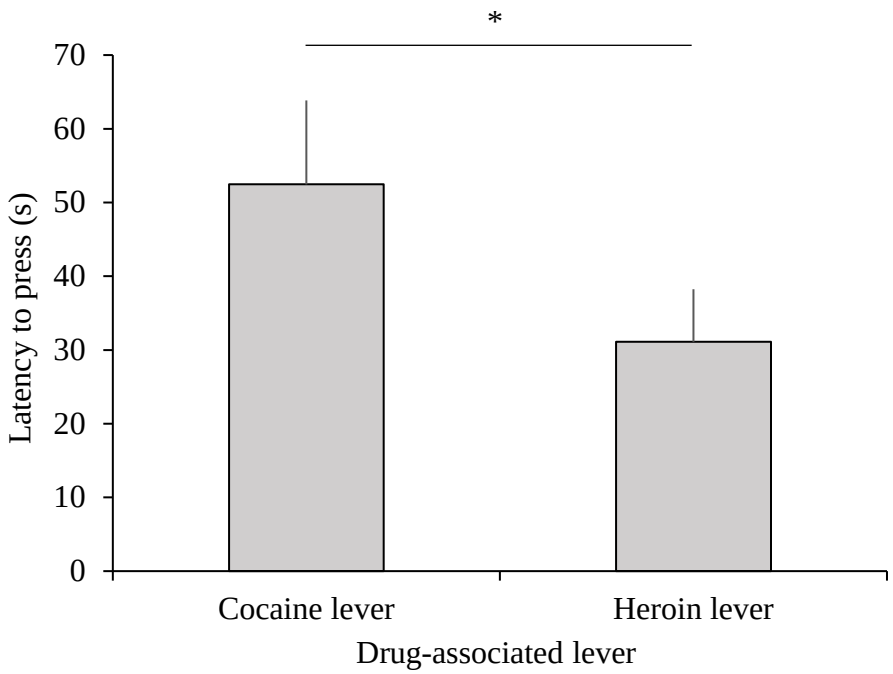

Fig. 5.11. Latency to press a lever as a function of the drug associated with it. Error bars represent ± 1 SE. *significantly different at the .05 level. *N* = 8 for each group.

Activity scale (Ellinwood et al. 1984)

Figure 5.12 (next page) shows the average level of activity as measured by the 9-point scale as a function of first drug infusion and time. For both heroin and cocaine, and the control group, the highest average level of activity was found in the first 5 min of period 1 (*M* = 7.69, *SE* = 0.25; *M* = 5.44, *SE* = 0.44 and *M* = 4.5, *SE* = 0.61, respectively). Then, activity gradually diminished over time for both drugs and the control group, but in all cases remained higher in the heroin group relative to the others: at 10 min, heroin *M* = 7.44, *SE* = 0.26, cocaine *M* = 4.5, *SE* = 0.6 and control *M* = 3.5, *SE* = 0.54; at 15 min, heroin *M* = 5.81, *SE* = 0.62, cocaine *M* = 4.44, *SE* = 0.84, and control *M* = 2.5, *SE* = 0.2; at 20 min, heroin *M* = 6.25, *SE* = 0.71, cocaine *M* = 3.88, *SE* = 0.97, and control *M* = 2.75, *SE* = 0.6; and, finally, at 25 min, heroin *M* = 6.38, *SE* = 0.73, cocaine *M* = 4.31, *SE* = 0.88, and control *M* = 3, *SE* = 0.84.

Shapiro-Wilk tests revealed lack of normality in several groups. Kruskal-Wallis tests were run to compare activity levels between drug groups and the control for each time bin, applying a Bonferroni correction (α = .01).

There was a significant difference between drug groups and control after the first 5 min of period 1, $H(2) = 11.71$, $p = .003$. This was also the case at the end of the 10 min bin, $H(2) = 11.65$, $p = .003$. There were no significant difference between drug groups and control for the rest of the time bins: 15 min, $H(2) = 6.81$, $p = .033$; 20 min, $H(2) = 4.92$, $p = .085$; and 25 min, $H(2) = 5.92$, $p = .052$.

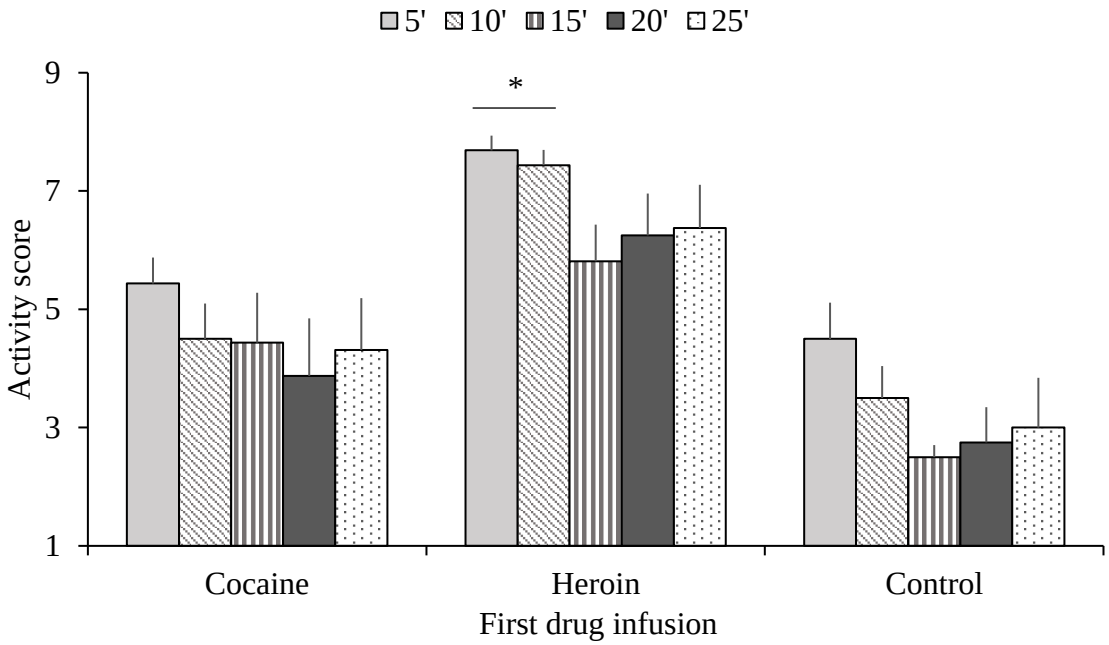

Fig. 5.12. Activity following the first drug infusion of the test session (period 1) as a function of drug and time. Error bars represent ± 1 SE. *significantly different from cocaine and control. $N = 4$ for control, 8 for drug groups, at each time point.

Post-hoc Mann-Whitney tests were run at $\alpha = .017$. The difference in the 5 and 10 min bins arose from the heroin group having significantly higher activity levels relative to both cocaine and control: for the 5 min bin, $U = 6$, $p = .005$, and $U = 0.5$, $p = .004$, respectively; for the 10 min bin, $U = 6$, $p = .005$ and $U = 0$, $p = .004$, respectively. The cocaine group was not significantly different from control in either case: 5 min bin, $U = 9$, $p = .283$; 10 min bin $U = 9.5$, $p = .283$.

Figures 5.13 (next page) and 5.14 (p. 128) show the activity levels in period 2 as a function of either second drug self-administered during the session or the combination of drugs received during the session, respectively.

Considering the second drug infusion only (fig. 5.13), the highest level of activity on average was found in the heroin group, *M* = 6.63, *SE* = 0.75, followed by cocaine, *M* = 5.06, *SE* = 0.45, and control, *M* = 2.75, *SE* = 0.25. Shapiro-Wilk tests revealed non-normal distributions across conditions.

A Kruskal-Wallis test indicated a significant difference between groups, *H*(2) = 9.03, *p* = .011, and Mann-Whitney tests (at α = .017) revealed that there was a significant difference between cocaine and control, *U* = 1.5, *p* = .008. The other two comparisons were not significant after the correction: *U* = 3.5, *p* = .028 for heroin vs control, and *U* = 0, *p* = .05 for heroin vs. cocaine.

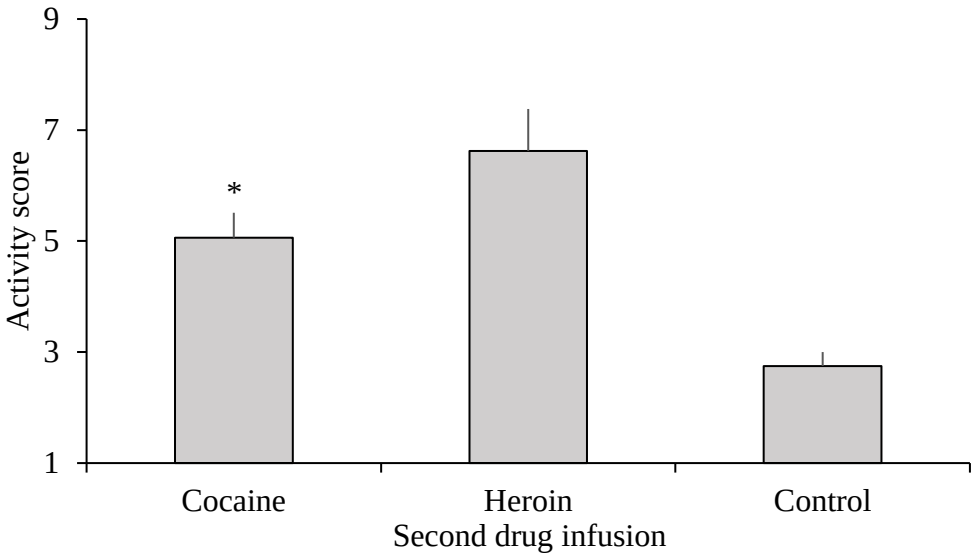

Fig. 5.13. Activity 5 min after the second drug infusion of the tests session (period 2) as a function of drug. Error bars represent ± 1 SE. *significantly different from control. *N* = 4 for control, 8 for drug groups.

Considering the combination of drugs self-administered (fig. 5.14), the highest level of activity was found in the cocaine-heroin group (*M* = 7, *SE* = 0.71), followed by the heroin-heroin group (*M* = 6.25, *SE* = 1.44), cocaine-cocaine (*M* = 5.13, *SE* = 0.66), heroin-cocaine (*M* = 5, *SE* = 0.71), and control (*M* = 2.75, *SE* = 0.25). Shapiro-Wilk tests revealed lack of normality in the heroin-heroin and control groups. A Kruskal-Wallis test indicated that there were no significant differences between groups, *H*(2) = 9.29, *p* = .054.

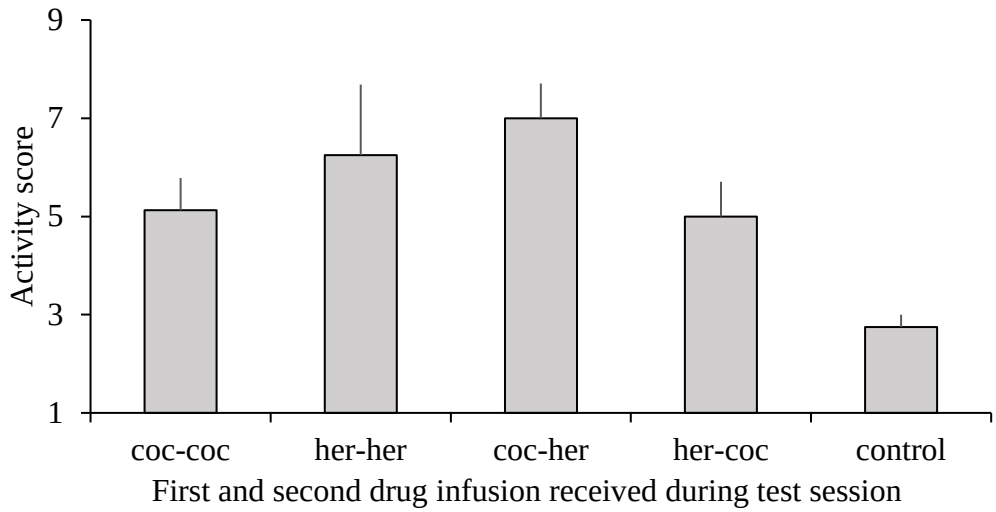

Fig. 5.14. Activity 5 min after the second drug infusion of the tests session (period 2) as a function of combination of drugs received. Error bars represent ± 1 SE. *N* = 4 for each group.

Table 5.3. **Summary of locomotion and stereotypy data as measured by crossovers and the Ellinwood et al. scale, respectively. Data is presented as the effect of first or second drug injection (h – heroin, c – cocaine): (↑) significant increase relative to controls following Bonferroni correction, (–) no sig. difference from control, (↑) a significant increase from control which did not survive Bonferroni correction.**

		Period 1 (1st inj.)					Period 2 (2nd inj.)		
		5’	10’	15’	20’	25’	overall	after h	after c
Locomotion (crossovers)	h	–		↑		↑	↑	–	–
	c	–		↑		–	↑	↑	↑
Ellinwood	h	↑	↑	–	–	–	↑	–	–
	c	–	–	–	–	–	↑	–	–

Categorical measures of behaviour

As in the previous section, categorisation of behaviour was done separately for the two periods after each drug infusion of the test session.

All comparisons between drug groups and controls were done using Fischer’s Exact Test (FET), since group sizes were too small to run chi-square tests. For comparisons between

drug groups within each 5 min bin of period 1 it was necessary to apply a Bonferroni correction for multiple testing (α = .01). Only one of the comparisons in period 1 survived this correction. Regardless, comparisons where p-values were significant at the .05 level are reported.

Figure 5.15 (below) shows the proportion of that exhibited locomotor behaviour as a function of time and first drug self-administered. Note the difference from the locomotor activity analysis as measured by number of crossovers. Differences between drug groups and control were significant at the .05 but not the .01 level only for the 5 min bin, p = .035. That is, 75% of the rats in the cocaine and control conditions exhibited locomotor behaviour at the 5th min of period 1, while only 12.5% of rats in the heroin condition did so.

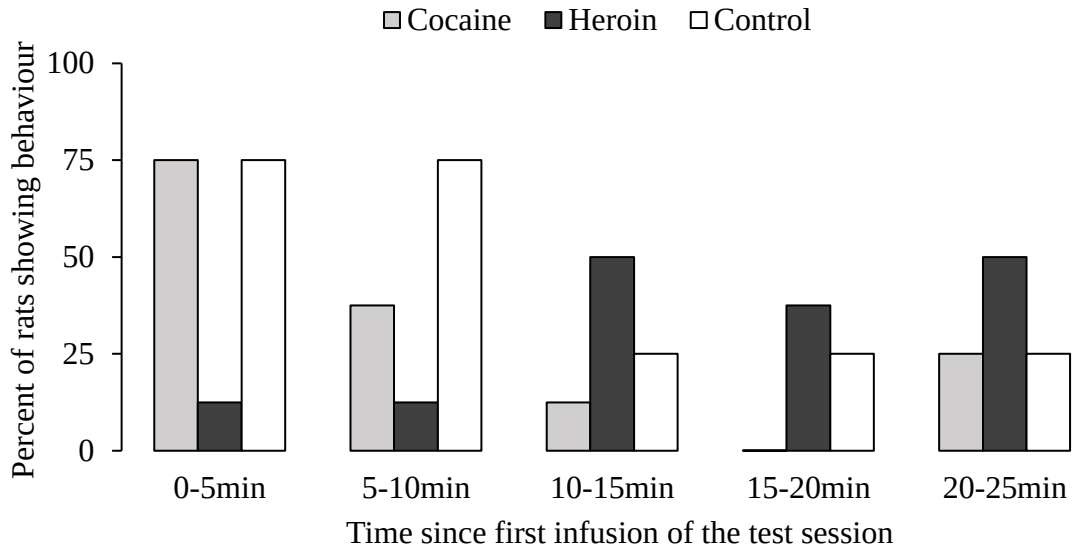

Fig. 5.15. Proportion of rats in each drug group showing locomotor behaviour in period 1 as a function of time. N = 4 for control, 8 for drug groups, at each time bin.

Figure 5.16 (next page) shows the proportion of rats that exhibited gnawing behaviour as a function of first drug and time. At the end of the 5-10 min of period 1, 62.5% of rats in the heroin group exhibited gnawing behaviour, compared to 25% in the control group and none in the cocaine group, p = .028.

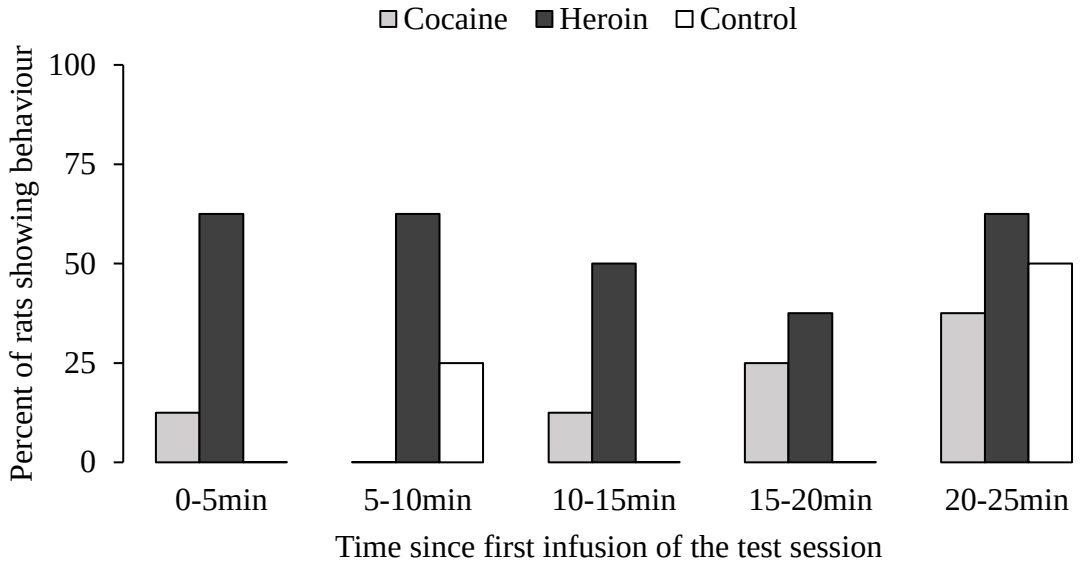

Fig. 5.16. Proportion of rats in each drug group showing gnawing behaviour in period 1 as a function of time. $N = 4$ for control, 8 for drug groups, at each time bin.

Figure 5.17 (next page) shows the proportion of rats exhibiting sniffing behaviour as a function of time and first drug. In the first 5 min, 75% of rats in the cocaine and control conditions exhibited sniffing behaviour, while none of the rats in the heroin group did, $p = .003$. In the 5-10 min of period 1, 62.5% of rats in the cocaine group and 50% of rats in the control group exhibited the behaviour, while none in the heroin group did, $p = .032$.

Figure 5.18 (next page) shows the proportion of rats exhibiting rearing behaviour. At the end of 10-15 min of period 1, 75% of rats showed rearing behaviour, while only 25% in the cocaine group and none in the control group did so, $p = .035$. At the end of 15-20 min of period 1, 50% of rats in the heroin group were rearing, while none in the cocaine and control groups were doing so, $p = .042$.

Figure 5.19 (p. 132) shows the proportion of rats exhibiting licking behaviour. At the end of 5-10 min of period 1, 50% of rats in the heroin group were exhibiting the behaviour, while none of the rats in the control and cocaine groups were doing so ($p = .042$).

Figures 5.20-5.24 (pp. 132-134) show the proportion of rats in each drug group and the control that were exhibiting head down, sway, groom, still and miscellaneous behaviours, respectively. For these behaviours, none of the comparisons between groups at any time bin were significant either before or after a Bonferroni correction.

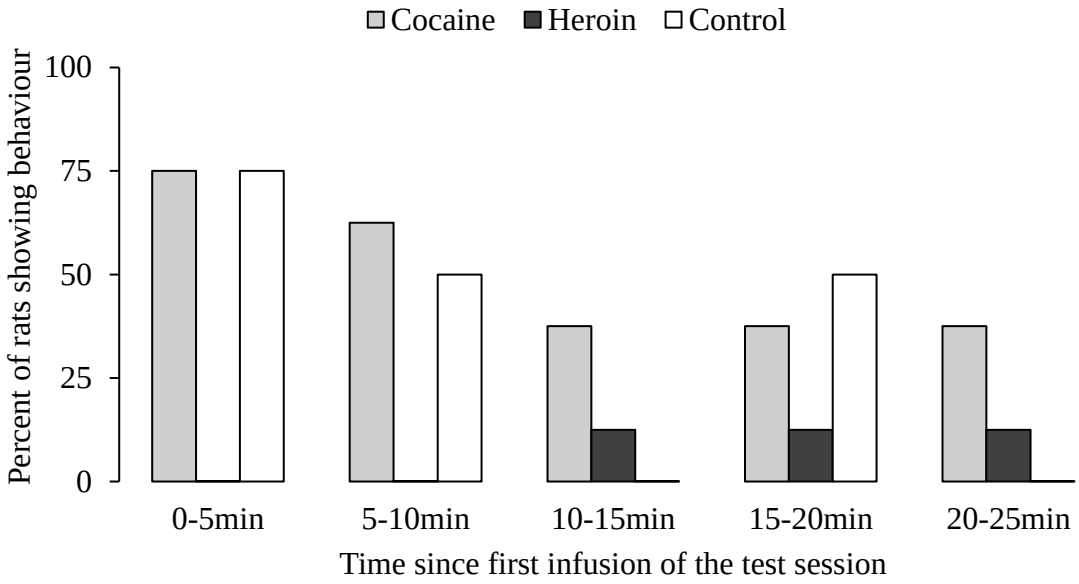

Fig. 5.17. Proportion of rats in each drug group showing sniffing behaviour in period 1 as a function of time. *N* = 4 for control, 8 for drug groups, at each time bin.

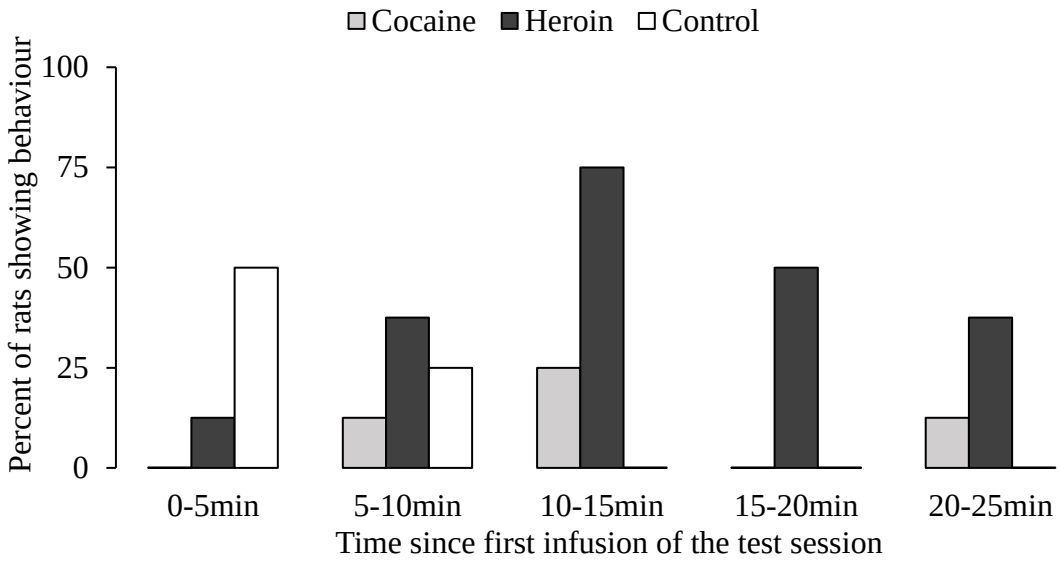

Fig. 5.18. Proportion of rats in each drug group showing rearing behaviour in period 1 as a function of time. *N* = 4 for control, 8 for drug groups, at each time bin.

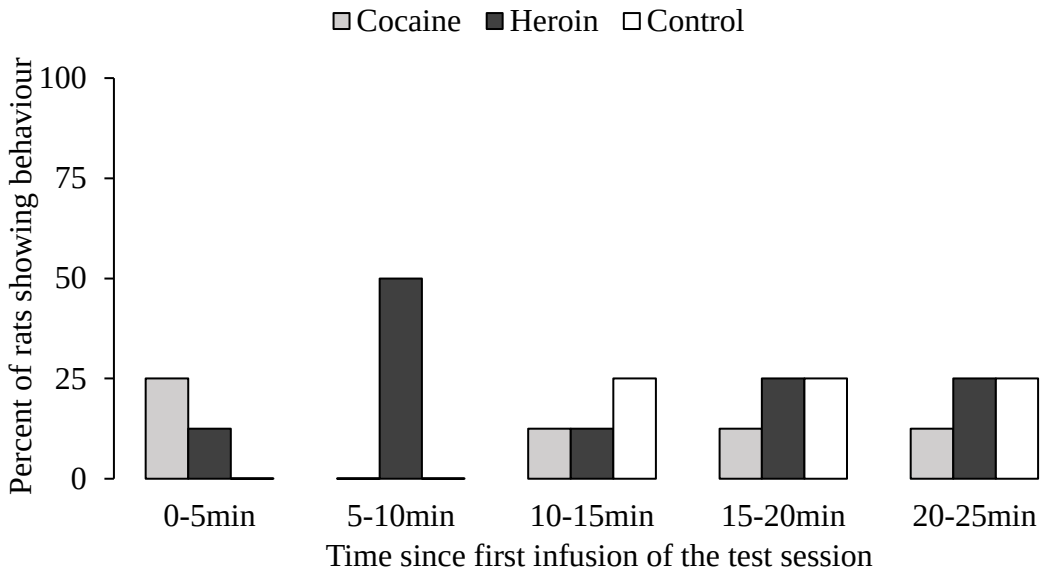

Fig. 5.19. Proportion of rats in each drug group showing licking behaviour in period 1 as a function of time. *N* = 4 for control, 8 for drug groups, at each time bin.

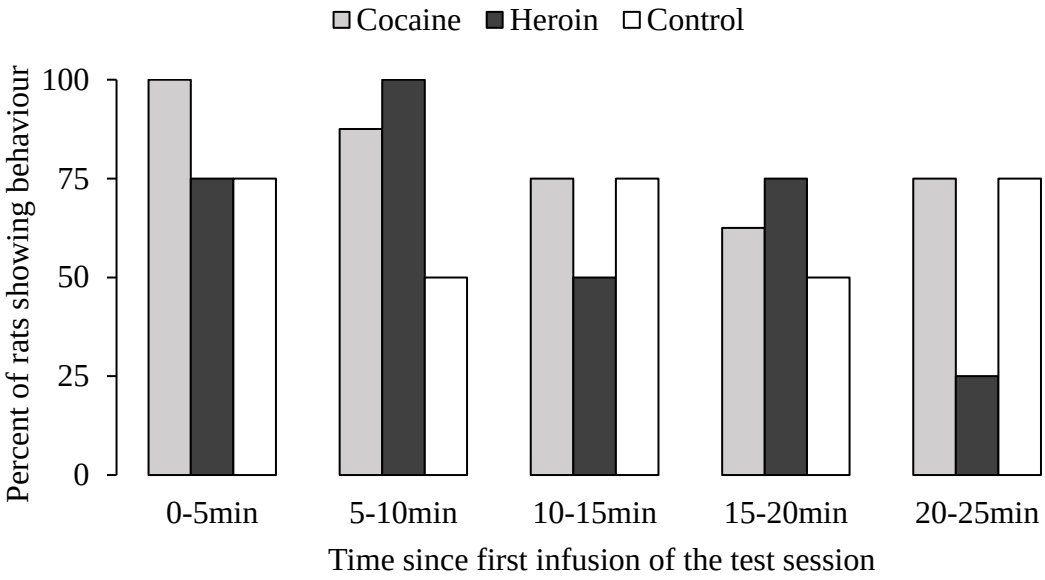

Fig. 5.20. Proportion of rats in each drug group showing head down behaviour in period 1 as a function of time. *N* = 4 for control, 8 for drug groups, at each time bin.

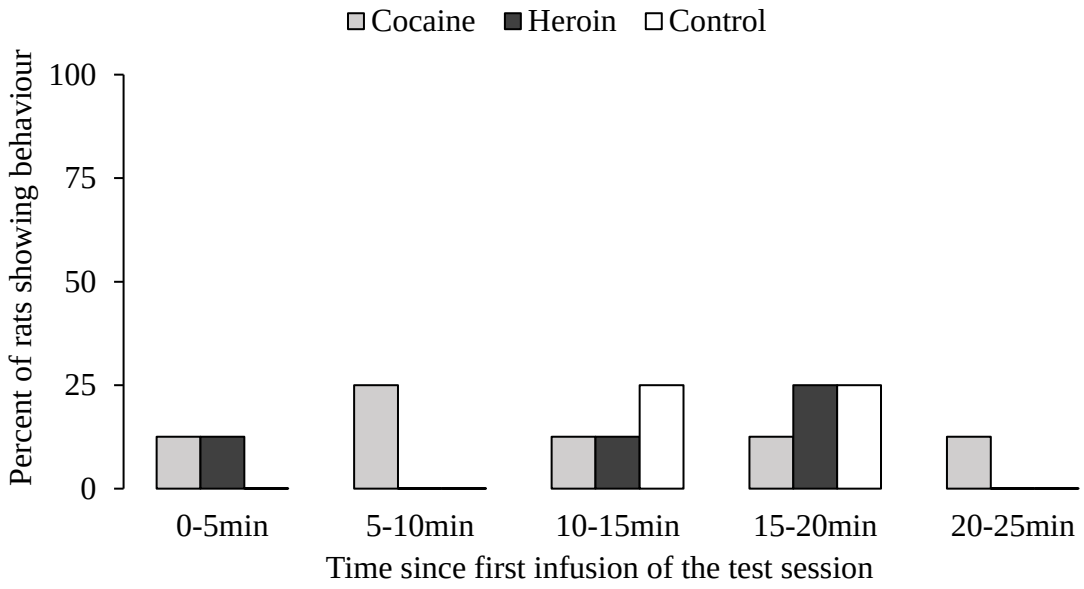

Fig. 5.21. Proportion of rats in each drug group showing sway behaviour in period 1 as a function of time. *N* = 4 for control, 8 for drug groups, at each time bin.

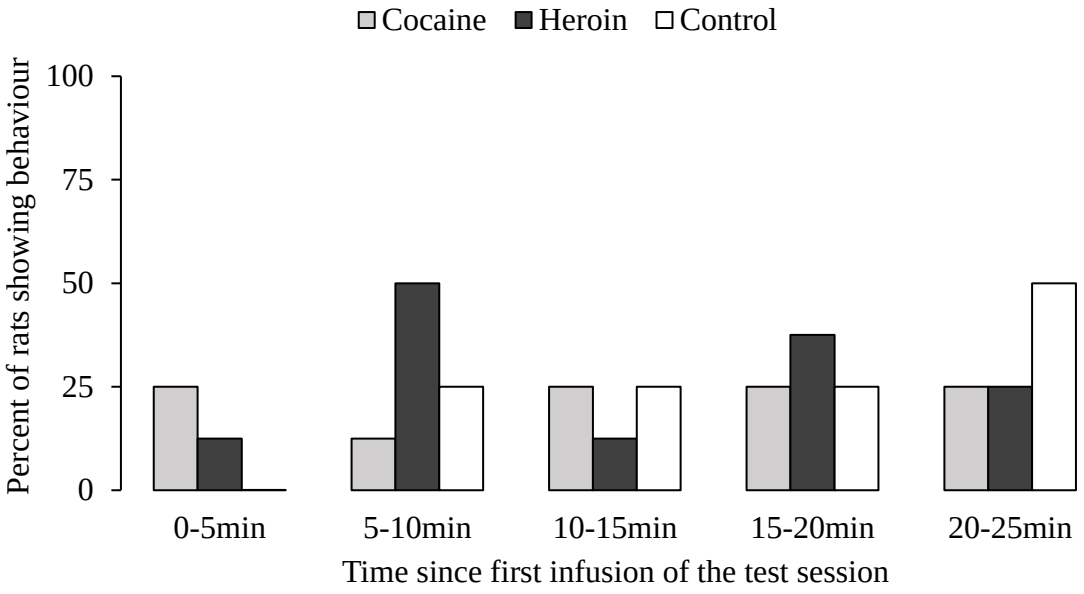

Fig. 5.22. Proportion of rats in each drug group showing grooming behaviour in period 1 as a function of time. *N* = 4 for control, 8 for drug groups, at each time bin.

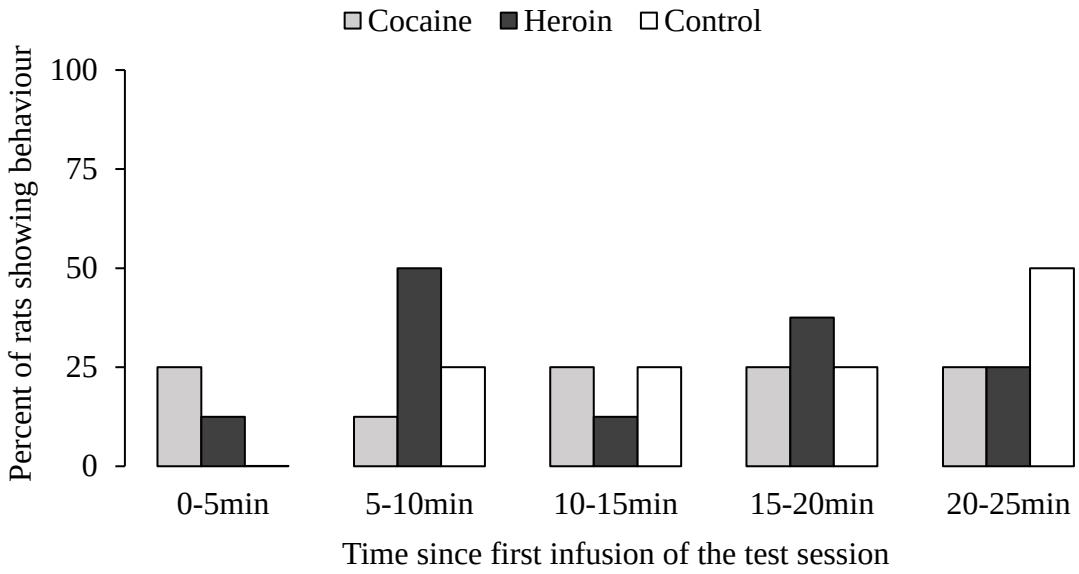

Fig. 5.23. Proportion of rats in each drug group staying still in period 1 as a function of time. *N* = 4 for control, 8 for drug groups, at each time bin.

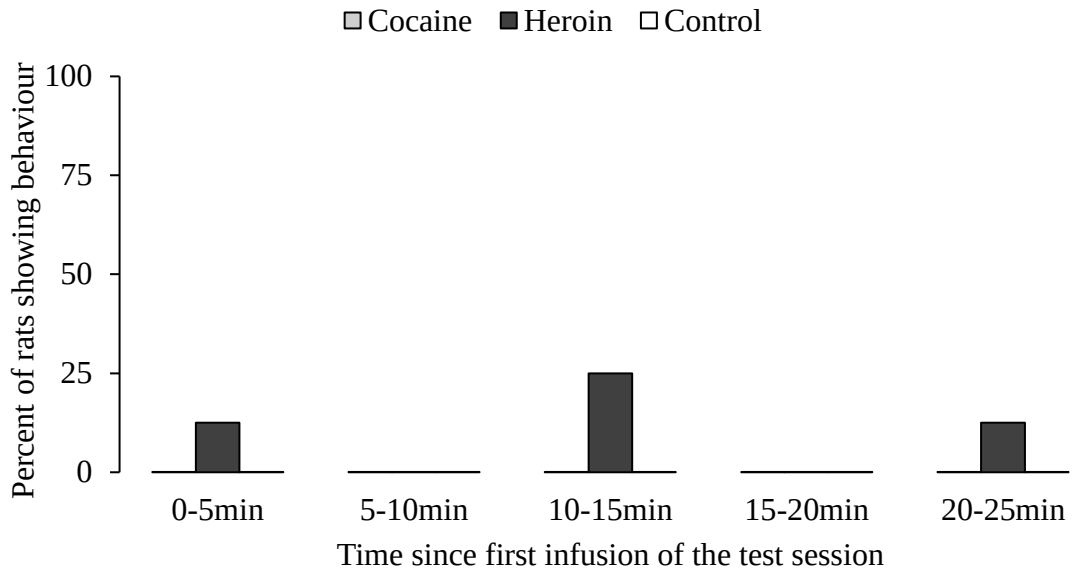

Fig. 5.24. Proportion of rats in each drug group showing miscellaneous behaviour in period 1 as a function of time. *N* = 4 for control, 8 for drug groups, at each time bin.

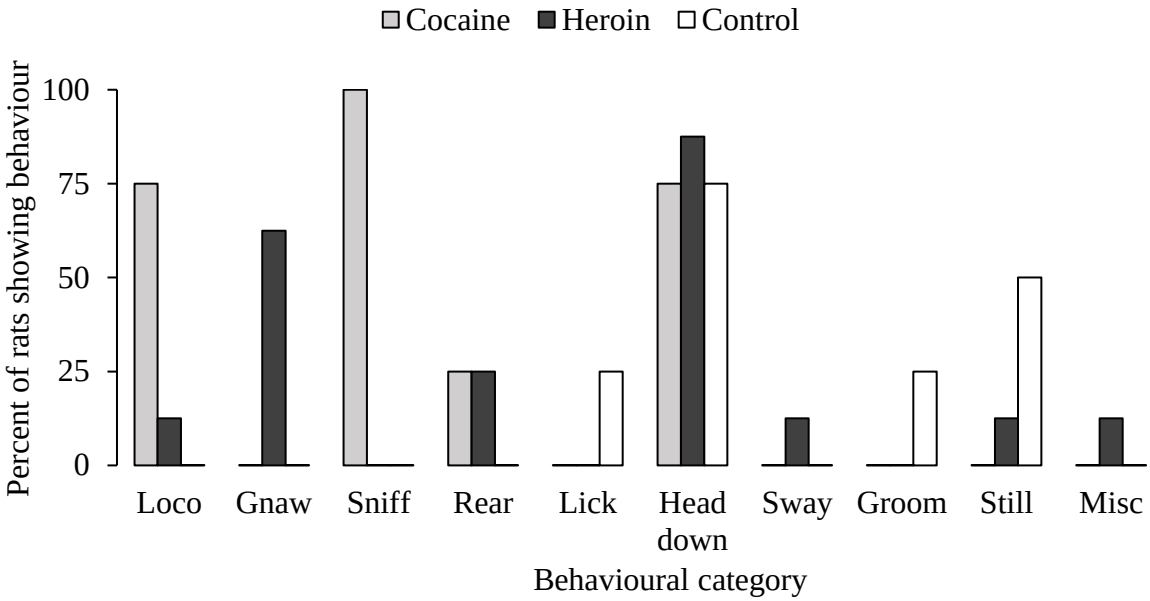

Fig. 5.25. Proportion of rats showing each of the listed behaviours following the second drug infusion during the test session (period 2). *N* = 4 for control, 8 for drug groups, at each time bin.

Figure 5.25 (above) shows the proportion of rats showing each of the categorised behaviours 5 min after the second drug infusion (period 2) as a function of drug. A Bonferroni correction for multiple comparisons was applied, α = .005. Only the group comparisons for sniffing behaviour survived this correction, as 100% of rats in the cocaine group exhibited this behaviour, while none in the heroin and control groups did, $p < .001$. For locomotion, 75% of rats in the cocaine group exhibited the behaviour, compared to 12.5% and 0% in the heroin and control groups, respectively, $p = .016$. Finally, for the gnawing behaviour, 62.5% of rats in the heroin group exhibited the behaviour, compared to none in the cocaine and control groups, $p = .008$. None of the comparisons for the other behaviours were significant at either the .05 or .005 levels.

Table 5.4. **Summary of categorical measures of behaviour. Data is presented as the effect of first or second drug injection (h – heroin, c – cocaine): (↑/↓) significant increase/decrease from control following Bonferroni correction, (–) no sig. difference, (↑/↓) a significant increase/decrease from control which did not survive Bonferroni correction. Behaviours for which no significant differences were found are not included.**

		Period 1 (1st inj.)					Period 2 (2nd inj.)
		5’	10’	15’	20’	25’	overall
locomotion	h	↓	–	–	–	–	–
	c	–	–	–	–	–	↑
gnawing	h	–	↑	–	–	–	↑
	c	–	–	–	–	–	–
sniffing	h	↓	↓	–	–	–	–
	c	–	–	–	–	–	↑
rearing	h	–	–	↑	↑	–	–
	c	–	–	–	–	–	–
licking	h	–	↑	–	–	–	–
	c	–	–	–	–	–	–

CatFISH

Please note that due to exceptionally low levels of IEG expression across the striatum following self-administration (see Appendix 3), quantitative analysis of the IEG data was carried out only for the PFC.

Infralimbic cortex (IL)

Figure 5.26 (next page) shows the number of *h1a*- and *arc*-positive nuclei in the infralimbic cortex as a function of drugs self-administered during the test session. On average, the highest number of *h1a*-positive nuclei was found in the heroin-heroin group (*M* = 48.13, *SE* = 11.47), followed by the control group (*M* = 48, *SE* = 5.01), then the cocaine-cocaine group (*M* = 45.81, *SE* = 5.32), the heroin-cocaine (*M* = 38.69, *SE* = 6.8) and the cocaine-heroin (*M* = 30.94, *SE* = 2.24) groups. On average, the highest level of *arc* expression was found in the control group (*M* = 32.44, *SE* = 12.54), followed by

heroin-cocaine (*M* = 24.06, *SE* = 3.87), then heroin-heroin (*M* = 23.81, *SE* = 6.9), cocaine-cocaine (*M* = 22.69, *SE* = 4.42) and, finally, the cocaine-heroin (*M* = 13.06, *SE* = 1.85) group.

Shapiro-Wilk tests revealed normal distributions of scores across groups for both *h1a*- and *arc*-positive nuclei. Levene's tests revealed homogenous variance across groups for *h1a, F*(4,15) = 2.03, *p* = .141, but not *arc*, *F*(4,15) = 8.28, *p* = .001. One-way ANOVAs revealed no significant difference between groups for either *h1a*, *F*(4,15) = 1.17, *p* = .364, or *arc*, *F*(4,15) = 0.98, *p* = .450. Welch and Brown-Forsythe tests were also non-significant.

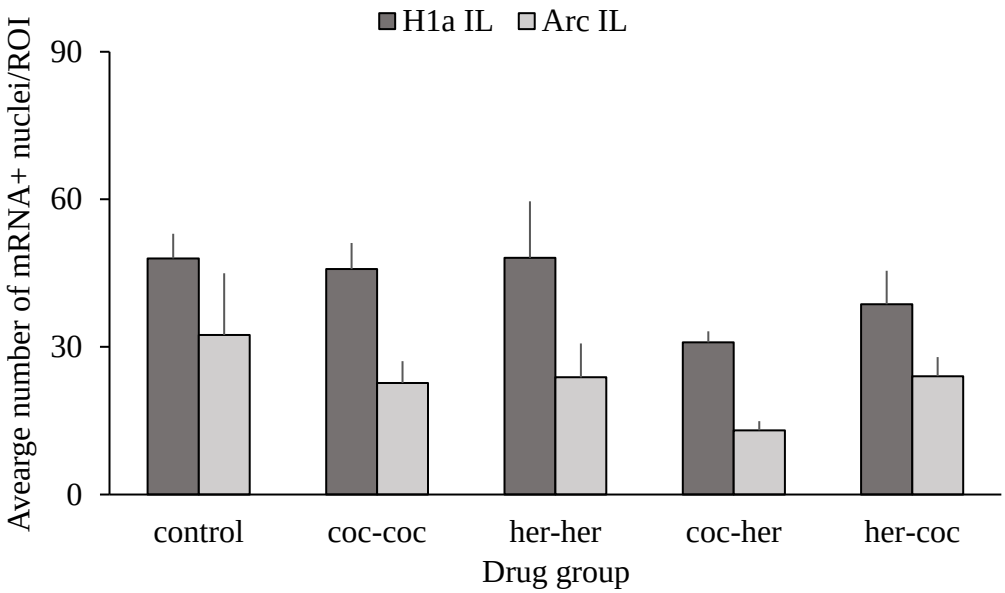

Fig. 5.26. Amount of *h1a*- and *arc*-positive nuclei in the infralimbic cortex (IL) as a function of drug group. Error bars represent ± 1 SE. *N* = 4 for each group.

Figure 5.27 (next page) shows the amount of nuclei in the infralimbic cortex co-expressing *h1a* and *arc* calculated as a percentage of all mRNA-positive nuclei in that region of interest (ROI). The amount of co-expressing nuclei across conditions, in descending order, was as follows: heroin-heroin (*M* = 22.22, *SE* = 2.41), cocaine-cocaine (*M* = 21.77, *SE* = 2.33), control (*M* = 21.45, *SE* = 3.88), heroin-cocaine (*M* = 20.55, *SE* = 2.25) and cocaine-heroin (*M* = 17.84, *SE* = 1.49).

Shapiro-Wilk tests revealed a non-normal distribution only in the heroin-heroin condition, *p* = .022. Levene's statistic was non-significant, *F*(4,15) = 0.96, *p* = .459. A one-way ANOVA revealed no significant differences between groups, *F*(4,15) = 0.45, *p* = .769.

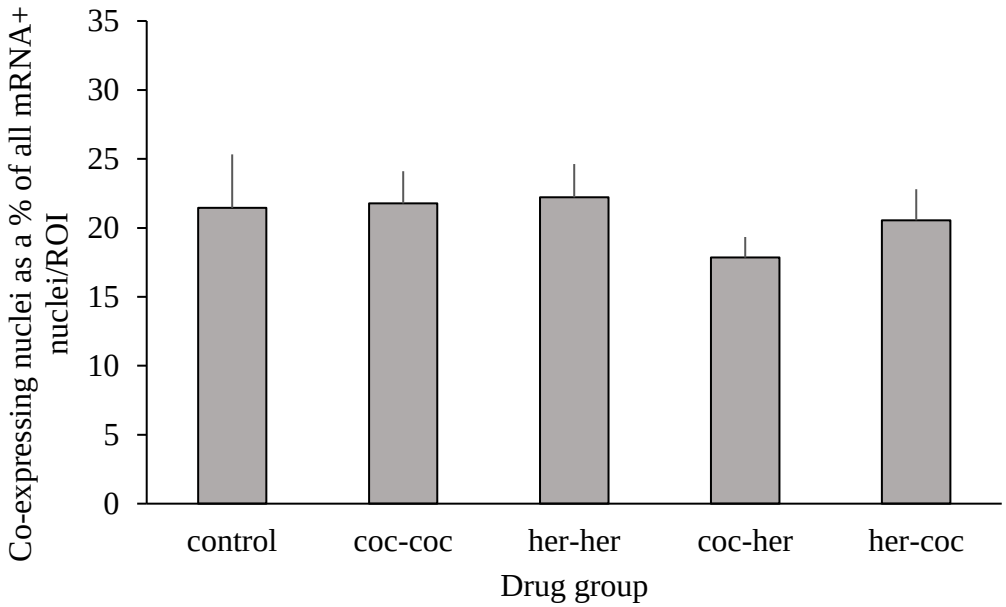

Fig. 5.27. Co-expressing nuclei as a percentage of all mRNA-positive nuclei in the infralimbic cortex (IL). Error bars represent ± 1 SE. *N* = 4 for each group.

Prelimbic cortex (PL)

Figure 5.28 (p. 140) shows the number of *h1a*- and *arc*-positive nuclei in the prelimbic cortex as a function of drug treatment. On average, the highest number of *h1a*-positive nuclei was found in the cocaine-cocaine group (*M* = 65.63, *SE* = 12.42), followed by the heroin-heroin group (*M* = 52.75, *SE* = 8.64), then the heroin-cocaine group (*M* = 48.94, *SE* = 7.01), the control (*M* = 45, *SE* = 4.4) and the cocaine-heroin (*M* = 36.69, *SE* = 5.04) groups. On average, the highest level of *arc* expression was found in the cocaine-cocaine group (*M* = 37.69, *SE* = 11.82), followed by control (*M* = 35.75, *SE* = 15.07), then heroin-cocaine (*M* = 34, *SE* = 5.64), heroin-heroin (*M* = 29.94, *SE* = 7.15) and, finally, the cocaine-heroin (*M* = 15.50, *SE* = 5.31) groups.

Shapiro-Wilk tests revealed predominantly normal distributions except in the control condition for *arc*-positive nuclei, *p* = .033. Levene's statistics revealed homogenous variance across groups for *arc* but not *h1a, F*(4,15) = 1.69, *p* = .204 and *F*(4,15) = 4.15, *p* = .018, respectively. One-way ANOVAs revealed no significant differences between groups for either *h1a, F*(4,15) = 1.17, *p* = .364, or *arc*, *F*(4,15) = 1.76, *p* = .189. Welch and Brown-Forsythe tests were also non-significant.

Figure 5.29 (next page) shows the amount of nuclei in the prelimbic cortex co-expressing *h1a* and *arc* calculated as a percentage of all mRNA-positive nuclei (per ROI). The amount of co-expressing nuclei across conditions, in descending order, was as follows: cocaine-cocaine (*M* = 26.99, *SE* = 2.8), control (*M* = 22.87, *SE* = 2.58), heroin-heroin (*M* = 22.10, *SE* = 3.44), heroin-cocaine (*M* = 20.38, *SE* = 2.49) and cocaine-heroin (*M* = 17.31, *SE* = 2.91).

Shapiro-Wilk tests revealed normal distribution of scores within conditions. Levene's statistic was non-significant, $F(4,15) = 0.07$, $p = .991$. A one-way ANOVA revealed no significant differences between groups, $F(4,15) = 1.54$, $p = .242$.

Anterior cingulate dorsal cortex (ACd)

Figure 5.30 (p. 141) shows *h1a*- and *arc*-positive nuclei in the ACd as a function of drug treatment. On average, the highest number of *h1a*-positive nuclei was in the cocaine-cocaine group (*M* = 67.81, *SE* = 8), followed by the heroin-cocaine group (*M* = 62.19, *SE* = 15.53), then the heroin-heroin group (*M* = 55.69, *SE* = 5.47), the control (*M* = 39.94, *SE* = 4.02) and the cocaine-heroin (*M* = 37.25, *SE* = 4.3) groups. On average, the highest level of *arc* expression was found in the cocaine-cocaine group (*M* = 34.94, *SE* = 8.02), followed by heroin-cocaine (*M* = 33.88, *SE* = 6.57), then heroin-heroin (*M* = 28.06, *SE* = 8.08), control (*M* = 26.63, *SE* = 10.77) and, finally, the cocaine-heroin (*M* = 17, *SE* = 4.49) groups.

Shapiro-Wilk tests revealed normal distributions within all drug groups. Levene's statistics revealed homogenous variance across groups for both *arc* and *h1a,* $F(4,15) = 0.72$, $p = .592$ and $F(4,15) = 2.41$, $p = .095$, respectively. One-way ANOVAs revealed no significant differences between groups for either *h1a,* $F(4,15) = 2.46$, $p = .090$, or *arc*, $F(4,15) = 0.83$, $p = .526$.

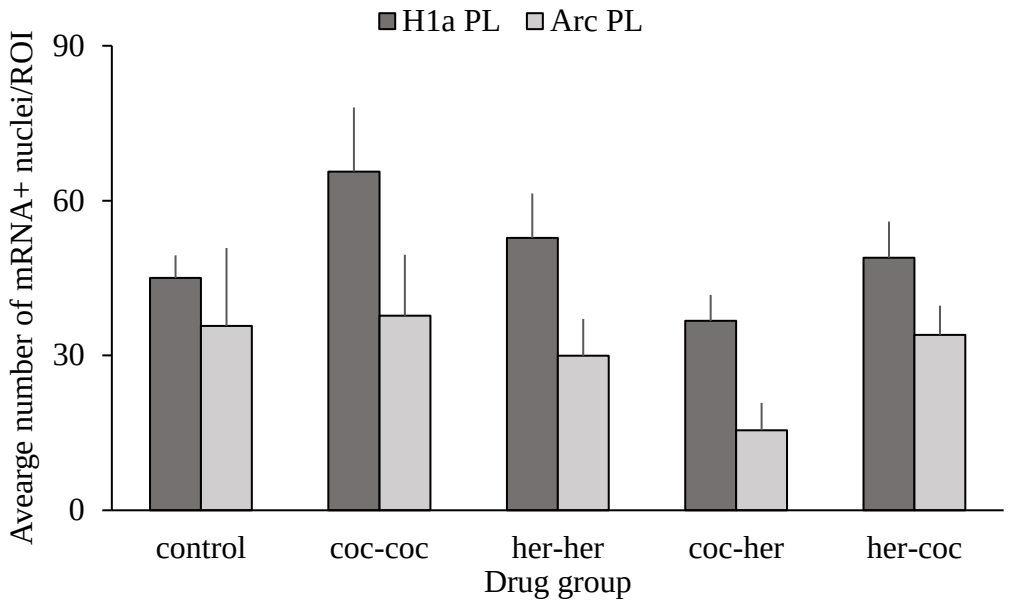

Fig. 5.28. Amount of *h1a*- and *arc*-positive nuclei in the prelimbic cortex (PL) as a function of drug group. Error bars represent ± 1 SE. *N* = 4 for each group.

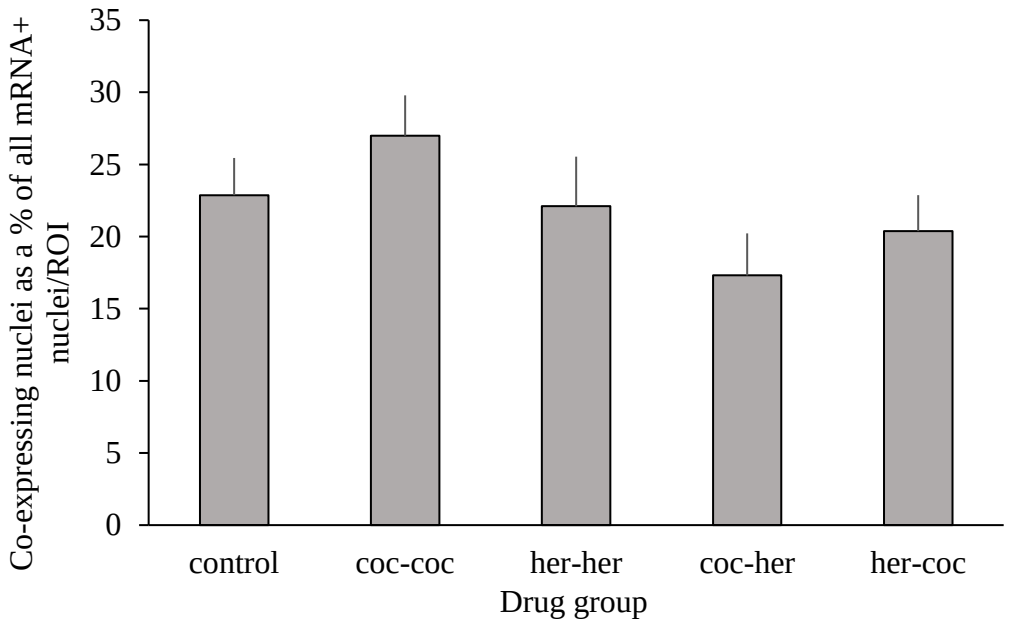

Fig. 5.29. Co-expressing nuclei as a percentage of all mRNA-positive nuclei in the prelimbic cortex (PL). Error bars represent ± 1 SE. *N* = 4 for each group.

Figure 5.31 (p.142) shows the amount of nuclei in the ACd co-expressing *h1a* and *arc* calculated as a percentage of all mRNA-positive nuclei (per ROI). The amount of co-expressing nuclei across conditions, in descending order, was as follows: cocaine-cocaine (*M* = 24.88, *SE* = 4.04), heroin-cocaine (*M* = 22.36, *SE* = 3.54), heroin-heroin (*M*

= 21.81, *SE* = 4.92), cocaine-heroin (*M* = 18.66, *SE* = 2.49) and control (*M* = 18.38, *SE* = 3.71).

Shapiro-Wilk tests revealed normal distribution of scores within conditions. Levene's statistic was non-significant, $F(4,15) = 0.57$, $p = .688$. A one-way ANOVA revealed no significant differences between groups, $F(4,15) = 0.51$, $p = .731$.

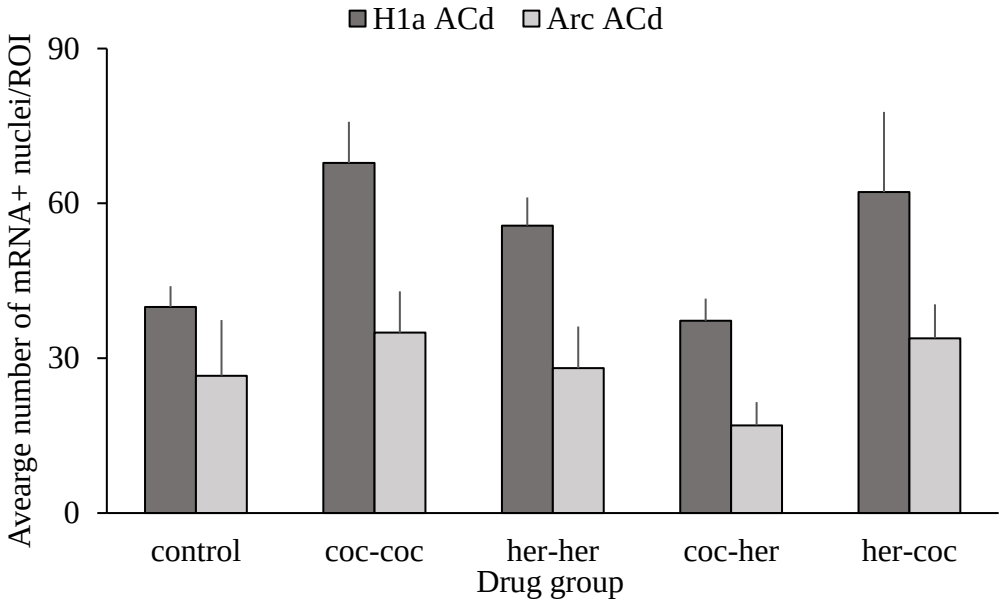

Fig. 5.30. Amount of *h1a*- and *arc*-positive nuclei in the anterior cingulate dorsal cortex (ACd) as a function of drug group. Error bars represent ± 1 SE. *N* = 4 for each group.

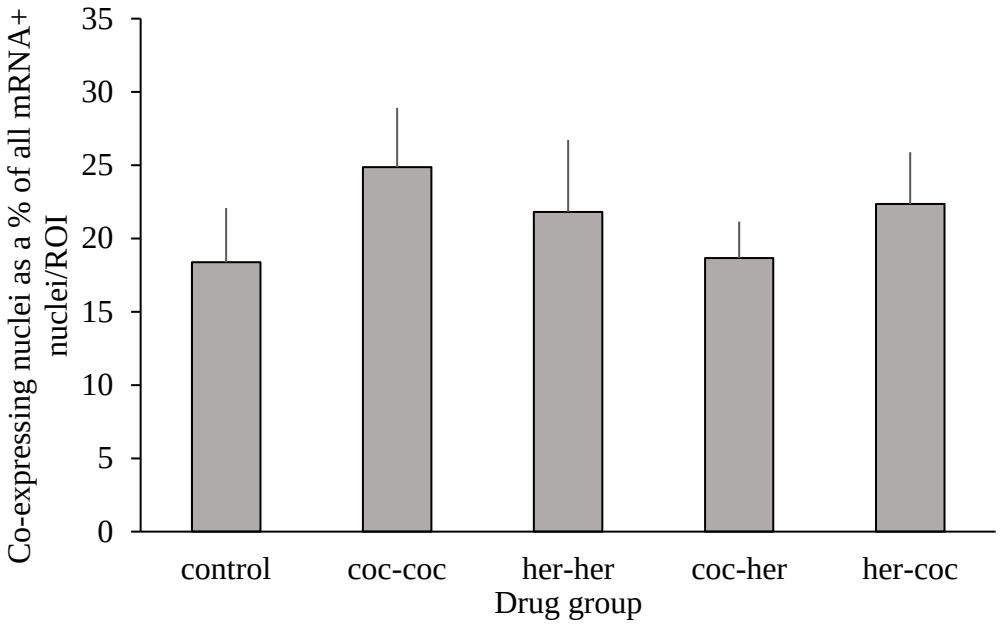

Fig. 5.31. Co-expressing nuclei as a percentage of all mRNA-positive nuclei in the anterior cingulate dorsal cortex (ACd). Error bars represent ± 1 SE. *N* = 4 for each group.

Discussion

The study reported here was a pilot study with a relatively small sample size and low statistical power. For this reason, parts of the following discussion revolve around the qualitative analysis of data, rather than statistically significant differences between treatment groups. This is particularly true for the categorical measures of behaviour (figs. 5.13-5.23) where statistical differences between drug groups were not found after corrections for multiple testing.

Self-administration and latency to approach drug-associated levers

All rats learned to self-administer heroin quickly, and escalated intake over time. Conversely, although all rats included in the analysis also lever-pressed for cocaine, lever press frequencies were much lower and with high variability between individual animals. Rats were also quicker to approach the heroin-associated lever during the final test session. When heroin and cocaine self-administration levels were presented as a percentage of available infusions that were self-administered, on average, rats self-administered a high proportion of the available heroin infusions, and a much lower proportion of available cocaine infusions. In fact, some rats had such low levels of cocaine self-administration that they could not be included in the final test session

groups where they had to press for cocaine. This resulted in the obvious methodological issue of having uneven levels of cocaine self-administration between groups in the final tests session. Taken together the data suggest that, under the conditions of this experiment, heroin was more effective reinforcer than cocaine, and rats were more motivated to obtain it.

The preference for heroin over cocaine found in this sample of rats was unexpected. One possible explanation for the poor reinforcing properties of cocaine could be a high dose leading to anxiogenic effects. Chronic or acute treatment with 20 mg/kg i.p. cocaine increases plasma corticosterone, and decreases time spent in an open field and locomotion relative to saline treatment (Yang, Gorman, Dunn, & Goeders, 1992). However, other studies have successfully trained rats to self-administer cocaine at a dose of 800 μg/kg and even higher. Rats self-administer cocaine doses of the range 0.5-2 mg/kg under both long and short access conditions (Mandt et al., 2012; Mantsch et al., 2001; Pettit & Justice, 1991; Roberts, Loh, & Vickers, 1989; Wee, Specio, & Koob, 2007). An alternative explanation could be a particularly stress-prone strain or batch of animals used in this study. Rats from the same supplier and strain can differ in behavioural traits (e.g. sign-tracking vs. goal-tracking Fitzpatrick et al. (2013)) depending on the barrier they are raised in. Thus, although Sprague-Dawley rats are widely used in cocaine self-administration studies, the rats in this study may represent a particularly stress-sensitive sample for which the 800 μg/kg dose of cocaine was anxiogenic and aversive. Issues with the training schedule itself or the drug-administration apparatus are unlikely, because these should have affected heroin similarly, but all animals learned to self-administer heroin easily. There is also the possibility that certain parameters of the training schedule and experimental setup might have been specifically necessary to facilitate cocaine but not heroin self-administration.

Locomotion and stereotypy

Following the first drug infusion of the test session, rats displayed a markedly different locomotor response depending on whether they received heroin or cocaine. Within the first 5 min following the first drug infusion, cocaine and heroin did not produce statistically significant increases in locomotion relative to saline, suggesting that activity during this time period cannot be distinguished from a conditioned effect of the self-

administration environment. However, over the following 20 min post-infusion, locomotor activity in rats receiving heroin remained high, while activity in the cocaine and control groups diminished substantially. Immediately after the infusion locomotor activity in the heroin group was also quite variable, although high on average. This change in locomotor activity following heroin was accompanied by an increase in the average score on the scale adapted from Ellinwood and Balster, suggesting an increase in stereotyped behaviour relative to cocaine and controls which occurred in many rats instead of locomotion. With time, this kind of behaviour decreased and was replaced by locomotor behaviour. Note that the high score on the stereotypy scale following the 1st injection of the tests session was mostly the result of heroin-induced gnawing behaviour, which is assigned a score of 8 on the Ellinwood scale (see table 5.1, p.113; definitions in this table are copied from the original paper). Categorical measures of behaviour confirmed this observation (discussed further on).

Cocaine- and heroin-induced locomotor behaviour are well-established phenomena, but are usually studied under conditions of *intermittent* non-contingent drug administration via the i.p. route. Under such conditions, both drugs elicit robust increase in locomotor activity, and this effect sensitizes with repeated drug administration, depending on environmental context and individual differences (Crombag, Jedynak, Redmond, Robinson, & Hope, 2002; Flagel, Watson, Akil, & Robinson, 2008; Paolone et al., 2007). The development of sensitisation (in the broader sense) following history of self-administration also depends heavily on circumstances such as the pattern of self-administration as well as the presence or absence of an abstinence period. In a study using very similar self-administration parameters to the ones presented here (6h sessions, 40 available infusions, 1.5 mg/kg/inf), cocaine self-administration led to reduced locomotor and stereotypy response to a cocaine challenge, reduced DA release in the accumbens core, reduced ability of cocaine to block the DA transporter, and reduced DA release and uptake after artificial stimulation, relative to saline self-administering animals, 24 hours after the final self-administration session (Calipari, Ferris, & Jones, 2014). Other studies have shown tolerance or lack of sensitisation effects within this time frame following self-administration (24h), which is in contrast to evidence for sensitisation following intermittent access self-administration paradigms

or after a period of abstinence (Hooks, Duffy, Striplin, & Kalivas, 1994; Kawa, Bentzley, & Robinson, 2016). It would have been of interest to examine the effects of the training schedule on tolerance and sensitisation in the present study given the lack of detectable IEG expression in the striatum, since the two phenomena are related (Chandra & Lobo, 2017). However, the design of the present study did not allow for the assessment of tolerance or sensitisation, because all rats had undergone drug self-administration – a limitation that could be addressed in the future.

Following the second drug injection, locomotor behaviour was highest in rats receiving cocaine, comparable to the levels following the first injection. Control levels of locomotion had subsided towards the end of the session, suggesting that the conditioned effect of the environment was transient. Locomotor activity after a second injection of heroin was not very different from that of controls. A breakdown of the effect into drug-combination groups revealed a trend towards increased locomotion following a second injection of cocaine, where the effect was more pronounced if the preceding injection was heroin. The diminished locomotion in the case of a second injection of heroin could reflect a sedative effect or an increase in stereotypy. Thus, there was a trend suggesting an interaction between the effects of the first and second injections on locomotor behaviour. Indeed, animals pre-treated with heroin show an increase in locomotion after a cocaine challenge, and the effect is dose-dependent and additive (Leri, Flores, Rajabi, & Stewart, 2003).

Stereotypy did not vary much between heroin- and cocaine-treated animals after the second injection of the test session, regardless of the first drug administered. Control animals scored low on the stereotypy scale, and heroin-treated animals tended to score higher than both cocaine-treated and controls. However, most differences did not reach statistical significance so definitive conclusions cannot be made.

Categorical measures of behaviour

Soon after the first drug injection of the test session (5-10 min), locomotor behaviour was present in the majority of animals receiving cocaine and those in the control group. By 15 min, a small proportion of these animals were showing this behaviour. For heroin, the pattern was the opposite – a small proportion of animals were exhibiting locomotor

behaviour early compared to later on following a heroin injection. Thus, categorical classification of behaviour confirmed the continuous measure of locomotion: cocaine-induced and conditioned locomotion were present in the beginning of the test session, while heroin-induced locomotion was mostly seen later on. Heroin-treated animals also exhibited a lot of rearing behaviour towards the end of the first period in the test session. A notable difference was also seen in the proportion of animals exhibiting gnawing and sniffing behaviours. The majority of animals exhibiting the former behaviour were those who self-administered heroin, while the latter behaviour was more characteristic of animals receiving cocaine. This is not surprising, since oral stereotypy is a known consequence of repeated opiate treatment at high doses (Pollock & Kornetsky, 1996). There were no notable differences between drug groups and controls for other types of behaviour, except miscellaneous behaviours exhibited only by a few animals, but all of them receiving heroin. These behaviours included jumping, holding on to the wall of the cage or displaying catatonia.

Following the second drug injection (or the last 5 min of the test session for control animals) the most prevalent types of behaviours were locomotion and sniffing (exploring) in cocaine-treated rats and gnawing stereotypy in the heroin-treated rats. The control group were mostly still, grooming or showing some other form of low activity. Thus, by the end of the test session, conditioned effects on behaviour had mostly disappeared, while heroin was still inducing stereotypy, and cocaine – increased locomotor activity and exploration.

Summary of behaviour

Heroin at a dose of 100 µg/kg was an effective reinforcer, maintaining escalating drug intake. Rats receiving heroin during the test session were the most active, expressing a mixture of locomotor and stereotyped behaviour in response to the drug. Stereotyped behaviour often took the form of gnawing, and occurred immediately (up to 5 min) after the heroin injection. After about 10 min had passed since the injection, these rats switched to locomotor and rearing behaviour.

Rats were less motivated for cocaine than heroin – they self-administered relatively few of the available infusions every session, and took longer to approach the lever than they

did with heroin. Cocaine-receiving animals were active only immediately after the cocaine injection, and this effect was not easily discernible from potential conditioned effects of the drug-associated context. In contrast to the case of heroin, animals did not exhibit stereotyped behaviour following cocaine, but instead showed increased exploratory behaviour (locomotion and sniffing).

There was evidence for interaction between the first and second injections of the test session in terms of drug-induced locomotor behaviour, but not other measures of behaviour. That is, locomotion was reduced with repeated cocaine or heroin administration. When a combination of the two drugs was self-administered, cocaine-induced locomotion was not reduced by preceding heroin (was even slightly enhanced), while heroin-induced locomotion was reduced by cocaine.

CatFISH

There was very low expression of *homer 1a* in the striatum (see Appendix 3), so catFISH was used only to analyse IEG expression in the PFC. *Arc* expression in the striatum was more prevalent, but no quantitative analysis was done to determine whether drug treatments elevated expression over baseline. In the PFC, both *h1a* and *arc* expression was easily detectable, but was not affected by drug treatment during the test session. As already mentioned in the introduction, Fumagalli et al. have shown that a single cocaine self-administration session can result in increased *arc* expression in the PFC. However, their control groups consisted of yoked animals receiving cocaine and saline, and these two groups had comparable, lower levels of *arc* expression. Thus, it is possible that the increase in *arc* expression reflected a learning process rather than a drug effect. Indeed, Gao et al. (2017) did not find increased levels of *arc* expression following 10 days of cocaine self-administration (although there was an increase after 60 days). The saline controls in their study were exposed to the same self-administration environment, but did not receive any cocaine. Finally, Li et al. (2013) found elevated levels of *arc* protein product following runway training for heroin, but both the task and the schedule of reward delivery was very different from the self-administration procedure used here. They had only a single trial (drug treatment) per day. Thus, the reasons why no reliable increase of IEG expression was found in any experimental groups could be either that (i) such increase accompanies early learning but is absent following extended training; (ii)

that the training schedule and the task were not adequate to elicit IEG expression; or (iii) the duration of self-administration training was not long enough to cause changes relative to drug-naïve animals. This experiment was not designed to address the final possibility directly, but future studies must take it into account.

Overlap was also not found to vary significantly between treatment groups, eliminating the possibility that some drug-specific activity pattern might be masked over by the high baseline activity.

Conclusions and future suggestions

Rats responded very differently to heroin and cocaine under the conditions of the present study, both in terms of self-administration behaviour and drug-induced behaviour such as locomotion and stereotypy. Low levels of *homer 1a* expression in the striatum and no change from baseline expression in the PFC prevented the use of catFISH to examine patterns of neuronal activity associated with any behaviour. The data presented here is not sufficient to exclude a possibility of using catFISH in this context. However, if findings reported in preceding chapters are to be related to drug self-administration behaviour, the parameters of the training schedule must be changed.

One necessary change would be to test for an effect of duration of the training schedule. IEG expression seems to be evident only during very short or very long periods of drug self-administration (Fumagalli et al., 2009; Gao et al., 2017; Li et al., 2013). Alternatively, other IEGs can be used for catFISH. One possible alternative would be using *c-fos* instead of *homer 1a* to identify active neurons in the striatum, since *c-fos* and *homer 1a* mRNA have similar temporal profiles of expression (peak ~30 min following activity). Previous studies have shown that *c-fos* expression in striatum and PFC is still elevated by drugs even after many days of self-administration training. Finally, catFISH can be used to mark the neuronal populations activated by two drug stimuli administered 1h apart rather than 25 min apart, in order to avoid interactions between them. This can be achieved by using nuclear *arc* signal to tag one neuronal population and cytoplasmic *h1a* signal to mark the other, since *h1a* diffuses to the cytoplasm ~1h following cellular activity.

The preference for heroin over cocaine shown by rats in this study is also intriguing. Future studies could address the question of whether this preference is the result of

availability of both drugs. That is, would the rats self-administer cocaine at higher rates if heroin was not available at all?

The interaction between heroin and cocaine in terms of their effect primarily on locomotion but not stereotypy is to be noted as well. Stereotypy was much more prevalent in animals receiving heroin. It is possible that activity produced by heroin in the dorsal striatum is increased following prolonged exposure to heroin, producing a different pattern of activity than that seen after non-contingent drug administration in naïve animals. The interaction effects of heroin and cocaine self-administration on locomotor behaviour may in turn reflect the interaction seen in terms of overlap in the Nacc following non-contingent drug administration. Future studies could answer these questions if the right training schedule and IEGs are used to apply the catFISH technique to the self-administration paradigm.

Chapter 6 – Discussion

1. Summary of findings and methodological considerations

Summary of findings

Non-contingent administration of heroin and cocaine at doses self-administered by rats elevated expression of the immediate-early genes *homer 1a* and *arc* in several parts of the striatum – a brain area involved in processing of reward and goal-directed behaviour. The temporal pattern of expression of the two genes was similar to the one found in the hippocampus following exploration of a novel environment. Levels of *homer 1a* expression were elevated within ~30 min following drug administration, while expression of *arc* was elevated as soon as 5 min after drug treatment. Thus, it was possible to take advantage of this difference and use the catFISH technique to search for neuronal populations activated by heroin and cocaine in the striatum, and to determine to what extent these populations are overlapping or distinct. This analysis revealed that cocaine activates the same population of neurons repeatedly in the Nacc core and the dorsal striatum (but not in the shell), which suggested the existence of a neuronal ensemble in those brain areas encoding for some of the effects of the drug. Conversely, the same was found for heroin only in the Nacc shell.

Although evidence for neuronal ensembles responding to heroin and cocaine was found only in certain striatal areas, IEG expression was affected by both drugs across the whole striatum. That is, cocaine treatment did have an effect on *homer 1a* and *arc* expression in the Nacc shell. However, it was not possible to determine whether there were neurons responding reliably to the drug in this brain area. Heroin did increase *homer 1a* and *arc* expression in other parts of the striatum than the Nacc shell. Yet, the effect varied depending on what drug was administered beforehand, whether expression was measured in the dorsal striatum or the Nacc, and there was no evidence for a neuronal ensemble responding to heroin in other striatal areas than the shell. Regardless of whether they represented neuronal ensembles or not, the neuronal populations

responding to heroin and cocaine overlapped significantly only in the Nacc shell. In all other areas of the striatum, the overlap did not exceed chance levels.

Finally, a self-administration experiment revealed that the dose of cocaine used for the non-contingent drug administration study did not maintain self-administration in a substantial proportion of animals. It was also evident that drug-induced behaviour such as locomotion and stereotypy persisted for at least 25 min following a self-administered drug injection; i.e. these behaviours were still evident by the time a second injection was administered for catFISH. This persistence of behaviour was more pronounced following 100 µg/kg heroin than 800 µg/kg cocaine. Following 14 days of heroin and cocaine self-administration IEG expression did not change in response to an injection of either drug in the PFC, and *homer 1a* expression was almost undetectable in the striatum.

Thus, catFISH revealed that heroin and cocaine engage different neuronal populations in most parts of the striatum except the Nacc shell, at least as far as *homer 1a* and *arc* expression are concerned. However, this observation must be considered taking into account factors such as drug dose, possible interactions between heroin and cocaine administered in succession, and previous experience with each of the two drugs.

Methodological considerations

It is important to keep in mind that the relationship between neuronal activity from an electrophysiological point of view (i.e. firing of action potentials) and IEG expression is not straightforward. Although there are a lot of commonalities between different IEGs in terms of the molecular mechanisms which trigger IEG expression, there are cell-type and regional differences in levels of IEG expression, as well as differences in the preferred transcription factors involved in IEG transcription. Generally, IEGs are transcribed after a strong depolarisation of a neuron leads to calcium influx through NMDA receptors and calcium voltage-gated channels. Calcium then interacts with protein kinases (e.g. CaMK and MAPK) which in turn activate transcription factors (e.g. CREB, SRF) to promote IEG transcription (Kawashima et al., 2014). Thus, the first point to consider is what constitutes strong activation sufficient to trigger calcium influx. It seems the rate of action potential firing is the most defining factor in this case. For example, Sgambato et al. have shown that cortical stimulation elicits the highest rate of

c-fos protein expression when a high number of shocks are applied over a given stimulation period, regardless of the temporal pattern of stimulation (i.e. single shock vs. trains, or high vs. low frequency) (Sgambato, Abo, Rogard, Besson, & Deniau, 1997). In addition, elevated levels of phosphorylated (i.e. activated) CREB is seen following both high- and low-frequency stimulation, but only prolonged stimulation at low frequencies can lead to transcription of *c-fos* (Bito, Deisseroth, & Tsien, 1996). CREB activity is tied to both LTP and LTD-inducing kinds of stimulation (Deisseroth, Bito, & Tsien, 1996). Second, if voltage-gated calcium channels are involved in IEG transcription, then IEG transcription induced by acute heroin administration may be both positively and negatively affected. Interestingly, activation of the μ-opioid receptor can both activate the MAPK pathway (Macey, Lowe, & Chavkin, 2006), and suppress activity of voltage-gated calcium channels. This presents a case of two competing mechanisms, one promoting and one supressing IEG expression in response to heroin. This could be one explanation for the substantially diminished expression of *arc* following repeated administration of heroin. Finally, expression of different IEGs may be more or less driven by one transcription factor or another, despite the fact that multiple converging intracellular cascades can promote transcription of the same IEG. This could explain, for example, why very low *homer 1a* transcription was found in the Nacc shell in response to cocaine, while *arc* transcription was high. The fact that heroin increased *homer 1a* in the shell further supports this point. Finally, several response elements may need to act in concert to allow c-fos expression (Robertson et al., 1995), and it is not unlikely that this is the case for other IEGs such as *arc* and *homer 1a*.

In summary, although IEGs are widely recognised as markers of neuronal activity, drug-induced IEG expression should be interpreted with caution. More precisely, this type of IEG expression is likely to reflect long-lasting neurobiological changes that occur as a result of early drug effects, and these changes occur under very specific circumstances that do not reflect all types of neuronal activity. The fact that changes in IEG expression were not found following a period of drug self-administration training further supports this claim.

Another consideration to be taken into account is what was considered a neuronal ensemble throughout the studies presented in this thesis. Some discussion of the

different definitions of a neuronal ensemble was provided in ch.1. Additionally, some authors distinguish between neuronal assemblies, neuronal ensembles, and the memory engram. This is particularly the case in the context of memory research, amygdalar and hippocampal function. A neuronal assembly is defined as a group of neurons which are activated in concert due to established connections between them. Neuronal ensembles, on the other hand, refers to groups of neurons involved in a particular computation. In electrophysiological research, the term also refers to populations of neurons which present correlated activity during a specific task, regardless of their causal role in behaviour or perception (Deadwyler & Hampson, 1997). Importantly, individual neurons within neuronal ensembles tend to be noisy, but together they produce a coherent output. Finally, the engram is a term particularly relevant to memory research, and refers to the physical representation of memory in the brain – the sum of the biochemical and biophysical changes induced upon learning (Holtmaat & Caroni, 2016; Tonegawa, Pignatelli, Roy, & Ryan, 2015). Regardless of the particular terms used, it is generally accepted that the coordinated function of distributed neuronal populations is what constitutes the coding language of the brain.

Calcium imaging has also proved a very useful tool for characterisation of neuronal ensembles, since it allows for the simultaneous recording of the activity of spatially distributed neurons of the cortex. Work in this field has corroborated findings using IEG-driven neuronal tagging. For example, calcium imaging has revealed that the visual cortex is comprised of groups of coactive neurons (assemblies) which exhibit synchronised spontaneous activity. During perception, these assemblies become active in a similar fashion, but time-locked to the stimulus in question (ensembles). In addition, the firing of neurons in these ensembles is a probabilistic phenomenon, and only a proportion of neurons exhibit the highest probability of firing (“core ensembles”) (J. E. Miller, Ayzenshtat, Carrillo-Reid, & Yuste, 2014).

Corroborating evidence for the function of the visual cortex has also been demonstrated in the field of IEG-tagged neuronal ensembles. In the primary visual cortex, upon initial experience with a light stimulus of a particular orientation, a large number of neurons express *arc*. Only a small subset of these neurons are recruited one day after. Thus, previously activated neurons are likely to be recruited again when the same stimulus is

presented a second time, thus forming a neuronal ensemble. Importantly, with repeated presentation of the stimulus, the ensemble becomes smaller and more reliably activated by the stimulus. Expression of *arc* is not necessary for this phenomenon, although it does improve the specificity of the resulting ensemble (Wang et al., 2006).

Obviously, whether the organisation of neuronal networks and encoding of stimuli and memories is the same between cortical and subcortical structures is a separate question. However, there is convincing evidence that this might be the case, at least in terms of contextual fear conditioning and motivated behaviour. More importantly, manipulating neuronal ensembles in subcortical structures through IEG tagging and optogenetics has revealed the causal role of neuronal ensembles in these forms of behaviour. For example, activity of neurons in the lateral amygdala during cocaine CPP is determined by the activity of CREB, such that neurons with higher levels of CREB are more likely to be recruited during learning. In addition, ablation of these neurons abolishes the expression of the CPP memory (Hsiang et al., 2014). Similarly, the expression of contextual fear memories can be elicited by the optogenetic stimulation of neuronal ensembles in the hippocampus (Liu et al., 2012).

One remaining question is whether there is evidence that memories of a drug effect are encoded similarly and independently of context. When cocaine is administered non-contingently (following a sensitising protocol), a large proportion of the Nacc neurons recruited following the drug administration are those that have been activated during previous injections (Mattson et al., 2008). Thus, the drug itself seems to influence neuronal activity the same way as a visual stimulus does in the cortex – by activating neurons that are intrinsically more excitable. However, this effect is not completely independent of context, since there is a much larger number of neurons active in a context previously paired with drug delivery (in the same study). To some extent, the circumstances of drug administration define which and how many neurons will be activated.

In summary, the concept of neuronal ensembles has a somewhat loose definition that varies depending on the specific field of research where it is used, and on the techniques used to define it. Nevertheless, there is general consensus that a population of neurons has to exhibit simultaneous activity time-locked to a given stimulus or behaviour in order

to be defined as an ensemble. In some cases, it is also required that the ensemble of neurons are causally involved in the expression of a behaviour, or are at least reliably activated by the repeated presentation of the same stimulus. In studies presented in this thesis, there were no available tools to infer causal role of the neuronal populations in behaviour. For this reason, a neuronal ensemble was defined as a population of neurons which is reliably active during the repeated presentation of the same drug stimulus.

Finally, on several occasions it was implied that the distinct neuronal populations activated by heroin and cocaine across the striatum are indicative of dissociated circuitry processing the acute effects of the two drugs. This claim warrants a clarification of what is meant by a "circuitry", and why distinct neuronal populations within the same brain area may be indicative of dissociated circuitries. The definition of circuitry used presently can be related (but not equated) to the Hebbian concept of "phase sequence": the serial activation of cell assemblies bridging the gap between sensory input and motor output (Hebb, 1949). More specifically, a circuitry here is defined as all neurons activated in response to heroin or cocaine administration, the activity of which encodes for any of their subjective or overt behavioural effects. Dissociation of circuitries in this case implies that such neurons are characterised by one or more of the following: they comprise distinct neuronal populations within the same brain areas, they are embedded in different brain areas, they receive excitatory input from different up-stream sources (or combinations of sources), and/or send afferent projections to different down-stream targets (or combinations of targets), and/or exhibit distinct genetic/neurochemical profiles (e.g. express different receptors, release different neurotransmitters, etc.). The data presented in this thesis provides direct evidence for the first prerequisite for dissociated circuitries processing heroin and cocaine, but there is already existing evidence that the striatum is functionally and structurally organised to accommodate for the rest of the prerequisites as well, as described in continuation.

First of all, MSNs and cortical pyramidal neurons are characterised by up- and down-states meaning they alternate between highly polarised (-80 mV) vs. depolarised (-55 mV) sub-threshold resting membrane potentials (J. A. Wolf, Schroeder, & Finkel, 2001). Action potential firing is rare during down-states and transition to up-states and spiking

activity is only possible with synchronised excitatory input coming from multiple sources converging onto single MSNs. Dopamine signalling through D1 receptors may further facilitate the transition to and the maintenance of up-states (O'Donnell, 2003). Thus, in order for MSNs to be excited (and to express IEGs), they must receive input from several sources which may include different combinations of amygdala, hippocampus, thalamus, PFC and VTA/SNc afferent inputs (Pennartz et al., 1994). Each of the brain areas sending these afferent projections i) may be affected differently by heroin, cocaine, and natural rewards (Chang et al., 1998; Mukherjee et al., 2018); ii) may contain neuronal ensembles involved in distinct functions (Warren et al., 2016; Zelikowsky et al., 2014), and iii) may be comprised of genetically distinct projection neurons. Thus, taking into account the integrative function of the striatum (i.e. the combined glutamatergic input it receives from multiple brain regions), the diverse connectivity and specialised functions of its input regions, and the necessity for synchronised excitatory input to elicit action potentials from MSNs, it is quite possible that distinct neuronal populations activated in the striatum represent activity within dissociated circuitries. Here it must be noted that, although the afferent inputs of the striatum from limbic and cortical areas are topographically organised in a ventromedial-dorsolateral fashion (see fig 1.1, p. 10), they are not constrained to perfectly defined striatal subregions, but are overlapping, with higher concentrations of certain afferents in e.g. shell vs core (Voorn et al., 2004).

Further support for the possibility that distinct neuronal populations in the striatum pertain to distinct circuitries comes from the diverse output targets of the striatum, some discussion of which was presented in ch.1. More specifically, the cortico-striatal-thalamocortical loops characteristic of the striatum are a good example of how parallel but distinct circuitries can pass through this brain area (Alexander, Crutcher, & DeLong, 1990; Pennartz et al., 1994).

It should also be taken into account that MSNs send collateral GABAergic projections to neighbouring MSNs. This mutual inhibition between MSNs is another functional-anatomical feature predisposing the accumbens and rest of striatum to accommodate neuronal ensembles embedded in distinct circuitries – whilst one particular ensemble is active, it can decrease the activity in other ensembles so that only a particular computation is taking place over others. Lateral inhibition can also help in the formation

of well-defined activity patterns *within* ensembles; i.e. reduce the number of neurons active within a given ensemble, leaving more available neurons for encoding of other functions (Pennartz et al., 1994).

Of course, all these characteristics of the striatum are only suggestive of the possibility that distinct circuitries process heroin and cocaine, in view of the data shown in this thesis. Further research is necessary to empirically ascertain the presence of such dissociated circuitries by incorporating i) viral tracing techniques to identify afferent and efferent input of heroin- and cocaine-activated neurons; ii) histochemistry – to assess for genetic differences between these neurons; and iii) opto- or chemogenetic techniques to look for functional differences of said neurons.

2. Implications of main findings for addiction theory and treatment practice

Theoretical implications

Current theories of addiction have shifted their focus away from the acute effects of drugs in order to explain addictive behaviour through aberrant learning processes and long-term plastic changes in the brain. For example, incentive-sensitisation theory proposes that the crucial factor in drug addiction is the interplay between sensitised brain systems controlling motivation, and drug-associated stimuli to which these systems become hyper-reactive. Sensitisation can take many forms, and includes, but is not limited to, changes in dopamine transmission from the VTA, and glutamatergic transmission from the prefrontal cortex to the Nacc (Berridge & Robinson, 2016; M. E. Wolf, 2010). Frontal-striatal dysfunction theory further expands this view by proposing that dopamine signalling in the amygdala might be involved in the strengthening of associations between drug effects and stimuli from the environment. In addition, it suggests that hypofunction of the prefrontal cortex may result in diminished ability to control impulses arising from a hyper-reactive motivational system (Jentsch & Taylor, 1999). Habit formation theory takes a somewhat different approach by suggesting that outcomes of behaviour in drug addiction become less and less able to control the behaviour, which becomes primarily dependent on stimulus-response associations (Everitt & Robbins, 2005). Regardless of the specific approach taken, there is an implied consensus between theories that the neurobiological and behavioural changes involving

the striatum and learning become relevant only following prolonged exposure to drugs. This framework has undoubtedly led to important insights into the psychobiological processes that underlie the maintenance of compulsive drug-seeking once it's established. However, it still remains unclear which psychobiological processes give rise to addiction in the first place (Wise & Koob, 2014). Arguably, the early acute effects of drugs are important in this regard, because drug-induced behavioural and neurobiological changes have to begin somewhere. Knowing where in the brain drugs exert their effects initially and how (or if) these effects lead to pathological drug-taking is an equally important theoretical question to that of how addiction is maintained once it's established. The studies presented here are a step towards answering these questions, especially considering the functional importance of IEG-expressing neurons. As discussed previously, neurons which express IEGs undergo changes in excitability, and are more likely to be incorporated in neuronal ensembles with a causal role in behaviour and perception. In addition, these neurons are more likely to undergo morphological changes. For example, silent synapses form preferentially in *c-fos*-positive neurons of the Nacc shell following repeated treatment with cocaine (Koya et al., 2012). Morphine treatment also results in the formation of silent synapses, albeit through different mechanisms (formation of new synapses vs. endocytosis of AMPA receptors at existing synapses, respectively (Graziane et al., 2016)). The findings presented here suggest that, at least initially, such changes may occur in an ensemble shared by heroin and cocaine in the Nacc shell. Conversely, different ensembles may undergo changes induced by each drug in the rest of the striatum.

The functional significance of these convergent and divergent neurobiological changes following heroin and cocaine remain an open empirical question. That is, it remains to be determined if separation of neuronal ensembles within the same brain area leads to distinct behavioural effects of heroin and cocaine. Recently, it was shown that distinct, intermingled neuronal ensembles within the vmPFC can drive both reward and extinction memories for food (Warren et al., 2016). Thus, a separation of neuronal ensembles may indeed signify functional differences.

Possible implications for treatment and prevention of drug abuse disorders

Treatments for addiction can be broadly classified in two main categories – behavioural psychotherapy and pharmacotherapy. Some types of behavioural therapies involve taking advantage of basic learning principles such as operant conditioning. For example, community-based approaches aim to encourage sobriety through positive social reinforcement (W. R. Miller, Meyers, & Hiller-Sturmhofel, 1999). Contingency management therapies reinforce abstinence through monetary or voucher prizes in exchange for drug-free samples (Petry, Martin, & Simcic, 2005). Cognitive-behavioural therapies usually involve training drug abusers to recognise and control triggers of drug craving, and craving itself (Carroll & Onken, 2005). Such approaches to addiction therapy do not have a direct connection to the neurobiology of the disorder. Yet, findings indicating dissociated neural substrates for different addictions may serve as proof of concept that heroin and cocaine addictions need to be conceptualised differently. For example, it has been shown that the environment can modulate the reinforcing (Caprioli et al., 2008; Caprioli, Paolone, et al., 2007) and subjective effects (Avvisati et al., 2016; Caprioli, Celentano, et al., 2007) of heroin and cocaine in opposite ways, as well as the propensity to relapse (Montanari et al., 2015). If behavioural therapies take into account high-risk factors for relapse and their management, there is good chance that these factors will be defined by drug-specific action in the brain at least to some extent.

Pharmacotherapy for addiction involves the controlled administration of substances that either mimic the pharmacological effects of the abused drug, or reduce its reinforcing properties through blockade of its target receptors. The best known example is methadone substitution therapy for opiate addiction (Joseph, Stancliff, & Langrod, 2000), but there are similar approaches for alcohol abuse (e.g. benzodiazepine treatment (Gatch & Lal, 1998)), as well as for nicotine addiction. More recently, research has focused on drugs that induce aversion to alcohol (e.g. ALDH inhibitors (Yao et al., 2010)) or suppress the reinforcing properties of drugs through blockade of NMDA receptors (Bisaga & Popik, 2000). NMDA antagonists are also used for disruption of memories which underlie the conditioned effects of reward-associated cues in laboratory animals (Exton-McGuinness & Lee, 2015). With this new interest in NMDA-targeting drugs, a good understanding of the precise way the brain is encoding the

specific effects of drugs has a lot of relevance to pharmacotherapy. Given that drug-induced brain changes and drug-related memories are a main driving force of drug addiction, and are dependent on the NMDA receptor, the ability to identify and target them with high specificity would be useful. In this context the separation of the neuronal populations encoding for heroin and cocaine effects is particularly relevant, as it implies that novel approaches may be more successful if they target drug-specific neural substrates instead of looking for a common substrate as a target.

3. Future suggestions

As already implied, the functional significance of the separation between neuronal populations responding to cocaine and heroin in the striatum remains to be determined. The Daun02 method and optogenetic manipulation of neurons activated during a specific event are useful tools in this regard. One suggestion for future research would be to test whether inactivation of neurons responding to cocaine can disrupt self-administration and reinstatement of drug-seeking behaviour for heroin (or vice versa).

In addition, it remains to be determined if there are any differences in terms of the genetic profile of neurons responding to the two drugs. A study using the fluorescence-activated cell-sorting technique (FACS) has shown that striatal neurons expressing *c-fos* in response to a single injection of cocaine also express *arc*, and include mostly dopamine D1 receptor containing neurons. These strongly activated neurons also have increased levels of the phosphatase Mkp1, and reduced levels of the kinase Map2k6, meaning that p38 MAPK signalling might be attenuated in cocaine-activated striatal neurons (Guez-Barber et al., 2011). The selective expression of IEGs such as *c-fos* and *arc in* D1-expressing MSNs is not surprising considering that IEGs are expressed as a result of neuronal activity which involves depolarisation of the cell membrane and calcium influx. Calcium-initiated IEG transcription is moderated by D1 and D2 dopaminergic receptors which stimulate and inhibit PKA, respectively (Tritsch & Sabatini, 2012), and PKA is necessary for the phosphorylation of CREB which controls *arc, homer 1a* and *c-fos* expression (Impey et al., 1998). In addition, activity at D1 receptors facilitates excitatory glutamatergic transmission in the striatum by increasing AMPA and NMDA receptor surface expression, enhancing currents through these receptors and inducing up-states in MSNs (Surmeier, Ding, Day, Wang, & Shen, 2007). Thus, it is likely

that activity at D1 receptors would have a facilitating effect on IEG transcription, and cells expressing D1 receptors would be more likely to express IEG compared to D2-expressing MSNs. Cocaine has a direct effect on DA transmission through inhibition of DA re-uptake, while heroin's effect is indirect and less pronounced (already discussed in chapter 1). Considering the separation of neuronal ensembles responding to cocaine and heroin as well, it is possible that the genetic profile of heroin-activated neurons will be different, and may include D2-receptor-expressing neurons. Future studies should address this question, because that would be the first step towards being able to identify and selectively target neurons that encode drug-specific information.

Another possible line of investigation would be to examine the afferent and efferent projections of neurons responding to heroin and cocaine administration. Retro- and anterograde labelling techniques can reveal whether the two drugs preferentially engage neurons receiving information from different brain areas. Finally, the electrophysiological profile of neurons responding to cocaine and heroin can be determined using transgenic animals that express GFP under the promoter of IEGs such as *c-fos* and *arc*.

Finally, it must be noted that, in the studies described so far in this thesis, no attempt was made to control for social factors on drug-induced IEG expression or reinforcement. The importance of social factors has recently begun to gain popularity in animal research of drug addiction (Heilig, Epstein, Nader, & Shaham, 2016), and studies modelling the effect of social factors on the reinforcing and motivating properties of heroin and cocaine have been taking place for a while. For example, a low dose of cocaine is more reinforcing, while a higher dose is less reinforcing in rats reared in isolation early in life (from postnatal day 21) relative to group housed rats, and isolated rats show higher levels of zif268 protein product in the Nacc shell and core, DMS and DLS following i.p. cocaine (Howes, Dalley, Morrison, Robbins, & Everitt, 2000). Accordingly, adult (42 days old) female rats who are housed individually have a higher break point on a progressive-ratio schedule of cocaine reinforcement relative to pair-housed females (Westenbroek, Perry, & Becker, 2013). Heroin self-administration rates are also higher in adult isolated rats relative to pair-housed ones (Bozarth, Murray, & Wise, 1989). Finally, social

isolation facilitates the reinstatement if heroin-induced CPP (Turner, Sunohara-Neilson, Ovari, Healy, & Leri, 2014).

While social isolation can promote the reinforcing and motivating properties of drugs of abuse, social interaction can serve as an alternative reinforcer acting in opposition to drug reinforcement. For example, social interaction can serve as a reinforcer in the CPP procedure, where one compartment can be paired with the presentation of a conspecific, while the other compartment is paired with drug administration. In such cases social interaction serves as a more potent reinforcer than cocaine, so that rats will choose the social-interaction-paired compartment over the cocaine-paired one. This effect is accompanied by reversal of cocaine-CPP-induced expression of the zif268 protein in the Nacc, amygdala and VTA (Fritz et al., 2011). Finally, cocaine self-administration is potentiated by the presence of a conspecific with access to cocaine during self-administration sessions, while decreased by the presence of an abstaining conspecific (Peitz et al., 2013; Smith, 2012). Thus, social factors can influence both the reinforcing properties of drugs and their ability to induce IEG expression.

In the studies presented in this thesis, all rats were tested following individual housing, which may have facilitated IEG expression in response to cocaine, but also reduced their propensity to self-administer a high dose of cocaine (Howes et al., 2000). One possible suggestion for future research would be to examine if such an effect is present for heroin-induced IEG expression, in particular *arc* and *homer 1a*, which have not been examined so far. Social effects on heroin self-administration as a function of dose has also not been studied, and it would be of particular interest since it may explain why rats in the self-administration study (chapter 5) showed better performance on acquisition of heroin vs. cocaine self-administration at the doses used.

4. Conclusion

The common goal of the series of experiments presented in this thesis was to compare the acute effects of heroin and cocaine – namely, the pattern of neuronal activity elicited by the two drugs in brain areas believed to be central to the neurological basis of drug addiction. The catFISH technique was the most appropriate method for this purpose. It is one of few currently available techniques which allows for direct comparisons of

neuronal activity elicited by two separate stimuli, within the same experimental animal, and in deep subcortical brain areas. Despite some caveats, such as the interaction between drug injections administered 25 min apart, and the different levels of IEG expression between brain areas, the results suggests a significant separation between the neuronal populations responding to heroin and cocaine in the striatum. Although the functional significance of this separation could not be determined, it is safe to suggest that it exists and it should be taken into account by future studies. Overall, this thesis provides a proof of concept that heroin and cocaine are processed differently by the brain, even within a brain area considered to be the common substrate for the addictive properties of the two drugs. Future research should focus on characterising the neuronal populations engaged by heroin and cocaine in terms of their genetic profile, connectivity, electrophysiological properties, and the kind of behaviours for which they encode.

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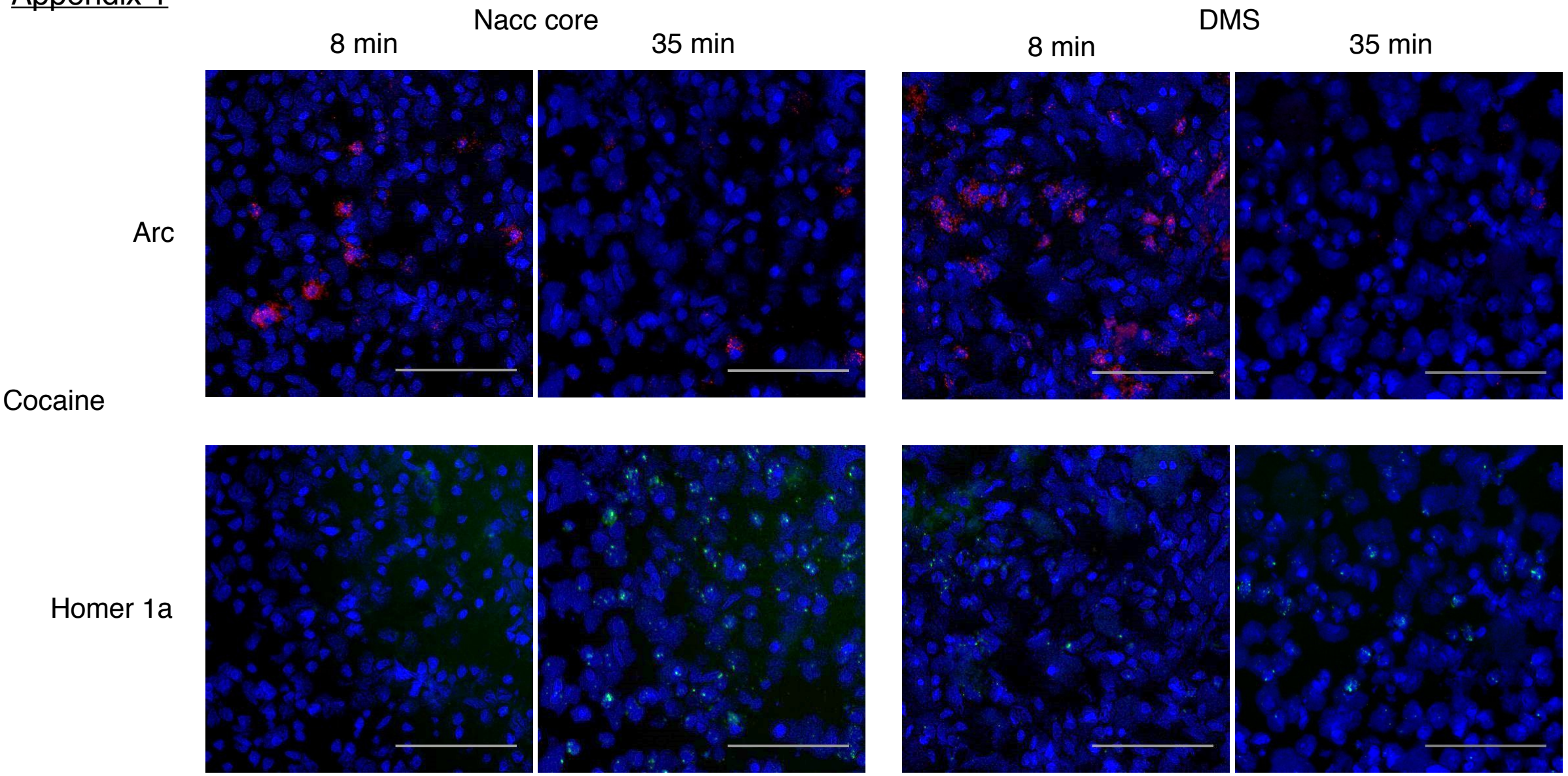
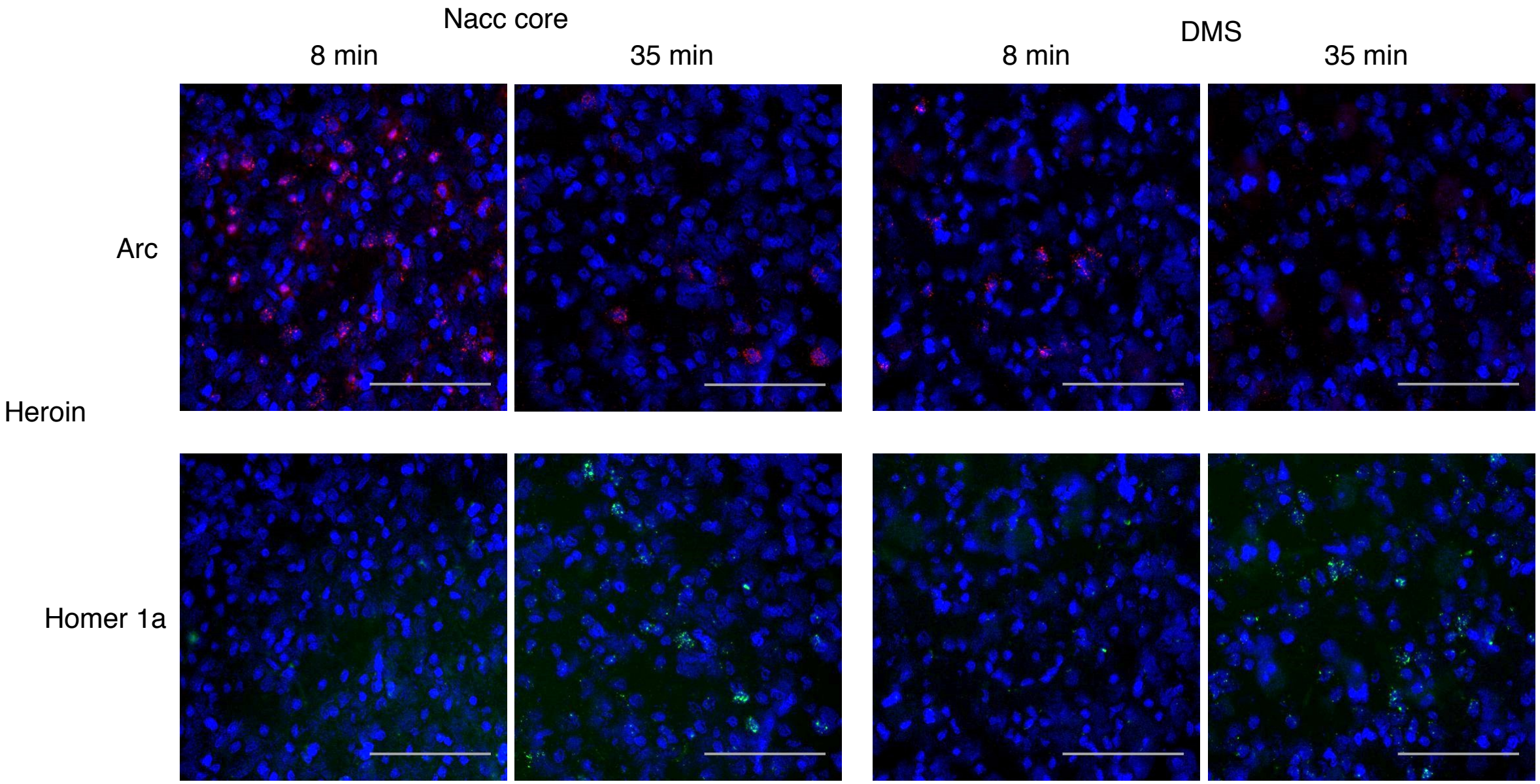
Appendix 1

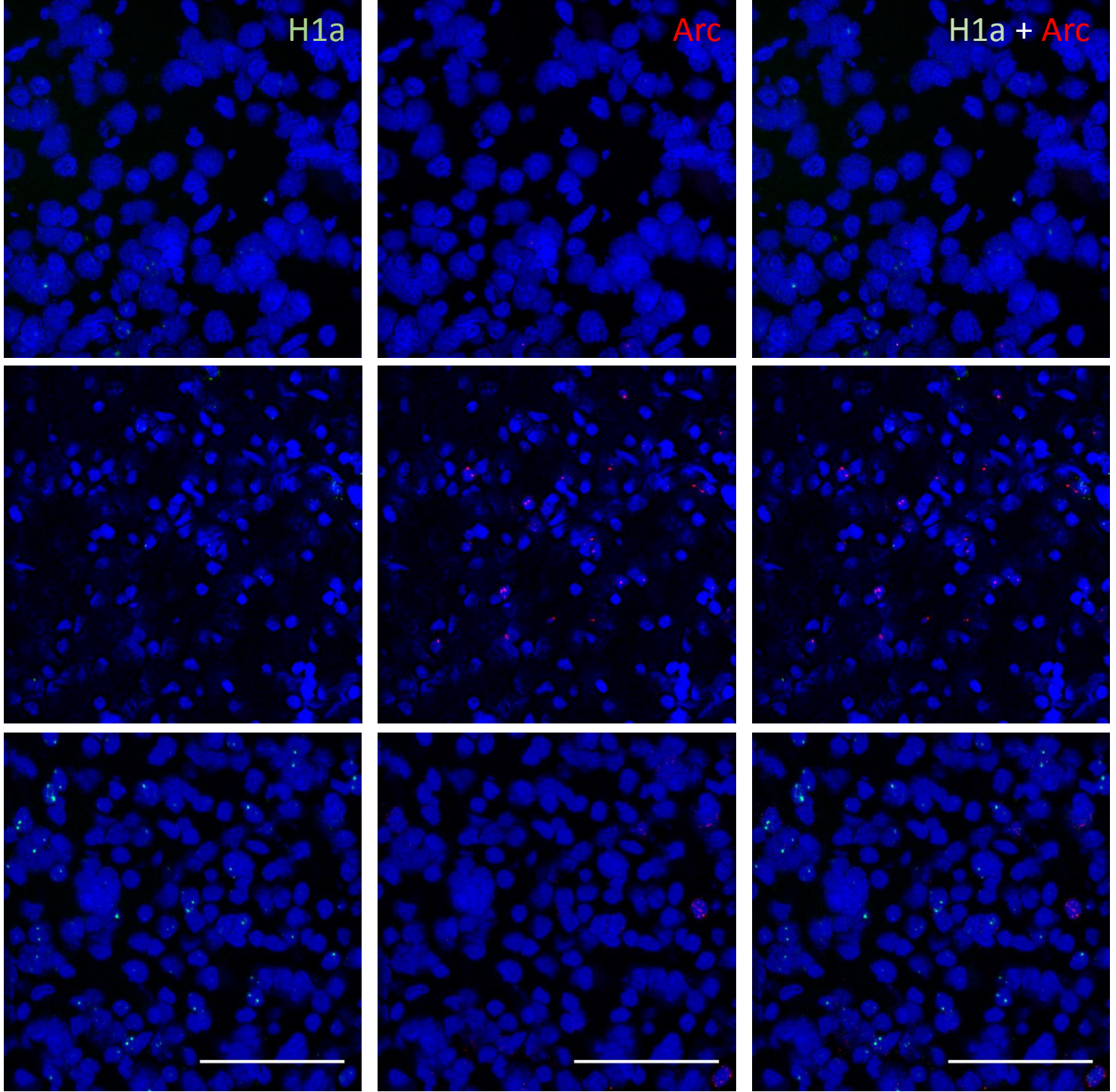


Representative images of cell nuclei (blue) expressing *homer 1a* (green) and *arc* (red) following cocaine administration. Scale bar = 100 μm

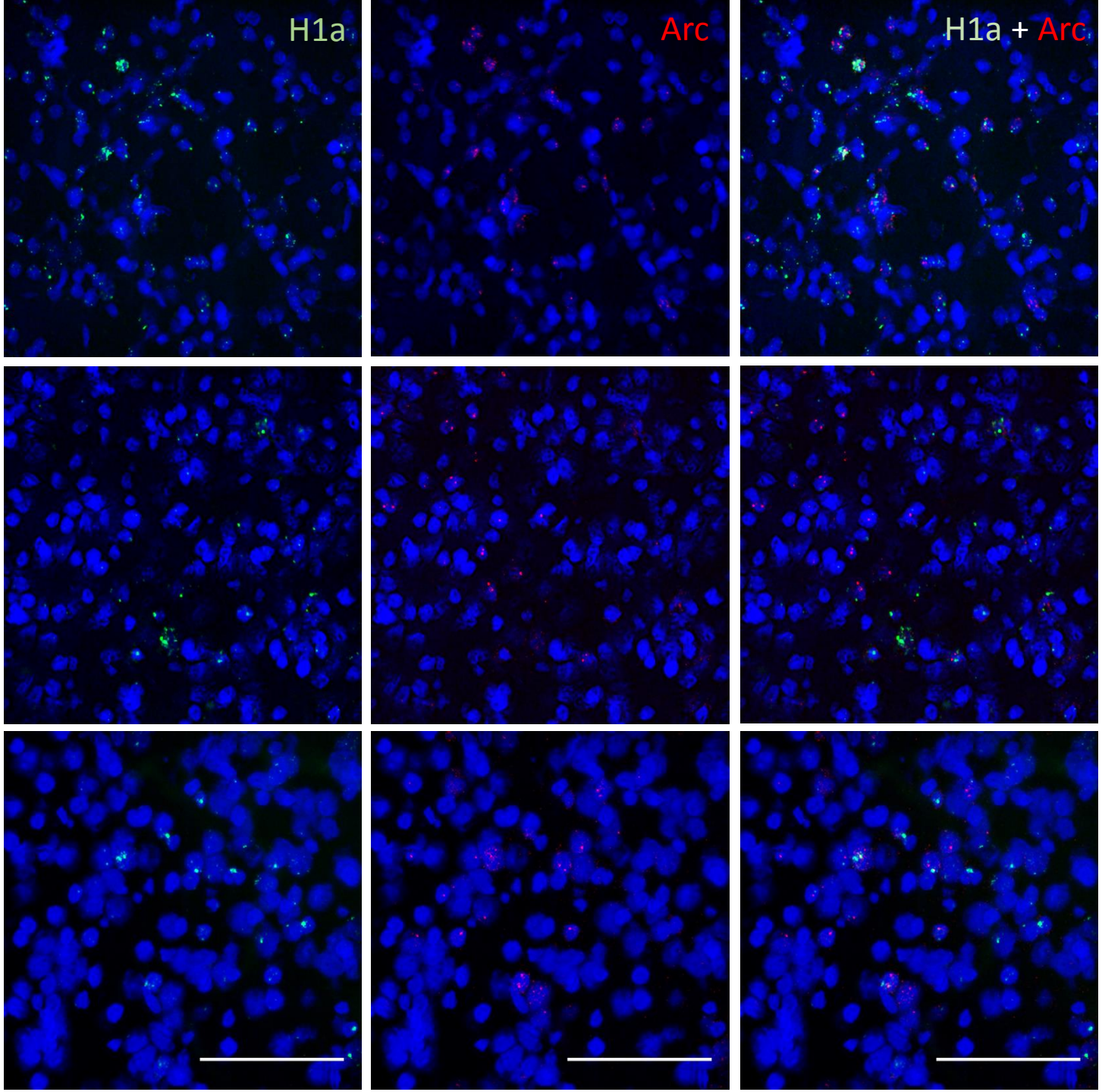


Representative images of cell nuclei (blue) expressing *homer 1a* (green) and *arc* (red) following heroin administration. Scale bar = 100 μm

Appendix 2



Representative images of cell nuclei (blue) expressing *homer 1a* (green) and *arc* (red) and merged images. Nacc core – saline control conditions. Scale bar = 100 µm.



Representative images of cell nuclei (blue) expressing *homer 1a* (green) and *arc* (red) and merged images. Nacc core – experimental conditions. Scale bar = 100 µm

Nacc core coc-coc 5Z1

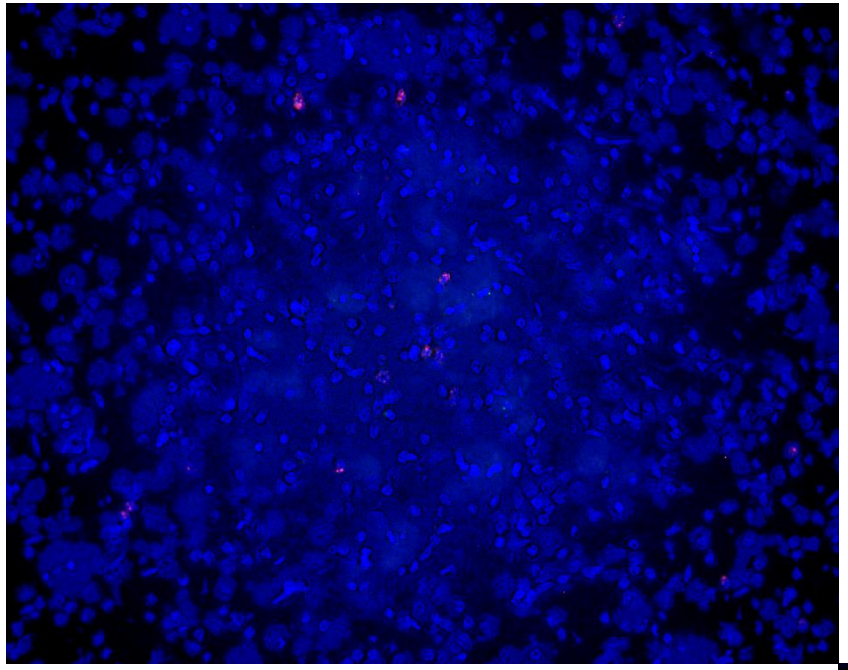
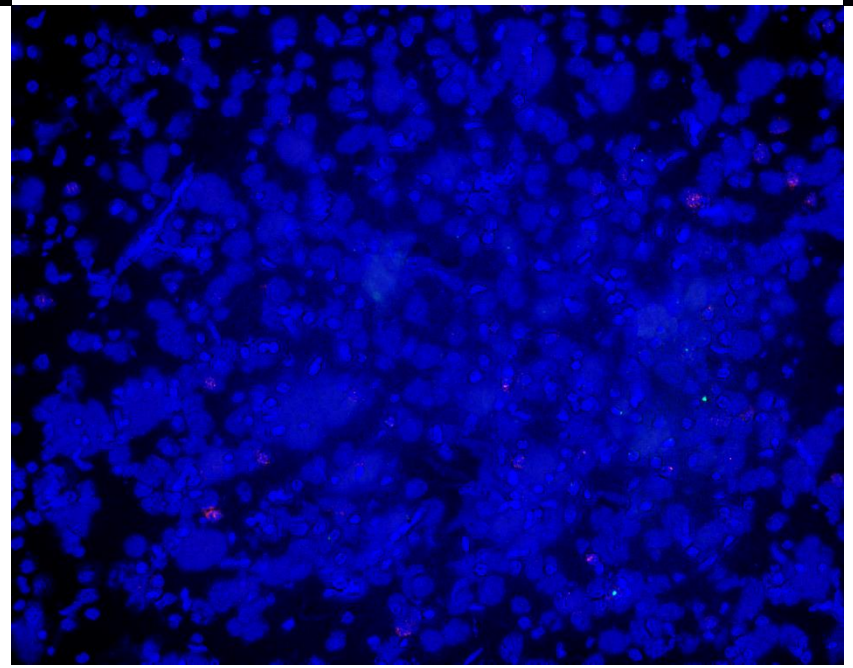


Image 1 (↑)
A representative image of the DMS of an animal that received two consecutive injections of cocaine after heroin and cocaine self-administration training. Note the lack of *homer-1a*-positive nuclei, and the low level of *arc* expression.

Image 2 (↓)
A representative image of the DLS of the same animal.



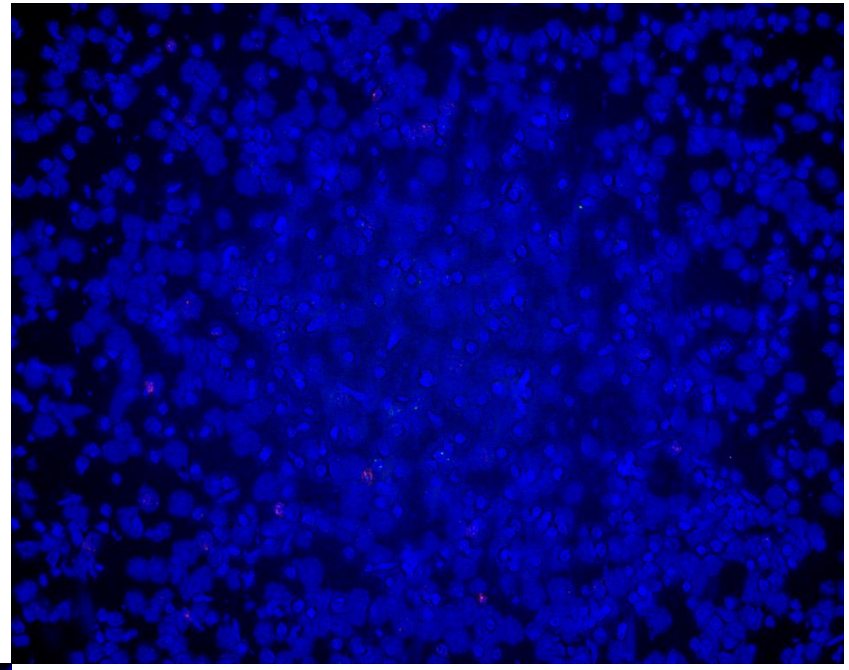


Image 3 (↑)
A representative image of the Nacc core of the same animal.

Appendix 3