eNeuro

Research Article: New Research | Disorders of the Nervous System

Distinct populations of neurons activated by heroin and cocaine in the striatum as assessed by catFISH

https://doi.org/10.1523/ENEURO.0394-19.2019

Cite as: eNeuro 2020; 10.1523/ENEURO.0394-19.2019

Received: 30 September 2019
Revised: 30 November 2019
Accepted: 19 December 2019

Distinct populations of neurons activated by heroin and cocaine in the striatum as assessed by catFISH

Philip Vassilev[1], Riccardo Avvisati[1], Eisuke Koya[1], Aldo Badiani[1,2]

[1] Sussex Addiction Research and Intervention Centre (SARIC), School of Psychology, University of Sussex, United Kingdom; [2] Department of Physiology and Pharmacology, Sapienza University of Rome, Italy.

Correspondence should be addressed to:
Aldo Badiani - Sussex Addiction Research and Intervention Centre (SARIC), School of Psychology, University of Sussex, Brighton, UK, BN1 9QH; aldo.badiani@gmail.com

Number of figures: 7
Number of tables: 2
Number of multimedia: 0
Number of words for abstract: 249
Number of words for significance statement: 95
Number of words for introduction: 664
Number of words for discussion: 1351

Acknowledgements: The study was supported by funding (Strategic Development Funds) to AB. We thank Dr. Sarah King and Dr. Hans Crombag for their expert advice. The authors report no conflict of interest.

Abstract

Despite the still prevailing notion of a shared substrate of action for all addictive drugs, there is evidence suggesting that opioid and psychostimulant drugs differ substantially in terms of their neurobiological and behavioural effects. These differences may reflect separate neural circuits engaged by the two drugs. Here we used the catFISH technique to investigate the degree of overlap between neurons engaged by heroin versus cocaine in adult male Sprague-Dawley rats. The catFISH technique is a within-subject procedure that takes advantage of the different transcriptional time-course of the immediate-early genes *homer 1a* and *arc* to determine to what extent two stimuli separated by an interval of 25 min engage the same neuronal population. We found that throughout the striatal complex the neuronal populations activated by non-contingent intravenous injections of cocaine (800 µg/kg) and heroin (100 and 200 µg/kg), administered at an interval of 25 min from each other, overlapped to a much lesser extent than in the case of two injections of cocaine (800 µg/kg), also 25 min apart. The greatest reduction in overlap between populations activated by cocaine and heroin was in the dorsomedial and dorsolateral striatum (~30% and ~22%, respectively, of the overlap observed for the sequence cocaine-cocaine). Our results point toward a significant separation between neuronal populations activated by heroin and cocaine in the striatal complex. We propose that our findings are a proof of concept that these two drugs are encoded differently in a brain area believed to be a common neurobiological substrate to drug abuse.

Significance statement

Despite significant advances in the substance use disorders field, effective prevention and treatment strategies are scarce and still under active development. Here we add to growing evidence indicating major differences in the neurobiological effects of opioid versus psychostimulant drugs, which is at odds with the still prevailing notion of a shared substrate of action for all addictive drugs. This suggests that, to be effective, development of prevention and treatment strategies should not look for a “silver bullet” solution to all drug addictions. Instead, they should be tailored to the specific drug preference of pathological users.

Introduction

Virtually all current theories of drug abuse posit that the addictive properties of drugs depend on common neurobiological processes, including hyper-reactivity of motivational systems (e.g., Wolf, 2010; Berridge and Robinson, 2016), impaired impulse control (e.g., Jentsch and Taylor, 1999), and aberrant learning (e.g., Everitt and Robbins, 2005). Regardless of the core process on which each theory focuses, the biological substrate of said processes involves the mesotelencephalic dopamine system projecting from ventral tegmental area (VTA) and substantia nigra (SN) to the striatal complex, including caudate and nucleus accumbens (NAcc), and to the prefrontal cortex (PFCx). Indeed, it is commonly assumed that all substances of abuse increase dopamine levels in the terminal regions of the dopaminergic system (Di Chiara and Imperato, 1988; Robinson and Berridge, 1993; Wise, 1996; Nestler, 2001, 2004; Hyman et al., 2006; Koob and Volkow, 2010; Berridge, 2012; Covey et al., 2014; Keiflin and Janak, 2015; Volkow and Morales, 2015; Berridge and Robinson, 2016; Keramati et al., 2017; Volkow et al., 2017) albeit via different mechanisms of action. Psychostimulant drugs, such as cocaine and amphetamines, produce dopamine overflow by binding the dopamine transporter (for reviews, see Kuczenski et al., 1982; Johanson and Fischman, 1989). Opioid agonists, such as heroin and morphine, are thought to increase dopamine concentrations indirectly by binding mu-opioid receptors located on inhibitory interneurons in the VTA, hence disinhibiting dopaminergic neurons (Gysling and Wang, 1983; Matthews and German, 1984; Johnson and North, 1992). Yet, the magnitude of drug-induced dopamine overflow differs enormously from one drug to another, even within the same pharmacological class. For example, some opioids produce dramatic increases in dopamine whereas others have very little effect (e.g., Gottas et al., 2014; Vander Weele et al., 2014). Furthermore, electrophysiological experiments have shown that neurons in the striatum respond in a very different manner to heroin versus cocaine self-administration (e.g., Chang et al., 1998; Wei et al., 2018), suggesting that the effects of the two drugs are encoded differently in this brain area.

The aim of the two experiments reported here was to further explore this hypothesis using the catFISH technique, which is a within-subject technique that takes advantage of the different transcriptional time-course of the immediate-early genes (IEGs) *homer 1a* and *arc* to

detect the activation of partly distinct neuronal populations in response to two temporally distinct stimuli (see Fig.1, Guzowski et al., 1999; Vazdarjanova et al., 2002; Vazdarjanova and Guzowski, 2004). To date, a few studies have looked at the effects of cocaine on *arc* (Caffino et al., 2011) or *homer 1a* expression (Unal et al., 2009), whereas there is no information on the effects of heroin administration on the expression of these two IEGs. As in the case of the IEG *c-fos*, which is known to be transcribed across the striatum in response to heroin and cocaine administration (Harlan and Garcia, 1998; Paolone et al., 2007; Celentano et al., 2009), both *arc* and *homer 1a* are activated by the transcription factor CREB; that is, they are transcribed following activation of the ERK/MAPK pathway, elevated cAMP activity, or calcium influx to the cell (Impey et al., 1998; Sato et al., 2001; Kawashima et al., 2014). Considering these shared mechanisms of expression, we expected that *arc* and *homer 1a* would be suitable markers of neuronal activity produced by drug administration. We predicted that intravenous (i.v.) injections of heroin and cocaine will produce a rapid and transient IEG transcription in the striatum. Indeed, we found that intravenous administration of low doses (i.e., those typically used in self-administration experiments) of heroin and cocaine produce temporally distinct increases in the expression of *homer 1a* (*h1a*) and/or *arc* suggesting that both drugs induce neuronal activity across the striatum. In a second experiment, we used the catFISH technique to establish to what extent this activity occurs in overlapping vs. drug-specific neuronal populations. Based on electrophysiological evidence suggesting distinct neuronal activity produced by heroin vs cocaine (Chang et al., 1998), we predicted that administration of heroin following cocaine would activate non-overlapping neuronal populations across the striatum.

Methods

Subjects

A total of 66 male Sprague-Dawley rats (n = 37 in Exp. 1 and n = 29 in Exp. 2) from ENVIGO (Netherlands) 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/dark cycle (lights on at 19:00 hours). The rats were housed in groups of 3 or 4 until surgery and individually thereafter. Food and water were provided *ad libitum* except during testing sessions.

All regulated procedures were carried out in accordance with the UK 1986 Animal Scientific Procedures Act (ASPA) and received approval from the relevant Animal Welfare and Ethics Review Board. After their arrival in the animal facilities, the rats were given a period of at least 7 days before undergoing experimental procedures.

Drugs

Anesthesia was induced with 110 mg/kg of ketamine (Anesketin, Dechra) and 2 mg/kg of xylazine (Rompun, Bayer HealthCare). Cocaine and heroin hydrochloride (Johnson Matthey-MacFarlan Smith, Edinburgh, UK) were dissolved in sterile saline and infused i.v. at the doses specified in the next paragraphs. Each infusion consisted of a volume of 40 µl of the appropriate drug solution delivered over 4 s. Saline-treated rats received equivalent volumes of saline.

Intravenous catheter surgery

The surgical procedures were similar to those recently described by Avvisati et al. (2019). Briefly, after anesthesia, an 11 cm silicone catheter (0.37-mm inner diameter and 0.94-mm outer diameter), sheathed at 3.4 cm from its proximal end by a silicone bead, was implanted in the right jugular vein, externalised at the nape of the neck, and attached to a cannula secured to the top of the skull with dental cement. Following surgery, rats were allowed to recover for at least 7 days. Catheter patency was maintained by flushing the catheters daily with 0.1 ml saline.

Catheter patency test

At the appropriate time (see next sections) the rats were killed via an i.v. infusion of pentobarbital (120 mg/kg in 200 µl of saline) through the catheter. This also served as a catheter patency test: the rats that did not become ataxic and die within 5 s would be excluded from the data analysis. All catheters were found to be patent.

Drug administration procedures

Experiment 1. After recovery, the rats received, while briefly restrained, an i.v. infusion of either 400 µg/kg cocaine (n = 18) or 50 µg/kg heroin (n = 19) in their home cage. These doses were selected based on the findings of previous self-administration experiments (Caprioli et al.,

2007b; Caprioli et al., 2008). The rats received the lethal pentobarbital injection and were then decapitated at different time points after the cocaine or heroin infusion: 0 min (*n* = 3 for both the cocaine and heroin groups), 8 min (*n* = 3 for both the cocaine and heroin groups), 16 min (*n* = 4 for both the cocaine and heroin groups), 25 min (*n* = 4 for both the cocaine and heroin groups), and 35 min (*n* = 4 and *n* = 5 for the cocaine and heroin groups, respectively).

Experiment 2. After recovery, the rats were moved to testing chambers used for self-administration experiments (PRS Italia; see Avvisati et al., 2019). In order to reduce the potentially confounding effects of environmental novelty on drug-induced IEG expression (Uslaner et al., 2001; Paolone et al., 2007) we let the rats habituate to these chambers for 18 h before tethering them to the infusion lines. Food and water were available *ad libitum* during this habituation period and were removed immediately prior to tethering. The use of self-administration chambers allowed us to deliver drug infusions remotely via a computer-controlled infusion pump. The infusion pumps were programmed to start automatically, in the absence of the experimenter, 1 h after tethering. This way we avoided the confounding effects usually associated to signalled drug administration (Crombag et al., 1996) and/or handling. All rats received two i.v. infusions, 25 min apart, of: saline–saline (*n* = 4), cocaine 800 µg/kg–saline (*n* = 6), cocaine 800 µg/kg–cocaine 800 µg/kg (*n* = 6), cocaine 800 µg/kg–heroin 100 µg/kg (*n* = 6), or cocaine 800 µg/kg–heroin 200 µg/kg (*n* = 7). To administer two separate injections through the same catheter, the infusion lines were back-filled with the appropriate drug solutions, separated by a tiny air bubble, just before tethering of the rats. The rationale for using higher doses of cocaine and heroin in Exp. 2 was to boost the magnitude of IEG expression. These doses were still within the range of those used in self-administration experiments (e.g. Zito et al., 1985; Dai et al., 1989; Roberts et al., 1989; Pettit and Justice, 1991; Shaham and Stewart, 1994; Wise et al., 1995; Mantsch et al., 2001; Wee et al., 2007; Mandt et al., 2012).

Five minutes after the second infusion, the rats were given 120 mg/kg pentobarbital, i.v., and, after decapitation, their brains were snap-frozen in isopentane at -50°C.

Brain slicing

The brains were excised and snap-frozen in 400 ml of isopentane cooled to -50°C and later sectioned on a cryostat at 16- or 20-µm thickness. In Exp. 1, sectioning started from the tip of the olfactory bulbs and brain sections were removed until the Sylvian fissure no longer reached the midline (+3.70 mm from bregma). At this point, either 100 or 80 sections were removed (when sectioning at 16 µm and 20 µm, respectively) to reach +2.00 mm from bregma at which point the sections contained anterior dorsal striatum and NAcc core (Fig. 2A). Two coronal sections per rat (16 or 20 µm-thick) were obtained at this point. An identical procedure was used in Exp. 2 in order to collect two coronal sections containing NAcc core and shell, DMS, and DLS at +1.70 mm from bregma (Fig. 3A).

In situ hybridization

Immediately after cutting, the brain tissue sections were mounted on Superfrost Plus slides. On the first day of staining, the slides 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 (5-min incubation in 50%, 70%, and 2x 100%). Following this, the 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® kit (Advanced Cell Diagnostics, ACDbio). *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). 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 *h1a* gene mRNA, spanning 5001-5625 base pairs, accession No. U92079.1.

The *arc* and *h1a* probes were 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

Fluorescent signal was detected using a Zeiss Axioskop 2 plus epifluorescent microscope, and images were acquired using an Axiovision software (Zeiss).

Greyscale images were taken from both hemispheres of 2 adjacent sections for each rat at 20x magnification. This yielded four images per brain area for each rat. Final counts of DAPI-, *arc*-, and *h1a*-positive nuclei were averaged from these four images. The resulting images 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 and Travo, 2006). Greyscale images were analysed separately for each channel – DAPI, Alexa 488 (*h1a*) and Cy3 (*arc*) – as follows. 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 binary mask image containing only objects fulfilling the aforementioned criteria.

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 binary image mask showing only the defined particles.

A Windows 10 Dell OptiPlex 7060 desktop computer ran a MATLAB script to overlay the three binary mask images and count instances where objects defined as DAPI nuclei coincided

with objects defined as either *arc* mRNA, *h1a* mRNA or both. The MATLAB code will be made available on request. Thus, IEG expression was measured by counting DAPI-positive (DAPI+) cell nuclei also positive for *h1a*, *arc*, or both.

Statistical analyses

The data from Exp. 1 were analysed by two-way mixed ANOVAs with time and IEG as fixed factors. Amount of IEG-positive cell nuclei (as percentage of all DAPI-stained nuclei) was the dependent variable. The data from Exp. 2 were analysed using a two-way ANOVA with brain area and treatment group as fixed factors. The outcome variable was overlap (expressed as percent of the cocaine-cocaine group). All analyses were carried out in SPSS 25 (IBM) software. An alpha value of 0.05 or less was used for determining statistically significant effects.

Results

Experiment 1 (time course of *arc* and *h1a* expression following i.v. drug administration)

Figure 2B shows the amount of *arc*- and *h1a*-positive nuclei in the NAcc core and DMS expressed as a percentage of all DAPI-positive nuclei and as a function of time elapsed since i.v. injections of cocaine and heroin. Table 1 shows the same data before conversion to percentage.

***Arc* and *h1a* expression in the NAcc core.** Cocaine administration increased both *arc* and *h1a* mRNA levels in the NAcc core, but at different time points. A two-way mixed ANOVA showed non-significant main effects of IEG $F(1,13) = 0.08$, $p = .782$, $\eta^2 = .006$, and time $F(4,13) = 1.62$, $p = .227$, $\eta^2 = .333$ but a significant time x IEG interaction, $F(4,13) = 7.93$, $p = .002$, $\eta^2 = .977$.

Heroin produced a similar pattern of mRNA expression, but the effect did not reach significance: a two-way mixed ANOVA showed non-significant main effects of IEG $F(1,14) = 2.32$, $p = .150$, $\eta^2 = .142$, and time $F(4,14) = 0.72$, $p = .596$, $\eta^2 = .17$, and a non-significant time x IEG interaction, $F(4,14) = 2.15$, $p = .129$, $\eta^2 = .38$.

***Arc* and *h1a* expression in the DMS.** As in the NAcc core, cocaine treatment increased IEG levels in a time-dependent manner. A two-way mixed ANOVA showed significant main

effects of IEG $F(1,13) = 18.93$, $p = .001$, $\eta^2 = .593$, and time $F(4,13) = 5.36$, $p = .009$, $\eta^2 = .623$, and a significant time x IEG interaction, $F(4,13) = 44.58$, $p < .001$, $\eta^2 = .932$.

Heroin produced a similar effect. A two-way mixed ANOVA showed non-significant main effects of IEG $F(1,14) = 3.17$, $p = .097$, $\eta^2 = .185$, and time $F(4,14) = 0.22$, $p = .924$, $\eta^2 = .059$, but a significant time x IEG interaction, $F(4,14) = 3.58$, $p = .033$, $\eta^2 = .506$.

Experiment 2 (overlap in neuronal populations activated by cocaine and heroin)

Table 2 shows the average number of *arc*-only, *h1a*-only and double-stained cell nuclei as a function of brain area and drugs administered in experiment 2. Figures 4-7 show representative images from all brain areas analysed using catFISH.

Figure 3B shows the extent of overlap between neuronal populations activated by cocaine and heroin as a percent change from the cocaine-cocaine group. Overlap was quantified as the number of nuclei co-expressing *arc* and *h1a* expressed as a percent of all mRNA-positive nuclei (single and double-labelled). In all four brain areas examined, there was a substantial reduction in overlap when cocaine and heroin were administered in succession, relative to the overlap seen when cocaine was administered twice, and regardless of heroin dose (figure 3). A two-way mixed ANOVA showed a significant main effect of treatment group $F(3,19) = 20.97$, $p < .001$, $\eta^2 = .768$ and brain area $F(3,57) = 3.40$, $p = .024$, $\eta^2 = .152$ but not treatment x brain area interaction $F(9,57) = 0.79$, $p = .619$, $\eta^2 = .112$.

Discussion

We have shown that intravenous injections of heroin and cocaine at doses typically self-administered by rats produce a quick and transient increase of *homer 1a* and *arc* expression across the striatum. More importantly, using the catFISH technique, we took advantage of the difference in timing of expression between the two IEGs to show that heroin and cocaine activate partly distinct neuronal populations in this brain area.

In line with our findings, previous studies have shown that heroin and cocaine increase *c-fos* levels in the ventral and dorsomedial striatum (Hope et al., 1992; Harlan and Garcia, 1998;

Uslaner et al., 2001; Ferguson et al., 2004; Paolone et al., 2007; Celentano et al., 2009). The IEG *c-fos* is a marker of neuronal activity expressed under similar conditions of *arc* and *homer 1a* (Guzowski et al., 2001). In addition, our findings indicate that this activity occurs in separate neuronal populations and may explain why only a small proportion of neurons show similar electrophysiological responses to heroin and cocaine (Chang et al., 1998).

It is likely that drug-induced IEG expression represents glutamatergic activity modulated by DA, because NMDA and DA D1 receptors play a key role in IEG expression through activation of CREB (Impey et al., 1998; Mattson et al., 2005; Surmeier et al., 2007; Guez-Barber et al., 2011; Tritsch and Sabatini, 2012), and both DA and glutamate levels are increased in the striatum following heroin and cocaine administration. Note, however, that DA release alone does not produce IEG expression in the absence of glutamatergic activity (Kreuter et al., 2004). In addition, NMDA receptor activity and DA transmission in the accumbens are necessary for food and cocaine self-administration, but not heroin self-administration (Ettenberg et al., 1982; Pettit et al., 1984; Pulvirenti et al., 1992; Kelley et al., 1997). Finally, D1 receptor-expressing MSNs in the dorsal striatum appear to be sufficient to sustain operant behaviour (Kravitz et al., 2012) and these neurons express IEGs (i.e. are activated) following cocaine administration. Thus, loss and gain of function studies have provided evidence that activity of cells in the striatum plays a key role for cocaine, but not heroin, reinforcement through DA and glutamate transmission. It remains to be determined what is the functional role of the distinct neuronal populations engaged by heroin relative to cocaine.

A case for drug-specific neural circuitries

Perhaps the most intriguing interpretation of the results presented here is that partly distinct neuronal populations activated by heroin and cocaine across the striatum are suggestive of dissociated circuitry processing the acute effects of the two drugs. There is already existing evidence that the striatum is functionally and structurally organised to accommodate circuits which operate in parallel but carry out separate functions. First, striatal medium spiny neurons (MSNs) are characterised by more or less excitable states, i.e. “up” and “down” states (Wolf et al., 2001; O'Donnell, 2003) and 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, PFCx and VTA/SNc afferent inputs (Pennartz et al., 1994). Each of the brain areas sending these afferent projections: i) is affected differently by heroin, cocaine, and natural rewards (Chang et al., 1998; Mukherjee et al., 2018); ii) contains neuronal ensembles involved in distinct functions (Zelikowsky et al., 2014; Warren et al., 2016), and iii) might be comprised of genetically distinct projection neurons. Thus, considering the integrative function of the striatum, 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 the activation of partly distinct neuronal populations in the striatum reflects the activation of 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, 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). It should also be considered 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 ensemble is active, it can decrease the activity in other ensembles so that only one computation is taking place over others (Pennartz et al., 1994). The experiments presented here are only suggestive of distinct striatal circuitry engaged by heroin and cocaine. Future studies should address this hypothesis by expanding on our findings in three ways. First, single-cell quantitative PCR studies can further elucidate phenotypic differences between neuronal populations activated by heroin and cocaine in terms of their genetic make-up (Hrvatin et al., 2018). Second, retro- and anterograde labelling studies in conjunction with immunohistochemistry can reveal whether these neuronal populations connect to distinct up- and downstream targets. And third, selective loss- and gain-of-function studies can be used to test whether inactivation of neurons responding to cocaine in the dorsal striatum and accumbens core would impair heroin reinforcement. The Daun02 technique (Koya et al., 2009; Koya et al., 2016) would be a useful technique in this regard, as well as other techniques which manipulate neuronal ensembles such as the TetTag approach using the Fos-

tTA mouse line combined with optogenetics (Reijmers and Mayford, 2009; Liu et al., 2012; Du and Koffman, 2017).

Methodological considerations

Two caveats to the experimental design used here are worthy of mention. There are known differences between the effects of non-contingent vs contingent exposure to heroin and cocaine (e.g. Galici et al., 2000; Lecca et al., 2007; Radley et al., 2015). In the present study we administered heroin and cocaine in a non-contingent but unsignalled manner as we were interested in comparing the acute pharmacological effects of these two drugs using IEG expression as a marker of neuronal activation. Contingent administrations (e.g., self-administration) require repeated exposure to drugs over several test sessions which has been shown to produce habituation to IEG expression (Hope et al., 1992; Unal et al., 2009). Of course, we recognise the value of studying the encoding of drug-related information in the striatum during and after periods of drug self-administration. Future studies could employ *in vivo* imaging techniques such as the UCLA/Inscopix© miniscope to address this question directly. A second, somewhat related caveat is that our paradigm includes a multi-substance component. It is possible that circuit activity may differ following polysubstance versus single-drug use histories. However, electrophysiological evidence from rats self-administering both substances is congruent with our findings (Chang et al., 1998). Also, we administered only two injections of cocaine and/or heroin to drug-naïve rats so it is unlikely that any long-term polysubstance use effects would have influenced our observations.

Conclusion

In summary, we found a significant dissociation in the neuronal populations responding to self-administration doses of heroin versus cocaine, as indicated by *arc* and *homer 1a* expression. Our findings provide a proof of concept that heroin and cocaine effects on the brain must be studied as separate phenomena, adding to the evidence of major differences between the various drugs of abuse (for a review, see Badiani et al., 2011). Although the functional significance of these differences remains to be fully explored, they might have implications for both research and treatment. It is remarkable, for example, that the functional or anatomical integrity of the dopaminergic system is required for the reinforcing properties of cocaine but

not of heroin (e.g., Ettenberg et al., 1982; Pettit et al., 1984; Pisanu et al., 2015), that distinct projections from the PFCx to the shell of the NAcc are implicated in the relapse to cocaine versus heroin seeking after abstinence (Peters et al., 2008; Bossert et al., 2012), and that basic environmental manipulations gate in opposite directions the reinforcing, affective, and neurobiological response to heroin versus cocaine in rats and humans (Uslaner et al., 2001; Ferguson et al., 2004; Caprioli et al., 2007a; Paolone et al., 2007; Caprioli et al., 2008; Caprioli et al., 2009; Celentano et al., 2009; Montanari et al., 2015; Avvisati et al., 2016; De Pirro et al., 2018; De Luca et al., 2019).

References

Avvisati R, Contu L, Stendardo E, Michetti C, Montanari C, Scattoni ML, Badiani A (2016) Ultrasonic vocalization in rats self-administering heroin and cocaine in different settings: evidence of substance-specific interactions between drug and setting. Psychopharmacology (Berl) 233:1501-1511.

Avvisati R, Bogen IL, Andersen JM, Vindenes V, Morland J, Badiani A, Boix F (2019) The active heroin metabolite 6-acetylmorphine has robust reinforcing effects as assessed by self-administration in the rat. Neuropharmacology 150:192-199.

Badiani A, Belin D, Epstein D, Calu D, Shaham Y (2011) Opiate versus psychostimulant addiction: the differences do matter. Nat Rev Neurosci 12:685-700.

Baecker V, Travo P (2006) Cell Image Analyzer - A visual scripting interface for ImageJ and its usage at the microscopy facility Montpellier RIO Imaging. In: ImageJ User and Developer Conference, 1 Edition, pp 105-110. Luxemburg.

Berridge KC (2012) From prediction error to incentive salience: mesolimbic computation of reward motivation. Eur J Neurosci 35:1124-1143.

Berridge KC, Robinson TE (2016) Liking, wanting, and the incentive-sensitization theory of addiction. The American psychologist 71:670-679.

Bossert JM, Stern AL, Theberge FR, Marchant NJ, Wang HL, Morales M, Shaham Y (2012) Role of projections from ventral medial prefrontal cortex to nucleus accumbens shell in context-induced reinstatement of heroin seeking. J Neurosci 32:4982-4991.

Caffino L, Racagni G, Fumagalli F (2011) Stress and cocaine interact to modulate Arc/Arg3.1 expression in rat brain. Psychopharmacology (Berl) 218:241-248.

Caprioli D, Celentano M, Paolone G, Badiani A (2007a) Modeling the role of environment in addiction. Prog Neuropsychopharmacol Biol Psychiatry 31:1639-1653.

Caprioli D, Paolone G, Celentano M, Testa A, Nencini P, Badiani A (2007b) Environmental modulation of cocaine self-administration in the rat. Psychopharmacology 192:397-406.

Caprioli D, Celentano M, Dubla A, Lucantonio F, Nencini P, Badiani A (2009) Ambience and drug choice: cocaine- and heroin-taking as a function of environmental context in humans and rats. Biol Psychiatry 65:893-899.

Caprioli D, Celentano M, Paolone G, Lucantonio F, Bari A, Nencini P, Badiani A (2008) Opposite environmental regulation of heroin and amphetamine self-administration in the rat. Psychopharmacology 198:395-404.

Celentano M, Caprioli D, Dipasquale P, Cardillo V, Nencini P, Gaetani S, Badiani A (2009) Drug context differently regulates cocaine versus heroin self-administration and cocaine- versus heroin-induced Fos mRNA expression in the rat. Psychopharmacology (Berl) 204:349-360.

Chang JY, Janak PH, Woodward DJ (1998) Comparison of mesocorticolimbic neuronal responses during cocaine and heroin self-administration in freely moving rats. J Neurosci 18:3098-3115.

Covey DP, Roitman MF, Garris PA (2014) Illicit dopamine transients: reconciling actions of abused drugs. Trends Neurosci 37:200-210.

418 Crombag HS, Badiani A, Robinson TE (1996) Signalled versus unsignalled intravenous
419 amphetamine: large differences in the acute psychomotor response and sensitization.
420 Brain research 722:227-231.
421 Dai S, Corrigall WA, Coen KM, Kalant H (1989) Heroin self-administration by rats: influence of
422 dose and physical dependence. Pharmacol Biochem Behav 32:1009-1015.
423 De Luca MT, Montanari C, Meringolo M, Contu L, Celentano M, Badiani A (2019) Heroin versus
424 cocaine: opposite choice as a function of context but not of drug history in the rat.
425 Psychopharmacology (Berl) 236:787-798.
426 De Pirro S, Galati G, Pizzamiglio L, Badiani A (2018) The Affective and Neural Correlates of
427 Heroin versus Cocaine Use in Addiction Are Influenced by Environmental Setting But in
428 Opposite Directions. J Neurosci 38:5182-5195.
429 Di Chiara G, Imperato A (1988) Drugs abused by humans preferentially increase synaptic
430 dopamine concentrations in the mesolimbic system of freely moving rats. Proc Natl
431 Acad Sci U S A 85:5274-5278.
432 Du JY, Koffman EE (2017) Labeling Aversive Memory Trace in Mouse Using a Doxycycline-
433 inducible Expression System. Bio-Protocol 7.
434 Ettenberg A, Pettit HO, Bloom FE, Koob GF (1982) Heroin and cocaine intravenous self-
435 administration in rats: mediation by separate neural systems. Psychopharmacology
436 (Berl) 78:204-209.
437 Everitt BJ, Robbins TW (2005) Neural systems of reinforcement for drug addiction: from actions
438 to habits to compulsion. Nat Neurosci 8:1481-1489.
439 Ferguson SM, Thomas MJ, Robinson TE (2004) Morphine-induced c-fos mRNA expression in
440 striatofugal circuits: modulation by dose, environmental context, and drug history.
441 Neuropsychopharmacology 29:1664-1674.
442 Galici R, Pechnick RN, Poland RE, France CP (2000) Comparison of noncontingent versus
443 contingent cocaine administration on plasma corticosterone levels in rats. Eur J
444 Pharmacol 387:59-62.
445 Gottas A, Boix F, Oiestad EL, Vindenes V, Morland J (2014) Role of 6-monoacetylmorphine in the
446 acute release of striatal dopamine induced by intravenous heroin. Int J
447 Neuropsychopharmacol 17:1357-1365.
448 Guez-Barber D, Fanous S, Golden SA, Schrama R, Koya E, Stern AL, Bossert JM, Harvey BK,
449 Picciotto MR, Hope BT (2011) FACS identifies unique cocaine-induced gene regulation in
450 selectively activated adult striatal neurons. J Neurosci 31:4251-4259.
451 Guzowski JF, McNaughton BL, Barnes CA, Worley PF (1999) Environment-specific expression of
452 the immediate-early gene Arc in hippocampal neuronal ensembles. Nat Neurosci
453 2:1120-1124.
454 Guzowski JF, Setlow B, Wagner EK, McGaugh JL (2001) Experience-dependent gene expression
455 in the rat hippocampus after spatial learning: a comparison of the immediate-early
456 genes Arc, c-fos, and zif268. J Neurosci 21:5089-5098.
457 Gysling K, Wang RY (1983) Morphine-induced activation of A10 dopamine neurons in the rat.
458 Brain research 277:119-127.
459 Harlan RE, Garcia MM (1998) Drugs of abuse and immediate-early genes in the forebrain. Mol
460 Neurobiol 16:221-267.

461 Hope B, Kosofsky B, Hyman SE, Nestler EJ (1992) Regulation of immediate early gene expression
462 and AP-1 binding in the rat nucleus accumbens by chronic cocaine. Proc Natl Acad Sci U
463 S A 89:5764-5768.
464 Hrvatin S, Hochbaum DR, Nagy MA, Cicconet M, Robertson K, Cheadle L, Zilionis R, Ratner A,
465 Borges-Monroy R, Klein AM, Sabatini BL, Greenberg ME (2018) Single-cell analysis of
466 experience-dependent transcriptomic states in the mouse visual cortex. Nat Neurosci
467 21:120-129.
468 Hyman SE, Malenka RC, Nestler EJ (2006) Neural mechanisms of addiction: the role of reward-
469 related learning and memory. Annu Rev Neurosci 29:565-598.
470 Impey S, Obrietan K, Wong ST, Poser S, Yano S, Wayman G, Deloulme JC, Chan G, Storm DR
471 (1998) Cross talk between ERK and PKA is required for Ca2+ stimulation of CREB-
472 dependent transcription and ERK nuclear translocation. Neuron 21:869-883.
473 Jentsch JD, Taylor JR (1999) Impulsivity resulting from frontostriatal dysfunction in drug abuse:
474 implications for the control of behavior by reward-related stimuli. Psychopharmacology
475 (Berl) 146:373-390.
476 Johanson CE, Fischman MW (1989) The pharmacology of cocaine related to its abuse.
477 Pharmacol Rev 41:3-52.
478 Johnson SW, North RA (1992) Opioids excite dopamine neurons by hyperpolarization of local
479 interneurons. J Neurosci 12:483-488.
480 Kawashima T, Okuno H, Bito H (2014) A new era for functional labeling of neurons: activity-
481 dependent promoters have come of age. Front Neural Circuit 8.
482 Keiflin R, Janak PH (2015) Dopamine Prediction Errors in Reward Learning and Addiction: From
483 Theory to Neural Circuitry. Neuron 88:247-263.
484 Kelley AE, Smith-Roe SL, Holahan MR (1997) Response-reinforcement learning is dependent on
485 N-methyl-D-aspartate receptor activation in the nucleus accumbens core. Proc Natl
486 Acad Sci U S A 94:12174-12179.
487 Keramati M, Ahmed SH, Gutkin BS (2017) Misdeed of the need: towards computational
488 accounts of transition to addiction. Curr Opin Neurobiol 46:142-153.
489 Koob GF, Volkow ND (2010) Neurocircuitry of addiction. Neuropsychopharmacology 35:217-
490 238.
491 Koya E, Margetts-Smith G, Hope BT (2016) Daun02 Inactivation of Behaviorally Activated Fos-
492 Expressing Neuronal Ensembles. Curr Protoc Neurosci 76:8 36 31-38 36 17.
493 Koya E, Golden SA, Harvey BK, Guez-Barber DH, Berkow A, Simmons DE, Bossert JM, Nair SG,
494 Uejima JL, Marin MT, Mitchell TB, Farquhar D, Ghosh SC, Mattson BJ, Hope BT (2009)
495 Targeted disruption of cocaine-activated nucleus accumbens neurons prevents context-
496 specific sensitization. Nat Neurosci 12:1069-1073.
497 Kravitz AV, Tye LD, Kreitzer AC (2012) Distinct roles for direct and indirect pathway striatal
498 neurons in reinforcement. Nat Neurosci 15:816-818.
499 Kreuter JD, Mattson BJ, Wang B, You ZB, Hope BT (2004) Cocaine-induced Fos expression in rat
500 striatum is blocked by chloral hydrate or urethane. Neuroscience 127:233-242.
501 Kuczenski R, Segal DS, Weinberger SB, Browne RG (1982) Evidence that a behavioral
502 augmentation following repeated amphetamine administration does not involve
503 peripheral mechanisms. Pharmacol Biochem Behav 17:547-553.

504 Lecca D, Valentini V, Cacciapaglia F, Acquas E, Di Chiara G (2007) Reciprocal effects of response
505 contingent and noncontingent intravenous heroin on in vivo nucleus accumbens shell
506 versus core dopamine in the rat: a repeated sampling microdialysis study.
507 Psychopharmacology (Berl) 194:103-116.
508 Liu X, Ramirez S, Pang PT, Puryear CB, Govindarajan A, Deisseroth K, Tonegawa S (2012)
509 Optogenetic stimulation of a hippocampal engram activates fear memory recall. Nature
510 484:381-U415.
511 Mandt BH, Johnston NL, Zahniser NR, Allen RM (2012) Acquisition of cocaine self-administration
512 in male Sprague-Dawley rats: effects of cocaine dose but not initial locomotor response
513 to cocaine. Psychopharmacology (Berl) 219:1089-1097.
514 Mantsch JR, Ho A, Schlussman SD, Kreek MJ (2001) Predictable individual differences in the
515 initiation of cocaine self-administration by rats under extended-access conditions are
516 dose-dependent. Psychopharmacology (Berl) 157:31-39.
517 Matthews RT, German DC (1984) Electrophysiological evidence for excitation of rat ventral
518 tegmental area dopamine neurons by morphine. Neuroscience 11:617-625.
519 Mattson BJ, Bossert JM, Simmons DE, Nozaki N, Nagarkar D, Kreuter JD, Hope BT (2005)
520 Cocaine-induced CREB phosphorylation in nucleus accumbens of cocaine-sensitized rats
521 is enabled by enhanced activation of extracellular signal-related kinase, but not protein
522 kinase A. J Neurochem 95:1481-1494.
523 Montanari C, Stendardo E, De Luca MT, Meringolo M, Contu L, Badiani A (2015) Differential
524 vulnerability to relapse into heroin versus cocaine-seeking as a function of setting.
525 Psychopharmacology (Berl) 232:2415-2424.
526 Mukherjee D, Ignatowska-Jankowska BM, Itskovits E, Gonzales BJ, Turm H, Izakson L, Haritan D,
527 Bleistein N, Cohen C, Amit I, Shay T, Grueter B, Zaslaver A, Citri A (2018) Salient
528 experiences are represented by unique transcriptional signatures in the mouse brain.
529 Elife 7.
530 Nestler EJ (2001) Molecular basis of long-term plasticity underlying addiction. Nat Rev Neurosci
531 2:119-128.
532 Nestler EJ (2004) Historical review: Molecular and cellular mechanisms of opiate and cocaine
533 addiction. Trends Pharmacol Sci 25:210-218.
534 O'Donnell P (2003) Dopamine gating of forebrain neural ensembles. Eur J Neurosci 17:429-435.
535 Paolone G, Conversi D, Caprioli D, Bianco PD, Nencini P, Cabib S, Badiani A (2007) Modulatory
536 effect of environmental context and drug history on heroin-induced psychomotor
537 activity and fos protein expression in the rat brain. Neuropsychopharmacology 32:2611-
538 2623.
539 Paxinos G, Watson C (1986) The rat brain in stereotaxic coordinates, 2 Edition. London:
540 Academic Press Limited.
541 Pennartz CM, Groenewegen HJ, Lopes da Silva FH (1994) The nucleus accumbens as a complex
542 of functionally distinct neuronal ensembles: an integration of behavioural,
543 electrophysiological and anatomical data. Prog Neurobiol 42:719-761.
544 Peters J, LaLumiere RT, Kalivas PW (2008) Infralimbic prefrontal cortex is responsible for
545 inhibiting cocaine seeking in extinguished rats. Journal of Neuroscience 28:6046-6053.
546 Pettit HO, Justice JB, Jr. (1991) Effect of dose on cocaine self-administration behavior and
547 dopamine levels in the nucleus accumbens. Brain research 539:94-102.

548 Pettit HO, Ettenberg A, Bloom FE, Koob GF (1984) Destruction of dopamine in the nucleus
549 accumbens selectively attenuates cocaine but not heroin self-administration in rats.
550 Psychopharmacology (Berl) 84:167-173.
551 Pisanu A, Lecca D, Valentini V, Bahi A, Dreyer JL, Cacciapaglia F, Scifo A, Piras G, Cadoni C, Di
552 Chiara G (2015) Impairment of acquisition of intravenous cocaine self-administration by
553 RNA-interference of dopamine D1-receptors in the nucleus accumbens shell.
554 Neuropharmacology 89:398-411.
555 Pulvirenti L, Maldonado-Lopez R, Koob GF (1992) NMDA receptors in the nucleus accumbens
556 modulate intravenous cocaine but not heroin self-administration in the rat. Brain
557 research 594:327-330.
558 Radley JJ, Anderson RM, Cosme CV, Glanz RM, Miller MC, Romig-Martin SA, LaLumiere RT
559 (2015) The Contingency of Cocaine Administration Accounts for Structural and
560 Functional Medial Prefrontal Deficits and Increased Adrenocortical Activation. J Neurosci
561 35:11897-11910.
562 Reijmers L, Mayford M (2009) Genetic control of active neural circuits. Front Mol Neurosci 2:27.
563 Roberts DC, Loh EA, Vickers G (1989) Self-administration of cocaine on a progressive ratio
564 schedule in rats: dose-response relationship and effect of haloperidol pretreatment.
565 Psychopharmacology (Berl) 97:535-538.
566 Robinson TE, Berridge KC (1993) The neural basis of drug craving: an incentive-sensitization
567 theory of addiction. Brain Res Brain Res Rev 18:247-291.
568 Sato M, Suzuki K, Nakanishi S (2001) NMDA receptor stimulation and brain-derived
569 neurotrophic factor upregulate homer 1a mRNA via the mitogen-activated protein
570 kinase cascade in cultured cerebellar granule cells. J Neurosci 21:3797-3805.
571 Shaham Y, Stewart J (1994) Exposure to mild stress enhances the reinforcing efficacy of
572 intravenous heroin self-administration in rats. Psychopharmacology (Berl) 114:523-527.
573 Surmeier DJ, Ding J, Day M, Wang Z, Shen W (2007) D1 and D2 dopamine-receptor modulation
574 of striatal glutamatergic signaling in striatal medium spiny neurons. Trends Neurosci
575 30:228-235.
576 Tritsch NX, Sabatini BL (2012) Dopaminergic modulation of synaptic transmission in cortex and
577 striatum. Neuron 76:33-50.
578 Unal CT, Beverley JA, Willuhn I, Steiner H (2009) Long-lasting dysregulation of gene expression
579 in corticostriatal circuits after repeated cocaine treatment in adult rats: effects on zif
580 268 and homer 1a. Eur J Neurosci 29:1615-1626.
581 Uslaner J, Badiani A, Day HE, Watson SJ, Akil H, Robinson TE (2001) Environmental context
582 modulates the ability of cocaine and amphetamine to induce c-fos mRNA expression in
583 the neocortex, caudate nucleus, and nucleus accumbens. Brain research 920:106-116.
584 Vander Weele CM, Porter-Stransky KA, Mabrouk OS, Lovic V, Singer BF, Kennedy RT, Aragona BJ
585 (2014) Rapid dopamine transmission within the nucleus accumbens: dramatic difference
586 between morphine and oxycodone delivery. Eur J Neurosci 40:3041-3054.
587 Vazdarjanova A, Guzowski JF (2004) Differences in hippocampal neuronal population responses
588 to modifications of an environmental context: evidence for distinct, yet complementary,
589 functions of CA3 and CA1 ensembles. J Neurosci 24:6489-6496.

590 Vazdarjanova A, McNaughton BL, Barnes CA, Worley PF, Guzowski JF (2002) Experience-
591 dependent coincident expression of the effector immediate-early genes arc and Homer
592 1a in hippocampal and neocortical neuronal networks. J Neurosci 22:10067-10071.
593 Volkow ND, Morales M (2015) The Brain on Drugs: From Reward to Addiction. Cell 162:712-725.
594 Volkow ND, Wise RA, Baler R (2017) The dopamine motive system: implications for drug and
595 food addiction. Nat Rev Neurosci 18:741-752.
596 Voorn P, Vanderschuren LJ, Groenewegen HJ, Robbins TW, Pennartz CM (2004) Putting a spin
597 on the dorsal-ventral divide of the striatum. Trends Neurosci 27:468-474.
598 Warren BL, Mendoza MP, Cruz FC, Leao RM, Caprioli D, Rubio FJ, Whitaker LR, McPherson KB,
599 Bossert JM, Shaham Y, Hope BT (2016) Distinct Fos-Expressing Neuronal Ensembles in
600 the Ventromedial Prefrontal Cortex Mediate Food Reward and Extinction Memories. J
601 Neurosci 36:6691-6703.
602 Wee S, Specio SE, Koob GF (2007) Effects of dose and session duration on cocaine self-
603 administration in rats. J Pharmacol Exp Ther 320:1134-1143.
604 Wei C, Han X, Weng D, Feng Q, Qi X, Li J, Luo M (2018) Response dynamics of midbrain
605 dopamine neurons and serotonin neurons to heroin, nicotine, cocaine, and MDMA. Cell
606 Discov 4:60.
607 Wise RA (1996) Neurobiology of addiction. Curr Opin Neurobiol 6:243-251.
608 Wise RA, Leone P, Rivest R, Leeb K (1995) Elevations of nucleus accumbens dopamine and
609 DOPAC levels during intravenous heroin self-administration. Synapse 21:140-148.
610 Wolf JA, Schroeder LF, Finkel LH (2001) Computational modeling of medium spiny projection
611 neurons in nucleus accumbens: Toward the cellular mechanisms of afferent stream
612 integration. P Ieee 89:1083-1092.
613 Wolf ME (2010) The Bermuda Triangle of cocaine-induced neuroadaptations. Trends Neurosci
614 33:391-398.
615 Zelikowsky M, Hersman S, Chawla MK, Barnes CA, Fanselow MS (2014) Neuronal ensembles in
616 amygdala, hippocampus, and prefrontal cortex track differential components of
617 contextual fear. J Neurosci 34:8462-8466.
618 Zito KA, Vickers G, Roberts DC (1985) Disruption of cocaine and heroin self-administration
619 following kainic acid lesions of the nucleus accumbens. Pharmacol Biochem Behav
620 23:1029-1036.

621

622

Table 1. Mean (SE) number of *arc*- and *h1a*-stained cell nuclei as a function of brain area and drug administered in Exp. 1. The brains were excised at different time points after drug administration: 0 min (n = 3 for both the cocaine and heroin groups), 8 min (n = 3 for both the cocaine and heroin groups), 16 min (n = 4 for both the cocaine and heroin groups), 25 min (n = 4 for both the cocaine and heroin groups), and 35 min (n = 4 and n = 5 for the cocaine and heroin groups, respectively).

Table 2. Mean (SE) number of *h1a*-only, *arc*-only and double-stained cell nuclei as a function of brain area and drugs administered, 25 min apart, in Exp. 2: saline-saline (n = 4), and cocaine (800 µg/kg)-saline (n = 6), cocaine (800 µg/kg)-cocaine (800 µg/kg) (n = 6), cocaine (800 µg/kg)-heroin 100 µg/kg, (n = 6), and cocaine (800 µg/kg)-heroin 200 µg/kg (n = 7).

Figure 1. The catFISH paradigm. Working hypothesis based on Guzowski et al. 2005: the expression of mRNA encoding for *h1a* and *arc* should be detectable at different time points after drug administration. **A)** *arc* mRNA expression in the nucleus should peak at about 5 min after drug administration, whereas *h1a* mRNA should peak at about 30 min. **B)** Overlap in the expression of Drug 1-induced *h1a* mRNA and *arc* mRNA should be observed at time 30 min (25 min after Drug 1 and 5 min after Drug 2).

Figure 2. Effect of single drug injections on IEG expression. Time-course of *arc* mRNA and *h1a* mRNA expression in experiment 1. **A)** Regions of interest (plate from Paxinos & Watson, 1986). **B)** Average number of *arc*- or *homer 1a (h1a*)-positive cell nuclei as a function of brain area and administered drug (expressed as a percentage of all DAPI-positive nuclei). The brains were excised at different time points after drug administration: 0 min (n = 3 for both the cocaine and heroin groups), 8 min (n = 3 for both the cocaine and heroin groups), 16 min (n = 4 for both the cocaine and heroin groups), 25 min (n = 4 for both the cocaine and heroin groups), and 35 min (n = 4 and n = 5 for the cocaine and heroin groups, respectively).

Figure 3. Overlap in the neuronal populations engaged by heroin and cocaine. Co-expression of *arc* and *h1a* mRNAs in experiment 2. **A)** Regions of interest. (plate from Paxinos & Watson, 1986). **B)** Overlap expressed as % of overlap in the cocaine-cocaine condition as a function of brain area and drugs administered, 25 min apart, in Exp. 2: saline-saline (n = 4), cocaine (800 µg/kg)-saline (n = 6), cocaine (800 µg/kg)-cocaine (800 µg/kg) (n = 6), cocaine (800 µg/kg)-heroin 100 µg/kg (n = 6), and cocaine (800 µg/kg)-heroin 200 µg/kg (n = 7).

Fig. 4. Representative microscope images taken from the NAcc core. DAPI-stained cell nuclei (blue) co-express either only *h1a* (green), only *arc* (red), or both. The columns show green and red channels separately and then merged. Taken from Nacc core. Scale bars correspond to 0.1 mm. Arrows point to mRNA-positive nuclei.

Fig. 5. Representative microscope images taken from the NAcc shell. DAPI-stained cell nuclei (blue) co-express either only *h1a* (green), only *arc* (red), or both. The columns show green and red channels separately and then merged. Scale bars correspond to 0.1 mm. Arrows point to mRNA-positive nuclei.

Fig. 6. Representative microscope images taken from the DMS. DAPI-stained cell nuclei (blue) co-express either only *h1a* (green), only *arc* (red), or both. The columns show green and red channels separately and then merged. Scale bars correspond to 0.1 mm. Arrows point to mRNA-positive nuclei.

Fig. 7. Representative microscope images taken from the DLS. DAPI-stained cell nuclei (blue) co-express either only *h1a* (green), only *arc* (red), or both. The columns show green and red channels separately and then merged. Scale bars correspond to 0.1 mm. Arrows point to mRNA-positive nuclei.

Table 1.

	NAcc				DS			
	Cocaine (400 µg/kg)		Heroin (50 µg/kg)		Cocaine (400 µg/kg)		Heroin (50 µg/kg)	
	Arc	H1a	Arc	H1a	Arc	H1a	Arc	H1a
0 min	19.50 (1.52)	12.50 (4.44)	18.42 (6.86)	11.33 (1.8)	30.10 (1.97)	16.42 (5.85)	21.17 (6.59)	14.83 (1.02)
8 min	25.50 (5.36)	9.75 (2.38)	26.92 (13.66)	8.83 (2.71)	44.58 (4.43)	11.83 (1.91)	26.92 (8.21)	14.58 (5.27)
16 min	16.31 (3.35)	14.81 (1.22)	13.88 (3.63)	8.63 (2.94)	21.81 (3.08)	32.19 (3.73)	12.31 (2.86)	20.88 (5.59)
25 min	9.94 (2.78)	17.81 (0.82)	18.19 (3.95)	18.69 (4.29)	15.00 (3.89)	48.88 (5.99)	16.44 (3.76)	31.75 (7.3)
35 min	11.00 (1.67)	26.25 (5.13)	8.00 (2.22)	14.6 (4.26)	11.25 (1.44)	58.50 (1.52)	7.50 (2.2)	28.35 (4.58)

Table 2.

		NAcc core	NAcc shell	DMS	DLS
1st saline 2nd saline	h1a	4.94 (0.66)	4.44 (1.61)	5.98 (2.18)	8.5 (2.54)
	arc	2.38 (0.94)	2.48 (0.75)	1.98 (0.95)	3.75 (1.59)
	double	0.13 (0.07)	0.38 (0.16)	0.13 (0.07)	0.13 (0.13)
1st cocaine (800 µg/kg) 2nd saline	h1a	21.5 (4.44)	8.67 (2.36)	51.1 (7.99)	65.1 (7.83)
	arc	4.25 (0.77)	3.13 (0.68)	5.58 (1.77)	4.33 (0.95)
	double	1.46 (0.25)	1.67 (0.35)	3.63 (0.43)	4.46 (1.49)
1st cocaine (800 µg/kg) 2nd cocaine (800 µg/kg)	h1a	20.54 (5.45)	11.00 (2.87)	42.17 (8.65)	49.71 (7.5)
	arc	8.08 (0.59)	5.13 (0.67)	12.54 (1.99)	15.33 (3.89)
	double	5.46 (1.04)	3.33 (0.77)	14.17 (3.17)	21.33 (5.19)
1st cocaine (800 µg/kg) 2nd heroin (100 µg/kg)	h1a	20.33 (3.72)	8.04 (2.36)	58.67 (16.42)	66.04 (11.36)
	arc	23.29 (9.55)	18.92 (9.13)	3.63 (0.70)	3.04 (1.06)
	double	5.00 (2.07)	2.46 (0.87)	4.17 (1.19)	3.63 (0.96)
1st cocaine (800 µg/kg) 2nd heroin (200 µg/kg)	h1a	18.96 (4.33)	7.57 (1.75)	50.68 (7.34)	56.46 (7.64)
	arc	12.11 (1.53)	12.61 (2.91)	5.17 (0.74)	4.32 (1.16)
	double	3.14 (0.59)	1.75 (0.49)	3.11 (0.46)	3.93 (0.93)