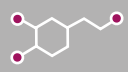
BEHAVIOURAL EFFECTS OF CHEMOGENETIC DOPAMINE NEURON ACTIVATION



Linde Boekhoudt

Brain Center
Rudolf Magnus

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Colophon

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(met een samenvatting in het Nederlands)

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te Groningen

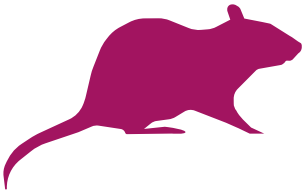
Promotor: Prof. dr. R.A.H. Adan

To my friends

CONTENTS

Too much of anything is never enough
- Pet Shop Boys, "Love etc"

CHAPTER ONE



GENERAL INTRODUCTION
USING DREADD TO UNDERSTAND THE ROLE OF DOPAMINE IN PSYCHIATRIC DISORDERS

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Challenges in the development of novel treatments for psychiatric disorders

Psychiatric disorders have a major impact on society. Lifetime prevalence for a mental disorder has been estimated at nearly 50% for the American population, ranging from 1% for schizophrenia to 17% for major depressive disorder (MDD) (Kessler et al, 2005; NIMH, 2016). In addition to direct detrimental effects on individuals suffering from these disorders, psychiatric disorders often severely impact their social environmental. Importantly, a large proportion of patients is resistant to current treatments, which severely compromises their quality of life (Kennedy et al, 2014; Mrazek et al, 2014). As such, there is a high unmet need for an improved understanding of the neurobiology underlying psychiatric states, in order to advance the development of target-specific and personalized treatments.

Over the past few decades, biological and clinical research has contributed to great advances in the understanding of medical conditions. However, in the field of psychiatry, this has not led towards a major progression in the development of novel treatments. For example, first-line pharmacological treatments for psychosis in schizophrenia are compounds that have been developed based on chlorpromazine, a compound that was accidentally discovered to have antipsychotic effects over 60 years ago (Kapur and Remington, 2001; van Os and Kapur, 2009).

Several issues may contribute to the complexity of developing more effective and target-specific treatments in psychiatry. A major challenge is that psychiatric disorders are heterogeneous, multi-faceted, and their underlying neurobiological substrates are poorly understood. Whereas a neurological disorder might be pinned down to a specific substrate, accompanied by an unambiguous clinical phenotype – e.g., degeneration of cells in the substantia nigra results in impaired control of voluntary movements in Parkinson's disease – this is typically not the case in psychiatry. Instead, psychiatric disorders encompass multiple symptoms, such as positive (psychotic), negative (e.g., motivational) and cognitive symptoms in schizophrenia (DSM-5, 2013). Furthermore, some of these symptoms are cross-diagnostic. For example, motivational deficits are present in MDD, as well as bipolar disorder and schizophrenia (Arrondo et al, 2015; Whitton et al, 2015). Finally, there may be substantial differences between individuals within a single diagnostic group. Importantly, these differences may represent distinct underlying pathophysiological substrates, and are likely to determine the individuals' response to pharmacological treatments (Demjaha et al, 2012; Farooq et al, 2013). This heterogeneity in the neurobiological substrates and treatment-responsiveness within a single disorder may severely obscure the discovery of a biomarker or novel effective

treatment (Scarr et al, 2015). Therefore, researchers and clinicians have argued for an adjusted approach, focusing on behavioural domains and specific symptoms, rather than diagnosis (Insel et al, 2010; Kas et al, 2011; Patrick and Hajcak, 2016).

In addition, the brain is the most complex organ in our body, and we have only just begun to unravel its anatomical, chemical, and genetic complexities. If we do not understand the fundamental functionality of the brain, this makes it extremely challenging to hypothesize what neurobiological substrates underlie specific symptoms or disorders, and what intervention could treat these dysfunctions. Neuro-imaging techniques have yielded major insights into the functional anatomy and network connectivity that is involved in sensory, motoric, and cognitive functions. However, basic neuroscientific research has revealed that even within a small region, many different cell types may be observed, all characterized by diverse anatomical, chemical, electrophysiological, and genetic properties. In addition, these neuronal populations are connected to numerous other cells, together comprising the immensely intricate neural circuitry that has evolved to adaptively guide behaviour. In order to fully understand the neuro-circuitry underlying normal and abnormal behaviours, we have to dissociate specific neuronal subpopulations and experimentally test their role in specified behavioural functions.

Thus, in search of more effective and target-specific treatments, it is essential to advance our understanding of the neurobiology underlying psychiatric disorders. This may be achieved by assessing the role of selective neuronal populations and neural circuitries in the regulation of specific behavioural domains, which can be translated across diagnoses, and across species. In this thesis, we aim to do exactly this. We focus on a small, yet vital, part of the brain: midbrain dopamine (DA) neurons.

DA neuronal activity in psychiatric disorders

Midbrain DA neurons release DA throughout the brain, and thereby regulate various major functions, including voluntary movement, reward-processing, and cognition. As such, disruptions in the DA system may severely affect these functions, and many psychiatric disorders have been associated with alterations in this system. Clinical neuro-imaging studies have shown that addiction, as well as obesity, is characterized by a decreased availability of DA D2-receptors (D2-R) in the striatum, the main output centre for midbrain DA neurons (Volkow et al, 2009; Wang et al, 2001). In ADHD and bipolar disorder, altered striatal DA transporter (DAT) expression has been observed, indicating a dysfunction in synaptic DA reuptake (Anand et al, 2011; Spencer et al, 2005). In addition, functional activity in the striatum during reward processing was found to be disturbed in ADHD, schizophrenia, and MDD (Arrondo et al, 2015; Furukawa et al, 2014;

Pizzagalli et al, 2009). The majority of these studies has focused on post-synaptic DA signalling (e.g., in the striatum), and relatively little is known about pre-synaptic activity of midbrain DA neurons. However, evidence suggests that for some disorders, including schizophrenia and ADHD, alterations in DA neuronal activity contribute significantly to various symptoms, including psychosis and dysfunctions in reward processing (Grace, 2015; Howes and Kapur, 2009; Maia and Frank, 2016; Tripp and Wickens, 2008; Winton-Brown et al, 2014).

For schizophrenia, it has long been hypothesized that elevated DA signalling is a core feature of the disorder. This was initially based on the antipsychotic effects of a DA-R antagonist, and later supported by findings of increased DA turnover in patients (Howes and Kapur, 2009). The role of DA in schizophrenia is emphasized by the observation that antagonistic properties at the D2-R appear are necessary, and maybe even sufficient, for antipsychotic efficacy (Kapur and Remington, 2001). Furthermore, a large-scale genome-wide association study recently identified the gene for the D2-R to be associated with schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics Consortium et al, 2014). The hypothesis that DA neuronal activity may be a key feature of the pathophysiology of schizophrenia, was based on studies using the radioactive ligand (18) F-DOPA to visualize pre-synaptic DA synthesis capacity. Increased (18)F-DOPA binding capacity has been observed in multiple studies (Fusar-Poli and Meyer-Lindenberg, 2013), and seems to be one of the most robust neurobiological substrates associated with psychosis in schizophrenia (Howes and Kapur, 2009). Elevated DA synthesis capacity was found to be already present in subjects at ultra-high risk for psychosis (Egerton et al, 2013), with higher levels in those subjects who later developed psychosis (Howes et al, 2011). Interestingly, an increase in (18)F-DOPA binding capacity was not observed in non-schizophrenic subjects experiencing hallucinations (Howes et al, 2013), or in schizophrenic subjects who did not respond to (anti-DAergic) antipsychotic treatment (Demjaha et al, 2012). The elevated pre-synaptic DA availability, and release, indicates an enhanced activity of midbrain DA neurons (Grace, 2016). The increasingly endorsed "aberrant salience hypothesis" proposes that aberrant DA neuronal activity is the common neurobiological substrate of psychosis in schizophrenia (Howes and Kapur, 2009), and may explain crucial aspects of positive symptoms. DA neurons typically show increased firing in response to salient stimuli, that require an animal's attention, such as an unexpected reward, a reward-predicting cue, or a threat (Schultz, 1992). As such, enhanced (spontaneous) DA neuronal firing is proposed to induce aberrant salience processing, attributing significance to irrelevant stimuli (Winton-Brown et al, 2014). Combined with an adverse cognitive schema, this may result in psychotic symptoms, including delusions and hallucinations (Howes and Murray, 2013). Interestingly, it has

been hypothesized that aberrant DA neuronal activity may also account (in part) for negative symptoms. Reduced responsivity to stimuli that do require salience signalling disrupts various reward-related processes (Maia and Frank, 2016), and may thereby result in motivational deficits, including impaired engagement in effortful actions (Gard et al, 2014). Thus, aberrant DA neuronal activity appears to be a core (although importantly not the sole) neurobiological substrate underlying symptoms in schizophrenia, and thus treatments targeted at DA neuronal activity may have great therapeutic potential (Howes and Nour, 2016).

In ADHD, DA is thought to play an essential role in both neurobiological substrates and the treatment of symptoms. Imaging studies have found increased striatal DAT densities in ADHD subjects (Spencer et al, 2005). This suggests that enhanced DA reuptake causes a DA deficit, which is treated by DAT-inhibiting psychostimulant drugs, such as methylphenidate. However, findings between studies have not been unanimous, and it is hypothesized that DAT expression may be upregulated in response to DA-stimulating treatment (Swanson et al, 2011). As such, it remains unknown whether ADHD results from reduced or enhanced DA function. An alternative hypothesis proposes that (at least part of) ADHD symptoms may be explained by abnormal DA neuronal activity (Tripp and Wickens, 2009). ADHD subjects were found to display diminished striatal activity in response to reward anticipation, along with increased activity in response to reward delivery (Furukawa et al, 2014). This suggests that the normally observed shift in DA neuronal activity, from reward delivery towards reward prediction (Schultz et al, 1997), does not take place in individuals with ADHD. This "dopamine transfer deficit" (DTD) may explain certain symptoms, including aberrant response to positive reinforcement (Tripp and Wickens, 2008). However, additional (preclinical) studies are needed to further evaluate the effects of altered DA neuronal activity on behaviour, and investigate whether it is sufficient to induce hyperactive, impulsive, and inattentive symptoms (Tripp and Wickens, 2012).

DA neuronal activity and regulation of behaviour

The DAergic midbrain is divided into two main populations: the ventral tegmental area (VTA) and substantia nigra (SN) (***Fig 1A***). These populations release DA throughout the brain, thereby regulating a wide spectrum of behavioural and cognitive functions. In the rodent brain, the VTA is located medially in the midbrain, and DA neurons are intermixed with inhibitory gamma-aminobutyric acid (GABA) neurons and a small proportion of excitatory glutamatergic neurons. The SN comprises the lateral part of the midbrain, and is divided into a dense population of DA neurons, the SN pars compacta (SNc), and the adjacent GABAergic pars reticulata (SNr). (In the primate brain, the organization

is somewhat different, see Haber and Knutson, 2010). Although there is no absolute border between the two populations, in general, they display different morphological, genetic, and electrophysiological properties (Roeper, 2013).

The VTA and SNc serve dissociative behavioural functions, based on their projections towards different brain regions (***Fig 1B***). The VTA sends its major DAergic projection to the ventral striatum, including the nucleus accumbens (NAc), which is often referred to as the mesolimbic reward circuit. Additional mesocorticolimbic projections ascend towards (amongst others) the amygdala and prefrontal cortex (PFC), areas involved in emotional processing and cognitive function, respectively. The nigrostriatal DA pathway, originating from DA neurons in the SNc, innervates the dorsal striatum. An essential function of this pathway is voluntary motor control, as is illustrated by the motoric deficits that occur following neurodegeneration of SNc DA neurons in Parkinson's disease (Kalia and Lang, 2015). The VTA and SNc are interconnected through loops between midbrain and striatal sub-regions (Haber and Knutson, 2010), together comprising a vital part of the basal ganglia.

From an evolutionary perspective, DA's role in the basal ganglia circuitry is to guide appropriate action selection (Grillner et al, 2013; Hills, 2006). This process is based on integration of information from sensory and memory systems, and is aimed to drive an animal's behaviour towards positive outcomes, and away from negative ones. For example, when you enjoy a particularly delicious meal at a newly opened restaurant, or a rat finds a scrumptious left-over cheesecake in a dumpster outside your house, both you and the rat will be more likely to approach these environments again. Conversely, had you or the rat encountered something aversive, such as food poisoning or a predator, this would instead promote future avoidance rather than approach. As such, the function of DA is inseparably associated with both reward processing and voluntary motor actions. When a certain stimulus or environment is experienced as rewarding, or is learned to be associated with something rewarding, this means that the animal will be more likely to approach this stimulus upon a next encounter.

In order to optimally guide adaptive action selection, the DA system is crucially involved in various aspects of reward-related processes, including salience signalling, associative learning, motivation, and behavioural activation (Berridge, 2007; Nicola, 2010; Robbins and Everitt, 2007; Salamone and Correa, 2012; Wise, 2004). DA populations in both VTA and SNc are thought to be involved in these processes, although their precise functions are not fully understood. Both populations display "reward prediction errors" in response to salient stimuli, and recent studies have shown that rodents will readily

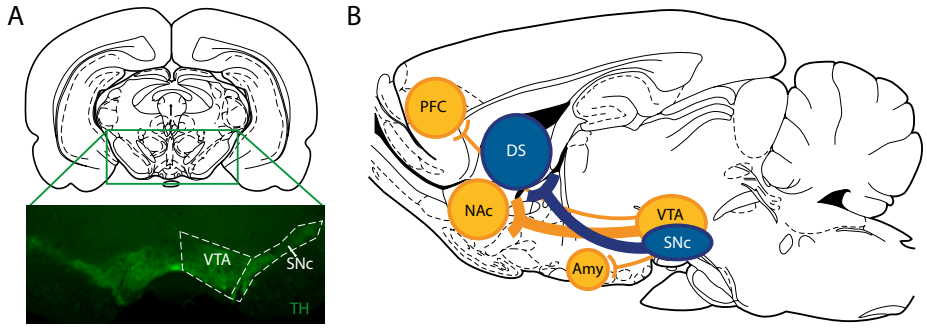

Figure 1. Dopaminergic pathways originating from ventral tegmental area (VTA) and substantia nigra (SNc). *A) Coronal section of the rat midbrain, showing DA neurons (TH+) in VTA and SNc. B) Sagittal section of the rat brain, showing midbrain DA pathways. VTA sends mesocorticolimbic projections towards nucleus accumbens (NAc), prefrontal cortex (PFC), and amygdala (Amy). SNc sends nigrostriatal projections towards dorsal striatum (DS). Schematic brain sections adapted from Paxinos and Watson, 1997.*

self-administer stimulation of DA neurons in either the VTA or SNc (Ilango et al, 2014; Rossi et al, 2013a), suggesting that activation of both these populations is rewarding (i.e., reinforcing). In addition, both populations express receptors for feeding hormones, and adjust their firing patterns in response to homeostatic state (e.g., hungry or fed) (Figlewicz et al, 2003; van der Plasse et al, 2015; Rossi et al, 2013b). Together, these findings indicate that DA neurons in both VTA and SNc are involved in the regulation of motivated behaviours, and that aberrant DA neuronal activity may contribute to various deficits, including neuropsychiatric symptoms. However, to what extent increased DA neuronal activity may indeed induce behavioural deficits, remains elusive.

Using DREADD technology to chemogenetically activate DA neurons

Over the last few years, a novel tool has been developed by the lab of Bryan Roth (University of North Carolina, Chapel Hill, USA) to investigate the effects of altered neuronal activity on behaviour (Rogan and Roth, 2011). Designer receptors exclusively activated by designer drugs (DREADDs) are human muscarinic receptors that have been mutated so that they are no longer activated by their endogenous ligand (acetylcholine), but instead by an otherwise inert “designer drug” (Armbruster et al, 2007). These DREADDs can be virally transduced into specific cell populations, using non-toxic adeno-associated viruses (AAV). Initially, two major classes of DREADDs were developed, the excitatory hM3Dq and inhibitory hM4Di, which are G-protein-coupled receptors (GPCRs) that couple to Gq- and Gi-proteins, respectively. Only upon (peripheral) administration

of the designer drug clozapine-N-oxide (CNO), the DREADDs are activated, and either enhance or reduce neuronal excitability.

An important feature of DREADD viruses is that they can be designed to be Cre-dependent. When the coding sequence for the DREADD is inserted into the viral vector using a double-floxed inverted open reading frame (DIO), Cre recombinase enzyme is necessary to recombine the sequence, and allow expression of the receptor. Cre recombinase is not endogenously present in animals, but can be introduced using transgenic animals, or viral infusions. In this thesis, we used TH::Cre rats, a transgenic rat line that expresses Cre in cells containing tyrosine hydroxylase (TH, the enzyme that is necessary to convert L-DOPA into DA) (Witten et al, 2011). Thus, by infusing a Cre-dependent virus containing hM3Dq into the midbrain of TH::Cre rats, we can specifically target DAergic neurons, and activate these neurons with CNO (***Fig 2A***).

A second approach that we have adopted, is the combination of DREADD with canine adeno virus 2 expressing Cre recombinase (CAV2Cre; Boender et al, 2014). Upon infusion into brain region "A" (e.g., ventral striatum), CAV2Cre is taken up by axon terminals, and induces Cre expression in neurons that project to A. In this case, wild type animals can be used, and by infusing a DREADD virus into brain region "B" (e.g., VTA), CNO will selectively activate neurons that project from B to A (VTA to striatum; ***Fig 2B***). Thus, this technique can be used to dissociate between behavioural functions of selective DAergic projections, including mesolimbic, mesocortical, and nigrostriatal pathways.

The use of DREADD technology, or "chemogenetics", has gained widespread popularity, especially in the field of neuroscience (Burnett and Krashes, 2016; Roth, 2016). Both chemogenetics and "optogenetics" – a technique using viral infusion of light-sensitive ion-channels rather than designer receptors (Deisseroth, 2015) – have been proven particularly powerful in the investigation of the neuronal circuitry underlying behaviour. These techniques allow for direct and transient manipulation of specific neuronal populations, which provide important advantages compared to more traditional neuroscientific methods, such as lesions, electrical stimulation, or pharmacological interventions.

First, as mentioned above, Cre-dependent DREADD viruses can be used to selectively manipulate neurons based on a specific cell-type, such as DA neurons or mesolimbic VTA neurons projecting to the striatum. Secondly, DREADD expression in the absence of CNO does not affect neuronal activity. Thus, in contrast to, e.g., lesions or transgenic mutations, behaviour can be assessed repeatedly within an intact animal, allowing for

within-subject comparison of behaviour with and without neuronal activation. This is highly desirable, since variations between animals may obscure the potential effects induced by experimental manipulations, and repeated within-subject testing elucidates the robustness of any observed effects. Thirdly, chemogenetics is relatively non-invasive and easily applied, since there is no need to implant chronic cannulas, optic fibres, or other devices. This enables the longitudinal assessment of behavioural effects following transient neuronal activation in freely moving animals. Similar to pharmacological manipulations, chemogenetic activation of excitatory DREADD hM3Dq with CNO affects neuronal activity for multiple hours (Alexander et al, 2009). Since DREADD technology is based on activation of GPCRs, the final effect on neuronal activity is dependent upon additional excitatory and inhibitory inputs. An interesting prospective is that chemogenetics can provide insights into the role of GPCR function in the brain, and may therefore contribute to the identification of novel therapeutic targets (Lee et al, 2014).

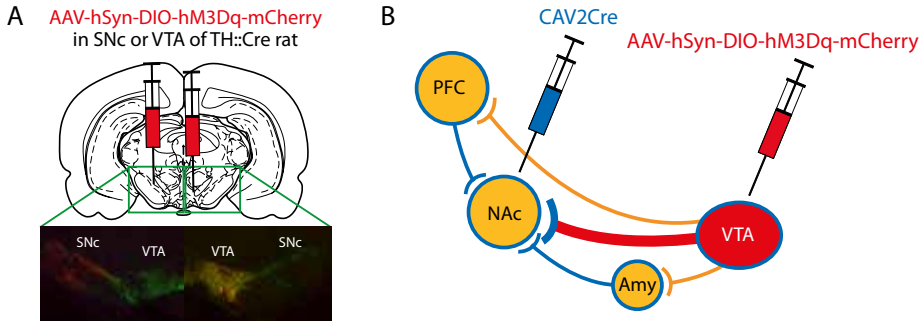

Figure 2. Using Cre-dependent DREADD to selectively target DA neuron subpopulations or neuronal pathways. *A) Infusion of Cre-dependent DREADD virus (AAV-hSyn-DIO-hM3Dq-mCherry) in TH::Cre rats for selective chemogenetic activation in DA neurons in VTA or SNc. Fluorescent pictures show DREADD expression (mCherry) in red and DA cells (TH) in green. B) Infusion of CAV2Cre into target region (e.g., NAc) and DREADD virus into midbrain (e.g., VTA) for chemogenetic activation of selective pathways.*

Too much of a good thing?

Brain DA signalling is necessary for a variety of functions, ranging from locomotion and feeding to reward processing and cognitive functioning. However, one can have too much of a good thing, and too much DA may have severely disruptive effects. The aim of this thesis is to elucidate the behavioural consequences of increased midbrain DA neuronal activity. We used DREADD technology to chemogenetically activate specific subsets of midbrain DA neurons, and determine the impact on behaviour. We chose to investigate behavioural domains that can be translated across species and across psychiatric disorders, and are relevant to disorders associated with aberrant DA signalling.

Outline of the thesis

Behavioural effects of chemogenetic dopamine neuron activation

In the following chapters, we present the results from chemogenetic activation of midbrain DA neurons on behaviour. In our experiments, we investigated the effects of enhanced DA neuronal activity in VTA compared to SNc, or in distinct neuronal pathways. We studied a broad set of behaviours, in which DA is known – to a lesser or greater extent – to play a role, and that are relevant to neuropsychiatric symptoms. In all cases, we chose to study behavioural outcomes that can be assessed multiple times within in the same animal, allowing us to make within-subject comparisons of behavioural output under baseline conditions compared to a state of increased DA neuron activation. By combining the effects on "basic" behaviours such as locomotion and feeding, with those on high-level cognitive performance, such as motivation and attention, we wish to provide a comprehensive behavioural profile of enhanced DA neuronal activity in the rat. With these findings, we hope to contribute to the understanding of neurobiological control of normal and abnormal behaviour, and provide valuable information for the development of novel treatments in the clinic.

Chapter 2: Chemogenetic activation of dopamine neurons in the ventral tegmental area, but not substantia nigra, induces hyperactivity in rats

DA in the basal ganglia is known to regulate locomotor activity. Psychostimulant drugs that enhance DA signalling induce locomotor hyperactivity (Canales and Iversen, 1998; Carr and White, 1987; Delfs et al, 1990; Ikemoto, 2002), a symptom that is present in multiple psychiatric disorders, including ADHD, schizophrenia, manic episodes in bipolar disorder, and anorexia nervosa (Angst et al, 2003; Beumont et al, 1994; Casper, 2006; Mehler-Wex et al, 2006; Perry et al, 2010). However, the causal relationship between DA neuronal activity and locomotor hyperactivity remains unclear. In this chapter, we tested whether enhanced excitability of DA neurons in either VTA or SNc is sufficient to induce hyperactivity. First, we validated our experimental approach by determining the effects of chemogenetic activation on DA neuronal activity in vitro. Next, we investigated which midbrain neuronal populations were responsible for inducing locomotor hyperactivity, by activating selective DA neuron subpopulations and pathways.

Chapter 3: Enhancing excitability of dopamine neurons promotes motivational behaviour through increased action initiation

A second major function of DA is the regulation of motivational behaviour. DA is thought to act as a mediator that translates incentive motivation into physical actions. Several psychiatric disorders, including MDD and schizophrenia, are characterized by motivational

deficits, which are thought to be related to aberrant DAergic activity (Maia and Frank, 2016; Salamone et al, 2016; Whitton et al, 2015). Although it is has been established that (particularly mesolimbic) DA signalling is crucial for motivational behaviour, it is unknown which specific aspects of motivation are regulated by DA neuronal activity. In this study, we aim to elucidate how increased DA neuronal activity drives behaviour to promote the exertion of effort. To this aim, rats were trained to lever press for sucrose under a progressive ratio schedule of reinforcement, in order to test how much effort the animals were willing to perform to obtain a palatable food reward. To investigate which aspects of instrumental performance were affected by DA neuron activation, we analysed lever press patterns over the course of increasing ratio requirements. In addition, we tested whether the effects of chemogenetic DA neuron activation on incentive motivation were dependent on pre-task access to sucrose, presence of reinforcement, or effort requirement. Together, this shows which features of motivational behaviour are driven by enhanced DA neuronal activity, and which are not.

Chapter 4: Does activation of dopamine neurons promote or reduce feeding?

DA signalling is necessary for feeding behaviour (Zhou and Palmiter, 1995). However, much debate remains concerning the role of DA in the control of food intake. Neurochemical and behavioural similarities have been observed between drug addiction and obesity, including comparable alterations in the DA system (Volkow and Wise, 2005). Furthermore, drugs that act on the DA system are known to affect food intake (Davis et al, 2012; Leibowitz et al, 1986). However, the relationship between DA neuronal activity and feeding behaviour remains elusive. One unresolved issue is whether enhanced DA neuronal activity would either stimulate or reduce feeding. Secondly, the respective roles of VTA and SNc DA neurons are not fully understood. In this study, we tested the effects of chemogenetic midbrain DA neuronal activation on free food consumption in the rats' home cage. We analysed the microstructure of feeding behaviour, including meal size and frequency, following activation of midbrain DA neurons in the VTA or SNc. To determine which neuronal pathways are involved in feeding behaviour, we selectively activated distinct mesocortical and mesolimbic pathways. Furthermore, we compared these effects to pharmacological DA stimulation by psychostimulant drugs, which are known to have anorexic effects.

Chapter 5: Chemogenetic activation of midbrain dopamine neurons affects attention, but not impulsivity, in the five-choice serial reaction time-task in rats

Cognitive deficits and impaired impulse control are key features of various psychiatric disorders, including ADHD, schizophrenia, and addiction. Although DA signalling has been shown to be involved in impulsive behaviours as well as attentional performance, it remains unclear whether aberrant DA neuronal activity underlies exaggerated impulsivity and/or attentional deficits. In *Chapter 5*, we investigated the impact of enhanced DA neuronal activity on performance of the 5-choice serial reaction time task (5-CSRTT). This instrumental task has been developed to assess a range of behavioural functions that are related to psychiatric symptomology, including impulsivity, compulsivity, and attentional accuracy (Carli et al, 1983). In this task, rats are trained to respond to a stimulus light, which is presented randomly in one of five nose poke holes, in order to earn a sucrose reward. Premature, perseverative, or incorrect responses reflect impulsive or compulsive actions, or impaired performance accuracy, respectively. In addition, errors of omission and response latency reflect the animals' task engagement. Numerous pharmacological studies have shown that striatal and cortical DA signalling affects impulsive and attentional performance in the 5-CSRTT (e.g., Agnoli et al, 2013; Economidou et al, 2012; Winstanley et al, 2010). Consistent with previous chapters, we tested the effects of chemogenetic DA neuron activation on behavioural outcome, thereby distinguishing between VTA and SNc. We hypothesized that enhanced activity of DA neurons in either VTA or SNc would induce impulsive actions, and disrupt sustained attention.

Chapter 6: Summary and general discussion: insights into the role of midbrain dopamine neuronal activity in behaviour, and implications for psychiatry

In this final chapter, we discuss how our findings from the experimental studies may be combined to provide a comprehensive view of the behavioural effects of chemogenetic DA neuron activation. Furthermore, we discuss how these findings contribute to an understanding of how increased DA neuronal activity regulates behavioural functions, and how this translates to psychiatric disorders. We also discuss the limitations of our studies, and suggest how future research might overcome these issues. Finally, we discuss how our results, and DREADD technology in general, may contribute to the development of novel, target-specific, and more effective treatments for psychiatric disorders.

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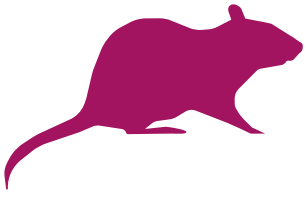
Keep runnin', runnin' and runnin', runnin'...
- Black Eyed Peas, "Let's get retarted"

CHAPTER TWO



CHEMOGENETIC ACTIVATION OF DOPAMINE NEURONS IN THE VENTRAL TEGMENTAL AREA, BUT NOT SUBSTANTIA NIGRA, INDUCES HYPERACTIVITY IN RATS

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Abstract

Hyperactivity is a core symptom in various psychiatric disorders, including attention-deficit/hyperactivity disorder, schizophrenia, bipolar disorders, and anorexia nervosa. Although hyperactivity has been linked to dopamine (DA) signalling, the causal relationship between midbrain DA neuronal activity and locomotor hyperactivity remains unknown. In this study, we test whether increased DA neuronal activity is sufficient to induce locomotor hyperactivity. To do so, we used designer receptors exclusively activated by designer drugs (DREADD) to chemogenetically enhance neuronal activity in two main midbrain DA neuron populations, i.e. the ventral tegmental area (VTA) and substantia nigra pars compacta (SN), in TH::Cre rats. We found that activation of VTA DA neurons induced a pronounced and long-lasting hyperactive phenotype, whilst SN DA neuron activation only modestly increased home cage locomotion. Furthermore, this hyperactive phenotype was replicated by selective activation of the neuronal pathway from VTA to the nucleus accumbens (NAC). These results show a clear functional difference between neuronal subpopulations in the VTA and SN with regards to inducing locomotor hyperactivity, and suggest that the DAergic pathway from VTA to NAC may be a promising target for the treatment of hyperactivity disorders.

Keywords

Dopamine – DREADD – chemogenetics – ventral tegmental area – substantia nigra – locomotor activity

Introduction

Hyperactivity is a core symptom of several psychiatric disorders, including attention deficit hyperactivity disorder (ADHD), schizophrenia, manic episodes in bipolar disorders, and anorexia nervosa (Angst et al, 2003; Beumont et al, 1994; Casper, 2006; Mehler-Wex et al, 2006; Perry et al, 2010). The neurobiological substrates underlying hyperactive behaviour are incompletely understood, which hampers the development of novel, more effective, treatments. Clinical imaging studies have shown alterations in dopamine (DA) signalling in individuals diagnosed with hyperactivity (Jucaite et al, 2005; Ludolph et al, 2008; Volkow et al, 2009), and preclinical studies have shown a strong link between DA signalling in the striatum and locomotor hyperactivity in rodents (Canales and Iversen, 1998; Carr and White, 1987; Delfs et al, 1990; Dickson et al, 1994; Gong et al, 1999; Kelly et al, 1975). However, both clinical and preclinical studies have mainly focused on DA signalling in the target areas of midbrain DAergic neurons (primarily the striatum), rather than direct manipulation of the DA neurons themselves. Hence, it remains unclear if there is a causal relationship between DAergic neuronal activity and locomotor hyperactivity, and, if so, which neuronal subpopulations are involved.

Within the midbrain, we distinguish DA neurons in the ventral tegmental area (VTA) and substantia nigra pars compacta (SN). These DA neuron populations send projections to ventral and dorsal parts of the striatum, respectively. Previous studies have shown that increasing midbrain DA neuronal activity (including both VTA and SN) induced locomotor hyperactivity in mice (Wang et al, 2013). Also, disinhibition of these neurons, by inhibiting midbrain GABAergic neuronal activity, increased locomotor activity (Vardy et al, 2015). However, these studies were not designed to distinguish between DA neuronal subpopulations in the VTA and SN, and thus their relative contribution to the hyperactive phenotype remains unknown.

Preclinical and clinical studies have shown evidence for a role of both mesolimbic and nigrostriatal DAergic pathways – emerging from VTA and SN, respectively – in locomotor activity. Imaging studies have shown alterations in DAergic signalling in ADHD subjects in both ventral and dorsal striatal subregions (Jucaite et al, 2005; Ludolph et al, 2008; Volkow et al, 2009), suggesting that DA neuronal activity in both VTA and SN might be affected. Initial pharmacological studies in rodents have shown that psychostimulant-induced hyperactivity mainly results from actions in the nucleus accumbens (NAC) (Canales and Iversen, 1998; Carr and White, 1987; Delfs et al, 1990; Dickson et al, 1994; Kelly et al, 1975), rather than in the dorsal striatum (Carr and White, 1987; Dickson et al, 1994; Kelly et al, 1975), suggesting a primary role for DA neurons in the VTA. Indeed, DA-deficient mice did not show psychostimulant-induced hyperactivity unless DAergic signalling in

the NAC was restored (Heusner et al, 2003). However, selective rescue of nigrostriatal DA signalling also enhanced locomotor behaviour in these mice, in the absence of a psychostimulant drug (Hnasko et al, 2006). Previously, we reported that chemogenetic activation of VTA neurons projecting to the NAC increased home cage locomotor activity in rats (Boender et al, 2014). In contrast, several studies using optogenetic activation of VTA DA neurons failed to observe effects on locomotor activity (Chaudhury et al., 2013; Gunaydin et al., 2014; Tye et al., 2013). Taken together, DAergic pathways emerging from both VTA and SN appear to be involved in regulating locomotor activity, but their respective roles in inducing hyperactivity remain to be resolved.

In this study, we sought to investigate whether chemogenetic activation of DA neurons in the VTA or SN induces locomotor hyperactivity. In order to directly manipulate neuronal activity of DAergic neurons, we used designer receptors exclusively activated by designer drugs (DREADD) in TH::Cre transgenic rats. Additionally, we targeted selective pathways to identify which midbrain neuronal subpopulations are crucially involved in inducing locomotor hyperactivity.

Experimental procedures

Subjects & surgical procedures

TH::Cre transgenic rats (Witten et al, 2011) were bred in-house, by crossing heterozygous TH::Cre$^{+/-}$ rats with wild type Long Evans mates. Cre-negative (Cre-) littermates served as control. All experiments were performed in accordance with Dutch laws (Wet op de Dierproeven, 1996) and European regulations (Guideline 86/609/EEC), and were approved by the Animal Ethics Committee of Utrecht University.

Surgical procedures All experimental animals were injected bilaterally with 1 µl of AAV5-DIO-hSyn-hM3Dq-mCherry ("Dq") (6.4–8.0*E12 virus molecules/ml; UNC Vector Core). Prior to surgery, rats were anaesthetized by intramuscular fentanyl/fluanisone (0.315 mg/kg fentanyl, 10 mg/kg fluanisone, Hypnorm, Janssen Pharmaceutica, Belgium), and xylocaine was sprayed on the skull to provide local anaesthesia (Lidocaine 100mg/ml, AstraZeneca BV, the Netherlands). All rats received three daily peri-surgical injections of carprofen (5 mg/kg, s.c., Carporal, AST Farma BV, the Netherlands), starting at the day of surgery. In order to allow for sufficient DREADD expression, there was a minimum of two weeks between surgery and electrophysiological recordings, and at least four weeks in between surgery and behavioural testing.

Experiment 1 (chemogenetic activation of DA neurons in vitro): In vitro electrophysiology experiments were performed in TH::Cre rats injected with virus at 3-4 weeks old, at

AP -4.8; ML +1.0 (5º angle); DV -7.1 for VTA, and AP -4.8; ML +1.9; DV -6.7 for SN. All coordinates are in mm relative to Bregma.

Experiment 2 (chemogenetic activation of VTA and SN DA neurons): Male TH::Cre rats (n=16; age 14 weeks at start of behavioural testing) and Cre- littermates (n=14) were injected into either the VTA (young rats, 156 ± 20 grams [mean ± SD], at AP -5.2; ML +1.1 [5º angle]; DV -7.4), or SN (adult rats, 337 ± 23 grams, at AP -5.4; ML +2.2; DV -7.7).

Experiment 3 (chemogenetic activation of VTA>NAC and SNm>DMS projections): To induce DREADD expression selectively in midbrain neurons projecting to either NAC or dorsomedial striatum (DMS), we used Cre-dependent DREADD combined with canine-adenovirus2 expressing Cre recombinase (CAV2Cre) (Boender et al, 2014; Hnasko et al, 2006). 1 µl of CAV2Cre (1.0*E12 molecules/ml, IGMM, France) was infused into either the NAC (AP +1.2; ML +2.8 [10º angle]; DV -7.5) or DMS (AP +1.2; ML +2.7 [10º angle]; DV -5.1) of adult male rats (n=13 wild type and n=3 Cre- Long Evans; 394 ± 34 grams; age 20 weeks at start of behavioural experiments), and AAV5-hSyn-DIO-hM3Dq-mCherry was injected into the VTA, at AP -5.6; ML +1.3 (5º angle); DV -8.2.

Until the onset of the behavioural experiments, animals were group housed, with ad libitum access to lab chow and water, on a 12-hour light-dark schedule (lights off 16:00). During the experiments, animals were singly housed, chow was available from 16:00 until 10:00, and water was available ad libitum.

Drugs

Clozapine-N-Oxide (CNO; kindly provided by Bryan Roth and NIMH) was dissolved in sterile saline (0.9% NaCl). All injections were given intra-peritoneally (i.p.) at 1 ml per kg bodyweight, and dissolved CNO was kept at 4 ºC in between injections, for a maximum of four weeks. For in vitro electrophysiology, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Tocris, UK), and bicuculline (Tocris, UK) were prepared as a stock solution.

Electrophysiology

Animals were anaesthetized by i.p. injection of sodium pentobarbital (Euthanimal), followed by transcardial perfusion with carbogenated modified artificial cerebrospinal fluid (ACSF) containing (in mM): 92 N-methyl-D-glucamine (NMDG), 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Napyruvate, 0.5 $CaCl_2.4H_2O$, and 10 $MgSO_4.7H_2O$, bubbled with 95% O_2 and 5% CO_2 (pH 7.3–7.4). Horizontal slices of the midbrain (300 µm) were prepared using a vibratome (Leica VT1200S, Leica Microsystems) in ice-cold modified ACSF. Slices were initially recovered

in the carbogenated modified ACSF for 15 min at 34 °C, and then transferred into a holding chamber with standard ACSF, containing (in mM): 126 NaCl, 3 KCl, 2 $MgSO_4$, 2 $CaCl_2$, 10 glucose, 1.25 NaH_2PO_4 and 26 $NaHCO_3$ bubbled with 95% O_2 and 5% CO_2 (pH 7.3) at room temperature, for at least 1h. Subsequently, slices were transferred to the recording chamber, superfused with standard ACSF continuously bubbled with 95% O_2 and 5% CO_2 at 30–32 ˚C. Whole-cell patch-clamp recording were performed from DA neurons in the VTA or SN, identified by mCherry fluorescence and visualized with an Olympus BX61W1 microscope using infrared video microscopy and differential interference contrast (DIC) optics. Patch electrodes were pulled from borosilicate glass capillaries, and had a resistance of 3-5 MΩ when filled with intracellular solutions. Internal solution contained (in mM): 140 K-gluconate, 1 KCl, 10 HEPES, 0.5 EGTA, 4 MgATP, 0.4 Na_2GTP, 4 phosphocreatine (pH 7.3 with KOH). Signals were amplified, filtered at 3 kHz and digitized at 10 kHz using an EPC-10 patch-clamp amplifier and PatchMaster v2x73 software. Series resistance was constantly monitored, and the cells were rejected from analysis if the resistance changed by >20%. No series resistance compensation was used. Resting membrane potential was measured in bridge mode (I=0) immediately after obtaining whole-cell access. CNO was dissolved in ACSF at a concentration of 5 µM and applied to the bath through perfusion. Data were analysed with Clampfit 10 (Axon Instrument) software.

Behavioural setup home cage locomotor activity

All rats were housed individually in PhenoTyper® 9000 home cages (Noldus IT, Wageningen, the Netherlands), 43x43x90cm, equipped with infrared video cameras in the top to monitor locomotor activity. CNO or saline was administered i.p. 30 minutes before onset of the dark phase, and locomotor activity was tracked from the moment of injection (experiment 2) or 30 minutes prior to injection (experiment 3). Subsequent injections of CNO were separated by at least one day. For the dose response test, all rats received all five doses (see Results section for doses used) in a Latin-square design, with one wash-out day in between each dose.

Tissue preparation and immunohistochemical analysis

After the behavioural experiments, rats were given a lethal dose of sodium pentobarbital, ca 0.1 ml/100g body weight, followed by transcardial perfusion with 0.9% NaCl followed by 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). Brains were post-fixed in 4% PFA for 48h, and afterwards saturated with 30% sucrose in PBS with 0.01% NaN_3. Brains were snap-frozen in -50 ºC iso-pentane and sliced at 40 µm on a cryostate. Tissue was preserved in cryo-protectant (25% glycerol and 25% ethylene-glycol in PBS) at -20 ºC.

Immunohistochemistry was performed by blocking the brain slices with 10% normal goat serum + 0.05% Tween-20 in PBS (1h), followed by incubation with primary antibodies Rabbit anti-dsRed (1/500, Clontech) and Mouse anti-TH (1/1000 experiment 2; 1/500 experiment 3; Millipore) in PBS-Tween (overnight at 4 ºC), then secondary antibodies Goat anti-Rabbit Alexa 568 (1/500, Abcam) and Goat anti-mouse Alexa 488 (1/1000 TH::Cre, 1/500 CAV2Cre; Abcam) in PBS-Tween (2h), and finally DAPI (30'). Slices were mounted on SuperFrost glasses (VWR, Leuven) with FluorSave (Millipore).

DREADD expression was quantified as fluorescence intensity in defined midbrain and striatal subregions, with background intensity subtracted. Expression was averaged for each subregion, i.e., bilaterally in three midbrain sections (anterior, middle, and posterior midbrain, ca 0.32 mm apart) or bilaterally in one striatal section (Bregma +1.7mm). Confocal images to study co-localisation of TH and mCherry were taken with an Olympus Fluoview 1000 Confocal microscope and FluoView software, and analysed using ImageJ. Specificity and transduction efficiency were assessed within mCherry-expressing regions.

Data analysis

Locomotor activity was recorded at 25 samples per second, and was analysed with EthoVision XT9 and XT11 (Noldus, Wageningen, the Netherlands). Movement tracks of the animals' centre point were smoothed by locally weighted scatterplot smoothing. Statistical analysis was performed in SPSS 16.0. Normality of data distribution was assessed using Kolmorogov-Smirnov test and additional inspection of Q-Q plots. When normality or equality of variance was violated, non-parametric tests were applied. Locomotion data showed a skewed distribution, with variance proportional to the mean, and were therefore natural log transformed to allow parametric testing. Effects of CNO on slice electrophysiology were tested with paired samples t-tests. Fluorescence intensity was compared to background using one sample t-test (corrected for testing multiple subregions), and compared between Dq+ groups with independent samples t-tests. In experiment 3, one DMS-injected rat was allocated to the NAC-group instead, due to high levels of DREADD expression in NAC (>7 SD's deviation from rest of the DMS group, and no difference in expression levels compared to NAC group). Effects of CNO compared to saline treatment on total distance moved were tested by repeated measures general linear model (RM-GLM), with Group as between-subjects factor, and Treatment and Time as within-subjects factors. Significant effects were followed up by post-hoc LSD pairwise comparisons. Correlations between DREADD expression levels in midbrain and striatum, in VTA:Dq+ and SN:Dq+ rats, were analysed with non-parametric Spearman's correlation coefficient. Comparison of proportions was assessed with

binomial testing. Statistical significance was set at α=0.05, and Bonferroni corrections were used to correct for multiple comparisons.

Results

Experiment 1: *in vitro* validation of chemogenetic activation of DA neurons in TH::Cre rats

To confirm that CNO activates hM3Dq-expressing (Dq+) DA neurons, targeted whole-cell current clamp recordings were made from Dq+ DA neurons in the VTA (identified by mCherry fluorescence), in slices prepared from TH::Cre rats injected with AAV-DIO-hSyn-hM3Dq-mCherry in the VTA (***Fig 1A***). Bath application of CNO (5 µM) to VTA slices depolarized Dq+ neurons by 6.1 ± 0.8 mV compared to baseline membrane potentials (t_8=7.662, P<0.0001; ***Fig 1C***) and increased the firing rate of spontaneous action potentials from 0.85 ± 0.09 Hz to 1.24 ± 0.17 Hz (t_8=3.018, P=0.0166; ***Fig 1C***). Furthermore, CNO increased the number of evoked action potentials in VTA Dq+ neurons (***Fig 1B,D***). CNO-induced depolarization was reversible following CNO washout (***Fig 1B***). CNO did not affect the membrane potential of non-hM3Dq-mCherry expressing (Dq-) neurons in the VTA (data not shown). Recordings from Dq+ DA neurons in the SN in midbrain slices, obtained from TH::Cre rats injected with AAV-DIO-hSyn-hM3Dq-mCherry in the SN, showed similar results: CNO application depolarized SN DA neurons by 4.6 ± 0.3 mV (t_6=13.69, P=0.0001) and increased the frequency of spontaneous action potentials to 145% ± 9.4% (t_5=4.11, P=0.0093) of baseline, which was reversed upon CNO washout (data not shown).

Experiment 2: chemogenetic activation of DA neurons in VTA, but not SN, induces hyperactivity

We selectively targeted DA neurons in the VTA or SN by injecting AAV-hSyn-DIO-hM3Dq-mCherry into these areas in TH::Cre rats (***Fig 2A***). Analysis of DREADD expression (mCherry) in subregions within the midbrain confirmed expression in VTA in VTA:Dq+ rats (***Fig 2A***; fluorescence levels significantly higher compared to background in medial VTA [mVTA] t_7=4.056, corrected P=0.02; and dorsolateral VTA [dlVTA], t_7=12.971, P<0.002) and expression in SN in SN:Dq+ rats (***Fig 2A***; fluorescence levels significantly higher compared to background in medial SN [SNm] t_7=4.369, P=0.012, and dorsal SN [SNcd] t_7=7.413, P<0.002). Both groups showed expression in the dlVTA and SNm, as can be expected given their close anatomical proximity (***Fig 2A;*** dlVTA in SN:Dq+ group t_7=5.518, P=0.004; SNm in VTA:Dq+ group t_7=4.311, P=0.016). In striatal projection areas, VTA:Dq+ rats showed DREADD expression primarily in ventral striatum (NAC Core and Shell) and DMS (all t_7>9.28, corrected P<0.002, higher compared to SN:Dq+ t>4.1, P<0.005), whilst SN:Dq+ rats primarily in dorsolateral striatum (DLS) (t_6=8.354, P<0.001,

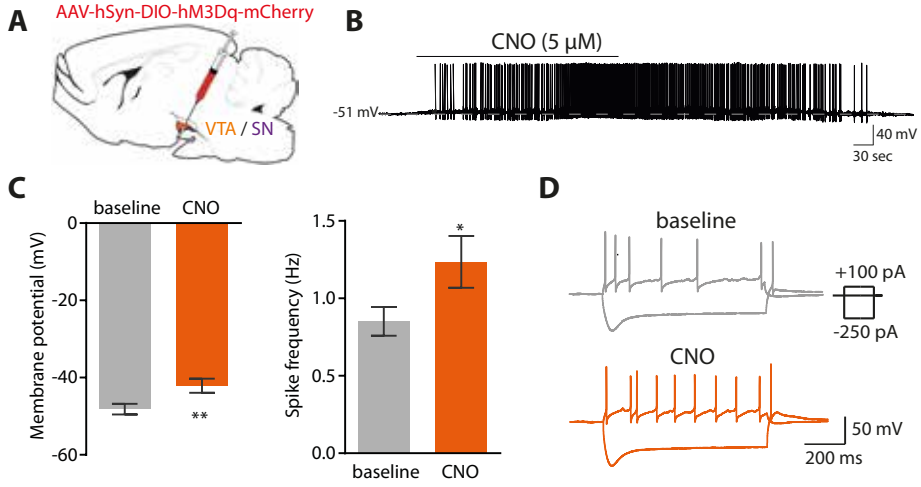

Figure 1. Effect of CNO-induced activation of hM3Dq-expressing DA neurons in the VTA of TH::Cre rats. *A) Infusion of Cre-dependent DREADD virus AAV-DIO-hSyn-hM3Dq-mCherry in either VTA or SN of TH::Cre rats. B) Sample current-clamp recording of an hM3Dq-expressing (Dq+) VTA DA neuron before, during and after CNO application. C) CNO elicited depolarization of the membrane potential and increased firing rate compared to baseline conditions. D) Sample current-recordings from an Dq+ DA neuron in response to hyperpolarizing and depolarizing current injections before and after CNO application. Error bars represent mean ± s.e.m. *P<0.05, **P<0.01, CNO compared to baseline.*

higher compared to VTA:Dq+ t_{13}=6.848, P<0.001) (***Fig 2A***). DREADD expression in the midbrain correlated positively with expression in striatal projection areas: fluorescence in VTA (both mVTA and dlVTA) correlated significantly with expression in the NAC Shell, Core, and DMS (all R>0.76, P<0.001), whilst expression in dorsal SN (SNcd) correlated significantly with DLS (R=0.843, P<0.001).

In vivo activation of DA neurons in the VTA induced a pronounced hyperactive phenotype (***Fig 2B***). CNO significantly increased total distance moved in VTA:Dq+ rats, but not VTA:Dq- controls (distance moved during 0:30-2:30h after i.p., Treatment*Group interaction $F_{1,13}$=82.641, P<0.0005; CNO vs saline for VTA:Dq+ P<0.0005; VTA:Dq- P=0.197) (***Fig 2B***). Activation of SN DA neurons also significantly increased total distance moved (Treatment*Group interaction $F_{1,13}$=4.706, P=0.049; CNO vs saline SN:Dq+ P=0.007, SN:Dq- P=0.363) (***Fig 2C***). However, the magnitude of the effect was remarkably different: VTA DA activation increased locomotor activity about 6-7 fold, compared to 1.5 fold following SN DA activation (***Fig 2B-C***). In VTA:Dq+ rats, hyperactivity was induced within 30 minutes after CNO injection, and lasted for at least 3.5-4 hours (***Fig 2B***; main effect of Treatment for 8 bins of 30 minutes, $F_{1,7}$=139.706, P<0.0005; Time*Treatment interaction

$F_{5.45,38.13}$=6.114, P<0.0005; post-hoc test for first time bin P=0.001, bin 2-8 all P<0.0005). In comparison, SN:Dq+ activation only increased locomotor activity 30 - 90 minutes after CNO injection (***Fig 2C***; main effect of Treatment for 8 bins, $F_{1,7}$=10.564, P=0.014; post-hoc test for first bin P=0.093; 2nd bin P=0.049; 3rd bin P=0.001; 4th bin P=0.015; 5th-8th bin all P>0.3). A dose response analysis within the VTA:Dq+ group showed a maximal hyperactive response with 0.1, 0.3 and 1.0 mg/kg CNO (6-7 fold increase in distance moved), and an intermediate effect with 0.03 mg/kg (3-4 fold increase, ***Fig 2D***; main effect of Treatment $F_{4,28}$=36.336, P<0.0005; Time*Treatment interaction $F_{5.3,37.2}$=1.698, P=0.156; post-hoc comparisons CNO vs saline: 0.03 mg/kg P=0.004; 0.1, 0.3, 1.0 mg/kg all P<0.002; post-hoc comparisons to 0.3 mg/kg CNO: 0.03 mg/kg P=0.045; 0.1 and 1.0 mg/kg P>0.22). CNO did not affect locomotor activity in the VTA:Dq- control group at any dose (***Fig 2D***; main effect of treatment $F_{4,24}$=1.056, P=0.399). Thus, chemogenetic activation of both VTA and SN DA neurons increased locomotor activity in TH::Cre rats, but only VTA DA neuron activation induced a pronounced hyperactive phenotype.

Experiment 3: activation of midbrain neuronal projections to the nucleus accumbens, but not dorsomedial striatum, induces hyperactivity

To investigate which neuronal projections are involved in inducing hyperactive locomotion, we used DREADD combined with CAV2Cre, a virus that infects neurons at terminals and retrogradely delivers Cre in neurons that project to the area of injection (Boender et al, 2014; Hnasko et al, 2006; Nair et al, 2013). Since VTA:Dq+ rats in experiment 2 showed DREADD expression in both the ventral and dorsomedial part of the striatum (***Fig 2A***), we injected CAV2Cre in either NAC or DMS, and Cre-dependent hM3Dq in the VTA, to selectively target midbrain neuronal projections to these striatal subregions (***Fig 3A***).

Analysis of DREADD (mCherry) expression in the midbrain confirmed NAC expression in NAC-targeted rats (***Fig 3B-C***; expression compared to background: NAC Core t_8=6.504, corrected P<0.001; Shell t_8=4.339, P=0.008), and DMS expression in DMS-targeted rats (***Fig 3B-C***; t_6=3.633, P=0.044). However, both groups showed equal levels of DREADD expression in the DMS (***Fig 3B***; NAC vs DMS group t_{14}=1.673, corrected P=0.468). Expression patterns in the midbrain were largely similar between the two groups, showing DREADD in mVTA, dlVTA, and SNm (***Fig 3B-C***; expression levels vs background all $t>_{4.6}$, P<0.01). However, the NAC group showed significantly higher levels of expression in the mVTA (***Fig 3B***; $t_{9.7}$=4.276, P=0.006). Expression levels in the mVTA were positively correlated with those in the NAC Core (R=0.806, P=0.001) and Shell (R=0.762, P<0.0005). Furthermore, there was a strong correlation between expression in the SNm and DMS (R=0.924, P<0.0005). In summary, the NAC group showed DREADD expression both

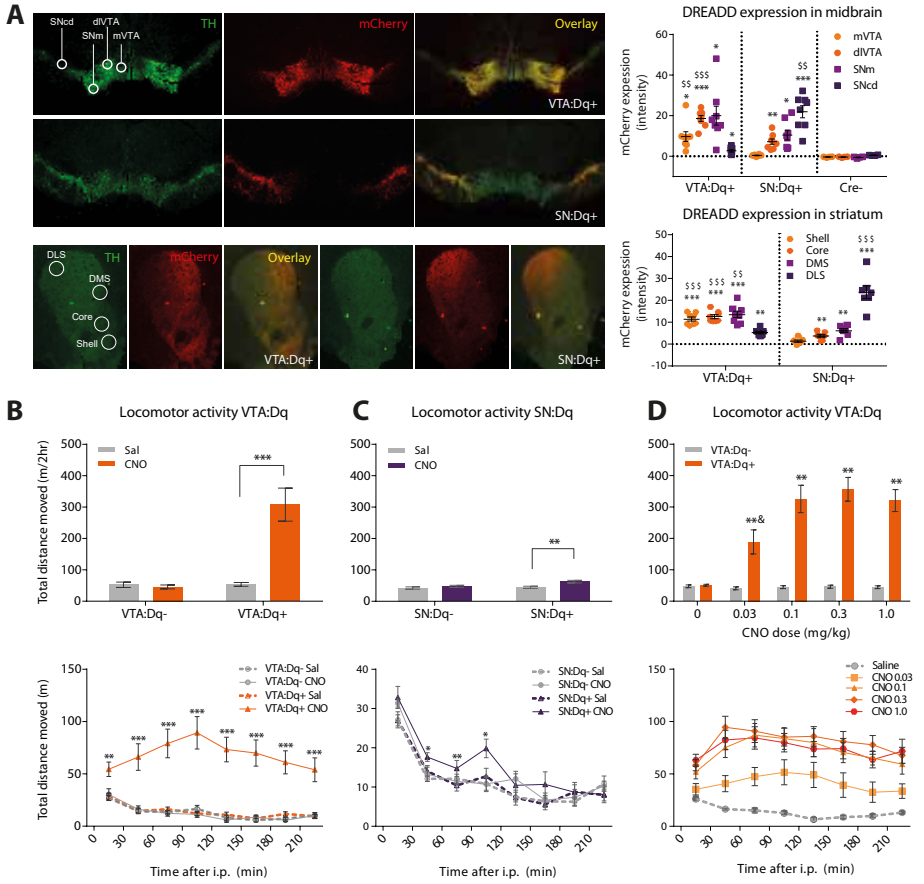

Figure 2. Locomotor response to chemogenetic activation of VTA or SN DA neurons. *A) Immunofluorescence of TH (green) and hM3Dq-mCherry (red) in midbrain (top panel) and striatum (lower panel) following virus injection into either VTA (VTA:Dq+) or SN (SN:Dq+). Right panel: quantification of fluorescence in four midbrain subregions and four striatal subregions shows dissociative patterns of DREADD expression in VTA:Dq+ and SN:Dq+ groups. B) CNO (0.3 mg/kg) induced hyperactivity in VTA:Dq+ rats, but not VTA:Dq- controls. C) CNO also significantly increased locomotor activity in SN:Dq+ rats, but not SN:Dq- controls. D) Dose response of CNO in VTA:Dq+ rats shows an intermediate increase in distance moved with 0.03 mg/kg, a maximal effect with 0.1, 0.3, or 1.0 mg/kg, and no effect in VTA:Dq- controls. B-D) Top panel: cumulative distance moved in 2 hours (0:30-2:30 after i.p. injection); lower panel: distance moved per 30 minutes. Error bars represent mean ± s.e.m. (n=7-8 per group) A: **P<0.01, ***P<0.001 difference compared to zero (i.e., background fluorescence); $$P<0.01, $$$P<0.001 difference between VTA:Dq+ and SN:Dq+ groups B-D: *P<0.05; **P<0.01; ***P<0.001 difference compared to saline; &P<0.05 difference compared to 0.3 mg/kg CNO.*

in VTA>NAC and SNm>DMS pathways, whilst the DMS group showed more selective expression, preferentially in SNm>DMS pathway. We refer to these groups as VTA>NAC and SNm>DMS, respectively.

Chemogenetic activation by CNO increased locomotor activity in both VTA>NAC and SNm>DMS groups, but only VTA>NAC activation induced a potent hyperactive phenotype, comparable to VTA DA neuron activation (***Fig 3D***; main effect of Treatment $F_{1,14}$=342.3, P<0.0005; Treatment*Group interaction $F_{1,14}$=179.2, P<0.0005; significant increase in distance moved following CNO vs saline in VTA>NAC, P<0.0005, and SNm>DMS, P=0.001). VTA>NAC and SNm>DMS activation increased locomotor activity approximately 7- and 1.4-fold, respectively (***Fig 3D***), similar to VTA and SN DA neuron activation (***Fig 2***). Comparable to VTA DA activation, CNO-induced hyperactivity in VTA>NAC rats already started within 30 minutes after injection, and lasted for at least 3.5 hours (***Fig 3D***; 2nd-8th bin all P<0.0005). SNm>DMS activation only significantly increased total distance moved 30-60 minutes after injection (***Fig 3D***; 3rd bin P=0.015). A dose response test showed that all doses, from 0.01 to 0.3 mg/kg CNO, induced hyperactivity in VTA>NAC animals, but only the highest dose increased locomotor activity in the SNm>DMS group (***Fig 3E***; main effect of Treatment $F_{4,56}$=48.4, P<0.0005, and Treatment*Group interaction $F_{4,56}$=11.8, P<0.0005; VTA>NAC all doses P<0.002; SNm>DMS 0.3 mg/kg P=0.028; 0.1 mg/kg P=0.056; 0.01 and 0.03 mg/kg P>0.1). CNO doses of 0.03 mg/kg and higher all induced a long-lasting hyperactive phenotype in the VTA>NAC group (***Fig 3E***). Similar to VTA DA neuron activation, a maximal effect was observed with 0.1 and 0.3 mg/kg CNO, whilst lower dosages induced an intermediate increase in locomotor activity (***Fig 3E***; only 0.01 mg/kg significantly smaller effect compared to 0.3 mg/kg CNO, P<0.002).

We compared DREADD expression levels with CNO-induced locomotor activity, and observed a clear distinction between the DMS and NAC group (***Fig 3F***). All rats in the DMS group showed low or no mCherry expression in the NAC, and only a modest increase in locomotor activity (see also ***Fig 3D-F***). On the other hand, all animals in the NAC group showed higher expression levels in NAC (***Fig 3B-C***), and a much more pronounced increase in locomotor activity (***Fig 3D-F***). These results suggest that increased activity of midbrain neuronal projections to the NAC specifically is necessary for inducing hyperactivity.

In summary, chemogenetic activation of midbrain neurons projecting to the NAC induced a pronounced hyperactive phenotype, whereas activation of midbrain neurons projecting to the DMS did not.

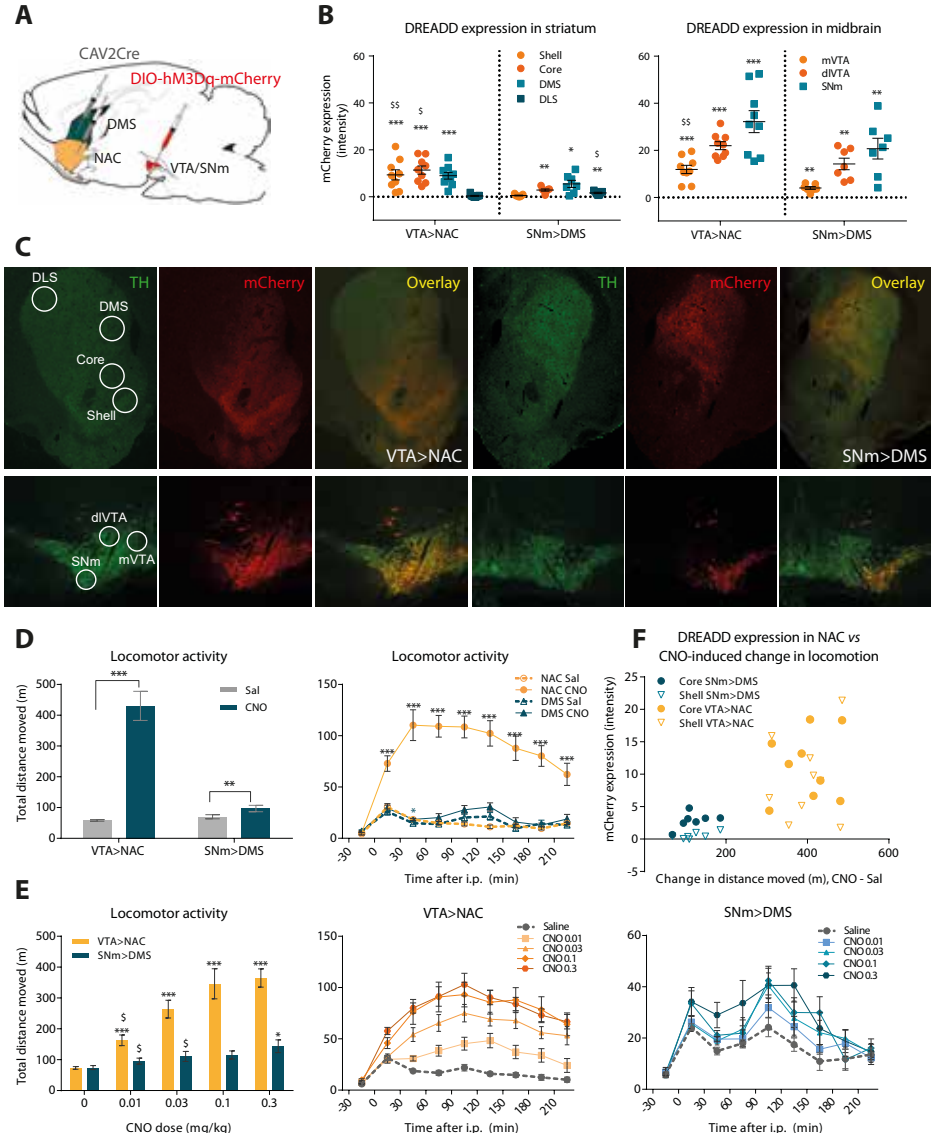

Figure 3. Locomotor response to chemogenetic activation of midbrain projections to NAC or DMS. *A) To selectively target neuronal projections, CAV2Cre was injected into NAC or DMS, and Cre-dependent DREADD virus into the midbrain (VTA/SNm). B) Quantification of fluorescence in subregions of the striatum and midbrain. C) Immunofluorescence of TH (green) and hM3Dq-mCherry (red) in striatum (top) and midbrain (lower panel) following injection of DREADD in VTA and CAV2Cre in NAC (left) or DMS (right). D) CNO (0.3 mg/kg) significantly increased distance moved in both VTA>NAC and SNm>DMS groups, but only VTA>NAC activation induced a pronounced hyperactive phenotype.* *(legend continues on next page)*

*E) Dose response curve. In the VTA>NAC group, total distance moved was significantly increased following all tested doses of CNO, 0.01 - 0.3 mg/kg. In the SNm>DMS group, only the highest dose increased total distance moved. F) DREADD expression in NAC Core and Shell, compared to CNO-induced changed in distance moved, shows distinction between DMS and NAC group. Error bars represent mean ± s.e.m. n=7-9/group. B: *P<0.05, **P<0.01, ***P<0.001 difference compared to zero; $P<0.05, $$P<0.01 difference between NAC and DMS groups. D-E: *P<0.05; **P<0.01; ***P<0.001 difference compared to saline; $P<0.05 difference compared to 0.3 mg/kg CNO.*

Neuronal populations targeted by DREADD in TH::Cre rats compared to DREADD-CAV2Cre combination

To compare the neuronal subpopulations that were targeted in experiment 2 and 3, we assessed the proportion of DREADD expressing (mCherry-IR) cells that was DAergic (TH-IR), as well as the proportion of DAergic neurons that expressed DREADD. In TH::Cre rats, 97% of all mCherry-IR cells were also TH-IR, compared to 79% in rats injected with CAV2Cre (P<0.0001 difference between groups, ***Table 1***). The proportion of DAergic cells expressing DREADD was also higher in TH::Cre rats compared to CAV2Cre: about 2/3 of TH-IR cells expressed DREADD, compared to 1/3 in CAV2Cre rats (P<0.0001, ***Table 1***). Thus, both the TH-specificity and the transduction efficiency for DAergic neurons was higher in TH::Cre rats compared to CAV2Cre-injected rats.

Table 1. Specificity and transduction efficiency of targeting dopaminergic cells with Cre-dependent DREADD in TH::Cre rats and with CAV2Cre. *Data represent total number of counted cells, collected from N=5 VTA:Dq+ rats, 4 SN:Gq+ rats, 3 VTA>NAC rats and 3 SNm>DMS rats. TH::Cre total: VTA:Dq+ plus SN:Dq+; CAV2Cre total: VTA>NAC plus SNm>DMS.*

	mCh-IR	TH-IR	**Specificity (% TH)**	TH-IR	mCh-IR	**Efficiency (% mCherry)**
TH::Cre VTA:Dq+	272	260	**95.6**	316	175	**55.4**
TH::Cre SN:Dq+	215	213	**99.1**	255	213	**83.5**
CAV2Cre NAC	274	228	**83.2**	261	106	**40.6**
CAV2Cre DMS	229	170	**74.2**	253	88	**34.8**
TH::Cre total	487	473	**97.1**	571	388	**68.0**
CAV2Cre total	503	398	**79.1**	514	194	**37.7**

Discussion

The present study is, to the best of our knowledge, the first to directly compare the effects of enhanced activity of DA neurons in the VTA and SN on locomotor behaviour, and we show that specifically the VTA DA neuron population is essential for inducing hyperactivity. Furthermore, we show that specifically activation of neuronal projections from VTA to NAC, but not to the DMS, induces hyperactivity in rats. These results are in line with earlier findings that chemogenetic activation of midbrain DA neurons in mice (Wang et al, 2013), or the VTA>NAC pathway in rats (Boender et al, 2014), induces a long-lasting increase in home cage locomotor activity. Our results complement these studies by showing that specifically increased neuronal activity of VTA to NAC pathway induces locomotor hyperactivity, whereas enhanced activity of midbrain neurons projecting to dorsomedial or dorsolateral striatum does not.

Previous pharmacological studies in rats and DA-deficient mice have also shown that psychostimulant-induced hyperactivity is dependent on the VTA>NAC DAergic pathway, rather than the nigrostriatal pathway (Carr and White, 1987; Creese and Iversen, 1974; Heusner et al, 2003; Kelly et al, 1975). In contrast, recent studies using optogenetic stimulation of VTA DA neurons found no effect on locomotor activity (Chaudhury et al., 2013; Gunaydin et al., 2014; Tye et al., 2013). These studies assessed short term novelty-induced locomotion in an open field, compared to long-term home cage locomotor activity in the present study, which may have affected the outcome. However, Wang et al. (2013) have shown that chemogenetic activation of midbrain DA neurons induced locomotor hyperactivity in the mice' home cage, as well as in an open field. This suggests that differences in behavioural setup (home cage versus novel chamber) are not the a major cause underlying different outcomes following chemogenetic compared to optogenetic stimulation of DA neurons. More importantly, although both techniques can be used to enhance neuronal activity, the actual physiological effect may be very different. It should be noted that, without direct experimental testing, one cannot directly compare the physiological effects of optogenetic versus chemogenetic stimulation. With regard to optogenetics, behavioural effects depend on the stimulation paradigm used. Due to the high temporal resolution, neuronal activity may be altered on a millisecond time scale. Depending on the time of stimulation, combined with the stimulation frequency, optogenetic activation of DA neurons may have varying effects on DA release. Indeed, it has been shown that low frequency optogenetic stimulation of VTA DA neurons was not sufficient to affect behaviour in rats, whereas high frequency stimulation was (Bass et al, 2010). Furthermore, short-term high frequency ("phasic") stimulation has been shown to affect behaviour differently compared to continuous low frequency ("tonic") stimulation (Chaudhury et al, 2013). Using DREADD technology,

CNO has a continuous effect on neuronal excitability. In this study, we showed that the effect of chemogenetic activation of VTA DA neurons on locomotor activity was dose-dependent, i.e., low doses of CNO resulted in submaximal increases in locomotor activity. Importantly, the dose response curve we observed in VTA:Dq+ and VTA>NAC groups is in line with predictions about in vivo DREADD functionality. In a recent paper, Roth suggested that, in the case of a high receptor reserve and low constitutive activity of the designer receptor, a maximal effect will be reached with a low dose of CNO (Roth, 2016). Indeed, we observed that 0.1, 0.3 and 1.0 mg/kg CNO all resulted in a maximal effect on locomotor hyperactivity. Only with very low doses, 0.03 mg/kg and lower, we observed an intermediate effect on locomotor activity. Furthermore, the time course of the behavioural effect also matches Roth's prediction – we found a significant increase in locomotor activity within 30 minutes following CNO administration, which lasted for at least 3.5 hours. These data support a high receptor reserve in our model, which therefore requires low doses of ligand for a maximal behaviour effect.

We found that chemogenetic activation of SN DA neurons, or midbrain neurons projecting to DMS, resulted in a modest increase in locomotor activity. This is in line with findings from DA-deficient mice, in which restoring the nigrostriatal DAergic pathway increased home cage locomotion approximately two-fold (Hnasko et al, 2006). Additionally, optogenetic stimulation of SN DA neurons in intact mice was shown to induce subtle movements (Barter et al, 2015). These results imply that DA neurons in both VTA and SN are involved in the regulation of locomotor activity, although there appears to be a dissociation in which aspects of locomotion are regulated by the different neuron populations. Our results, combined with literature, support a key role for VTA DA neurons in the regulation of hyperactivity, whilst SN DA neurons seem to be involved in other aspects of locomotion, such as motor coordination. Neurodegeneration of SN DA neurons, the core feature of Parkinson's disease neuropathology, is associated with deficits in motor control, including the initiation and switching of motor actions (Kalia and Lang, 2015; Matar et al, 2014; Smulders et al, 2015). Recordings from SN DA neurons in rodents have shown that firing activity increases during action initiation and switching (Jin and Costa, 2010), and encodes direction vectors for movement and posture (Barter et al, 2014, 2015). However, relatively little is known about the effects of enhanced SN DAergic activity on (motor) behaviour, and the current study is one of the first to determine these effects. Our results, together with those of Barter and colleagues (2015), suggest that either chemogenetic or optogenetic activation of SN DA neurons promotes locomotor actions. However, future studies are needed to further characterize which specific aspects of locomotor activity are affected by increased SNc DAergic activity.

In this study, we modulated midbrain neuronal activity by either targeting midbrain DAergic neurons in TH::Cre transgenic rats, or by targeting specific midbrain neuronal projections using CAV2Cre – both combined with a Cre-dependent DREADD virus. Our immunohistochemical results indicated that, with both approaches, we did not target completely distinct populations – i.e., all four experimental groups showed DREADD expression in the medial SN, as well as DMS. This overlap of expression patterns likely results from spreading of the virus, and from CAV2Cre possibly infecting the DMS via the injection needle tract towards the NAC. As such, we have not exclusively targeted VTA neurons projecting to NAC in these experiments. However, our results show that DREADD expression in VTA>NAC neurons was necessary in order to induce locomotor hyperactivity by CNO. When DREADD was only in expressed in more lateral regions of the midbrain (SN), projecting to more dorsal parts of the striatum, CNO merely caused a mild increase in locomotion. Although we cannot exclude that other neurotransmitters are involved, our data support a major role for DA in locomotor hyperactivity. Taken together, we provide evidence for a crucial role of DA neurons in the VTA that project to the NAC in inducing locomotor hyperactivity.

Our results suggest that hyperactivity as it is seen in psychiatric disorders may result from increased DAergic activity in the mesolimbic pathway, rather than the nigrostriatal pathway. For ADHD as well as schizophrenia, alterations in mesolimbic DA neuronal activity have been postulated (Furukawa et al, 2014; Howes and Nour, 2016; Tripp and Wickens, 2008). Thus far, these alterations have been primarily linked to deficits in reward processing and psychotic symptoms, respectively. Our results indicate that also hyperactivity symptoms may be related to aberrant VTA>NAC DA neuronal activity. However, this relationship needs to be validated within clinical populations. Inclusion of hyperactivity symptoms in the analysis of imaging studies could yield valuable information concerning the relationship between mesolimbic DA activity and functional outcome. We suggest that VTA>NAC DA neurons may be a promising target for future treatments in order to normalize hyperactivity symptoms, although further verifications are required.

In conclusion, we show that chemogenetic activation of VTA DA neurons, or VTA>NAC pathway, potently induces locomotor hyperactivity in rats. These results provide a fundamental basis for understanding the neuronal circuitry underlying hyperactivity, and may offer promising therapeutic targets for the treatment of hyperactivity. Future studies should address whether hyperactivity in a preclinical or clinical setting may be normalized by reducing VTA>NAC DA neuronal activity.

Author Disclosures

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Contributors

LB, AO, GvdP, and RAHA designed the study. LB, AO, MCML, IGW, and EW performed the experiments. LB and AO performed statistical analysis of results. LB, AO, GvdP and RAH wrote the manuscript. All authors have approved the final manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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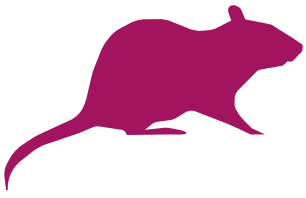
CHAPTER THREE

Hey ho, let's go!
- The Ramones, "Blitzkrieg bop"



ENHANCING EXCITABILITY OF DOPAMINE NEURONS PROMOTES MOTIVATIONAL BEHAVIOUR THROUGH INCREASED ACTION INITIATION

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Abstract

Motivational deficits are a key symptom in multiple psychiatric disorders, including major depressive disorder and schizophrenia. A likely neural substrate for these motivational deficits is the brain dopamine (DA) system. In particular the mesolimbic DA pathway, ascending from ventral tegmental area (VTA) towards the nucleus accumbens, has been identified as a crucial substrate for effort-related and activational aspects of motivation. However, the exact relationship between VTA DA neuronal activity and motivational behaviours is not fully understood. In this study, we used designer receptors exclusively activated by designer drugs (DREADD) in TH::Cre rats, in order to determine the effects of chemogenetic DA neuron activation on different aspects of motivational behaviour. We found that chemogenetic activation of DA neurons in the VTA, but not substantia nigra, significantly increased responding for sucrose under a progressive ratio schedule of reinforcement. This effect was dependent upon effort requirements and action-outcome contingencies, but was not affected by sucrose pre-feeding. Furthermore, increases in responding were characterized by enhanced initiation of reward-directed actions. Together, our findings indicate that enhanced activity of VTA DA neurons promotes the initiation of reward-seeking actions, thereby increasing motivational behaviour.

Keywords

Dopamine – DREADD – ventral tegmental area – motivation – action initiation

Introduction

Motivational processes regulate an animal's drive to overcome costs and effort in order to obtain a desired goal. Motivational deficits are a key symptom of multiple psychiatric disorders, including major depressive disorder and schizophrenia, and they strongly interfere with functional outcome and quality of life (Fervaha et al, 2015, 2016). In order to successfully treat motivational deficits, it is essential to understand the neurobiological substrates underlying distinct aspects of motivational behaviour. It has been hypothesized that alterations in dopamine (DA) neuronal activity may cause aberrant reward processing, resulting in the motivational deficits observed in psychiatric disorders (Maia and Frank, 2016; Whitton et al, 2015). Although the brain DA system is known to be crucially involved in regulating motivation (Berridge, 2007; Salamone and Correa, 2012; Wise, 2004), the exact relationship between midbrain DA neuronal activity and motivational behaviour remains incompletely understood.

DA signalling in the nucleus accumbens (NAc) has been identified as a crucial substrate for motivation (Nunes et al, 2013; Salamone and Correa, 2012). Blockade of NAc DA neurotransmission, through DA depletion or treatment with DA-receptor (DA-R) antagonists, has been shown to strongly diminish responding for rewards under a progressive ratio (PR) schedule of reinforcement (Aberman et al, 1998; Aberman and Salamone, 1999; Bari and Pierce, 2005). Conversely, enhanced NAc DA signalling, through DA transporter knockdown or acute D2-R overexpression, increased motivational behaviour in rodents (Peciña et al, 2003; Trifilieff et al, 2013). The mesolimbic DA pathway, from midbrain ventral tegmental area (VTA) DA neurons towards NAc, is thought to be particularly involved in effort-related and activational aspects of motivational behaviour (Salamone et al, 2016). However, it remains unknown to what extent these motivational aspects are regulated by VTA DA neuronal activity.

It has been suggested that, in addition to VTA, also midbrain DA neurons in the substantia nigra pars compacta (SNc) play an important role in motivation (Ikemoto et al, 2015; Palmiter, 2008). Both VTA and SNc DA neurons display reward prediction errors in response to presentation of a reward or reward-predicting cues (Barter et al, 2015; Schultz et al, 1997). Furthermore, mice have been shown to self-administer optogenetic stimulation of DA neurons in VTA as well as SNc, suggesting that activation of either of these neuronal populations is sufficient to induce reinforcement (Ilango et al, 2014; Rossi et al, 2013). We previously showed that enhanced activity of VTA mesolimbic neurons increased responding for sucrose under a PR schedule of reinforcement in rats (Boender et al, 2014; de Jong et al, 2015). However, direct evidence for a role of SNc DA neuron activity in motivation is lacking. Furthermore, it remains unknown which

aspects of behaviour are affected by VTA DA neuronal activity that drive the increase in motivation.

In the present study, we determined the effects of chemogenetic activation of midbrain DA neurons on motivational behaviour in rats, using designer receptors exclusively activated by designer drugs (DREADD) (Rogan and Roth, 2011). We selectively activated DA neurons in either the VTA or SNc, and determined the effects on responding for sucrose under a progressive ratio (PR) schedule of reinforcement. In order to elucidate which aspects of motivational behaviour were affected by enhanced DA neuronal activity, we analysed lever press patterns over the course of increasing ratio requirements, and tested whether the elevated responding was dependent on prior access to the reinforcer, presence of the reinforcer, or effort requirement. Together, we showed that selective chemogenetic activation of DA neurons in the VTA, but not SNc, increased motivational responding for palatable food. Furthermore, we provide evidence that enhanced VTA DA neuronal activity primarily drives motivational behaviour by promoting the repeated initiation of reward-seeking actions.

Experimental procedures

Subjects

In total, 49 male rats were used for the experiments. TH::Cre transgenic rats (Witten et al, 2011) were bred in-house, by crossing heterozygous TH::Cre$^{+/-}$ (Cre+) rats with wild type Long Evans mates. TH::Cre$^{-/-}$ (Cre-) littermates were used for control groups. Animals were socially housed in Macrolon type III or IV cages, with a wood block for cage enrichment. The rats had ad libitum access to regular chow and water in their home cage, and were kept on a reversed 12-hour light-dark schedule (lights off 7:00) to allow for behavioural testing in the dark phase. All experiments were performed in accordance with Dutch laws (Wet op Dierproeven, 1996) and European regulations (Guideline 86/609/EEC), and were approved by the Animal Experiments Committee of Utrecht University.

Surgical procedures

Rats were injected bilaterally with AAV2.5-DIO-hSyn-hM3Dq-mCherry (DIO-hM3Dq) (1.0–7.6*E12 molecules/ml) purchased from UNC Vector Core. Rats were allocated to one of three experimental groups: 1) VTA:Dq+: Cre+ rats injected with DIO-hM3Dq into the VTA; 2) SN:Dq+: Cre+ rats injected with DIO-hM3Dq into the SNc; and 3) Cre-: Cre- rats injected with DIO-hM3Dq into either the VTA or SNc (control group).

Prior to stereotactic surgery, rats were anesthetized by intramuscular fentanyl/fluanisone (0.315 mg/kg fentanyl, 10 mg/kg fluanisone, Hypnorm, Janssen

Pharmaceutica, Belgium). To minimize discomfort, xylocaine was sprayed on the skull for local anaesthesia (Lidocaine 100mg/ml, AstraZeneca BV, the Netherlands), and rats received subcutaneous injections of carprofen (5 mg/kg, Carporal, AST Farma BV, the Netherlands) on the day of surgery and the two days afterwards. Adult animals were housed individually for a week, to recover from surgery. Young animals (5 weeks old) were housed socially as soon as possible, typically from the day after surgery. There was a minimum of seven weeks in between surgery and behavioural testing.

Animals underwent surgery and were tested in four cohorts. Injection coordinates were adjusted to the rats' body weight. The first group (n=6 Cre+ + 6 Cre-, 13 weeks old, mean body weight 482 gram) received viral injections into the VTA: AP -5.8, ML +1.3 (5° angle), DV -8.4; 1.5 µl per hemisphere. The second group (n=7 Cre+ + 5 Cre-, 10 weeks old, mean body weight 327 gram) received injections into the SNc: AP -5.4, ML +2.2, DV -7.7; 1 µl per hemisphere. The third group (n=19, 5 weeks old, mean body weight 143 gram) was injected into either the VTA (n=6 Cre+ and 6 Cre-; AP -5.4, ML +1.1 (5° angle), DV -7.4), or SNc (n=6 Cre+; AP -5.2, ML +2.0 (5° angle), DV 7.0). The fourth group (n=8 Cre+, 11 weeks old, mean body weight 334 gram) received injections into the VTA (AP -5.6, ML +1.3 (5° angle), DV -8.2), 1 ul per hemisphere. All coordinates are in mm relative to Bregma.

Based on post-mortem detection of DREADD expression, two rats (one in the VTA:Dq+ group and one in the SN:Dq+ group) were excluded from behavioural analysis because of poor expression.

Drugs

The selective DREADD ligand clozapine-N-oxide (CNO; kindly provided by Bryan Roth (University of North Carolina, Chapel Hill NC, USA) and the NIMH Chemical Synthesis and Drug Supply Program) was dissolved in sterile saline (0.9% NaCl). For all experiments, 0.3 mg/kg CNO was used. This dose was based on previous work (Boender et al, 2014), and efficacy was verified for the present set-up. Dissolved CNO was kept at 4 ºC in between injections, for a maximum of four weeks. All injections were given intra-peritoneally (i.p.) at 1 ml per kg bodyweight.

Behavioral apparatus and testing

Rats were trained and tested in twelve sound-attenuated and ventilated operant conditioning chambers (30.5*24.22*1.0cm; MedAssociates, Georgia, VT, USA), equipped with two retractable levers, a cue light above each lever, and a food receptacle in between the levers. Following recovery from surgery, rats were habituated to the experimental set-up in a 60 minute shaping session, in which presentation of the cue light was

associated with delivery of a sucrose pellet (45 mg, TestDiet, USA). Subsequently, rats were trained for 30 minutes under fixed ratio 1 (FR1) schedule of reinforcement, during which a single lever press on the active lever (ALP) resulted in reward delivery. Presses on the inactive lever (ILP) were recorded, but had no programmed consequences. Completion of a ratio (i.e., pressing the active lever once) resulted in retraction of both levers, presentation of the cue light above the active lever, and delivery of a sucrose pellet into the food receptacle. Following a 20 second time-out (inter-trial interval), the cue light went off, and the levers were presented again. Inactive lever presses were recorded, but had no programmed consequences. Position of the active lever (left/right) was counter-balanced between animals. When rats had successfully acquired performance under the FR1 contingency, a PR schedule of reinforcement (first three cohorts) or FR5 schedule of reinforcement (fourth cohort) was introduced. Under the PR schedule, ratio requirements were progressively increased following each reward (n=1, 2, 4, 9, 12, 15, 20, 25, etc.) (Richardson and Roberts, 1996). PR sessions ended when the animal had not received a reward for 30 minutes. FR5 sessions lasted for 60 minutes. Primary readouts for instrumental performance were total number of ALP and ILP. Additionally, total number of rewards and breakpoint were assessed for performance under a PR schedule, which are directly related to the number of ALP. Furthermore, the percentage ILP was calculated (ILP/[ALP+ILP]*100%), in order to compare ILP across different levels of ALP.

When all rats showed stable performance under the PR or FR5 schedule (i.e., earning n±1 rewards on three consecutive days), the testing phase started. CNO or saline was administered 30 minutes before onset of the task, separated by at least 24 hours, and treatment order was counter-balanced between animals. To test the effects of reduced reward value on PR performance, outcome devaluation was established by allowing the rats to free-feed on sucrose pellets (identical to those earned in the instrumental task) for 60 minutes, in a separate cage, before starting the instrumental task as usual. Prior to testing, rats were habituated to the sucrose free-feeding set-up, to ensure they would consume the pellets. Before testing the effects of CNO treatment on instrumental performance in extinction, rats were exposed to three extinction training sessions, in order to minimize potential learning effects between testing days. PR task with reversed lever contingencies was tested in the third experimental cohort only. In this group, rats were re-trained on the PR schedule for three days following extinction, during which they reached their previous PR baseline performance. During the two reversal sessions, the active and inactive levers were reversed compared to all prior sessions. Thus, in order to receive a sucrose pellet, rats had to press the former inactive lever. The contingency was reversed only once, and did not change during the reversal sessions. To avoid learning

effects, all rats received CNO on the first day of reversal learning. On the following day, all rats were treated with saline.

To assess the effects of enhanced DA neuronal activity on lever press patterns during performance of the PR schedule, ALP were divided into separate bouts. A "bout" was defined as one or more lever presses, at least 2 seconds separated from adjacent bouts. This definition was based on the study of Ko and Wanat (2016). For each ratio requirement of the PR task, the following parameters were calculated: number of ALP bouts, average number of ALP within a bout (total ALP / number of bouts), bout interval (time between consecutive bouts), time to complete ratio, lever press rate (total ALP / time to complete ratio).

Statistical analysis

Statistical tests were performed in SPSS 16.0. Since behavioural effects of CNO treatment compared to saline did not differ between experimental cohorts, the data were pooled for analysis. Normal distribution of the data was assessed using Kolmogorov-Smirnov test and Q-Q plots. Number of ALP and ILP were transformed by natural log and square root, respectively, to allow for parametric testing. Repeated measures general linear model (RM-GLM) was used to test effects of Group (Cre-/VTA:Dq+/SN:Dq+) and Treatment (saline/CNO). Following a significant Group*Treatment interaction, post hoc tests were carried out to test treatment effects per group. For follow-up tests on the effects of VTA:Dq+ activation on behaviour, SN:Dq+ rats as well as SN:Dq- controls were excluded from the analysis, since no effects were observed on responding following chemogenetic activation of SNc DA neurons. Comparisons between groups (for reversal test) were made with Mann-Whitney U Test (ALP, ILP), or two sample t-test (%ALP). %ALP was compared to 50% test value with one-sample t-test. Statistical significance was set at $\alpha = 0.05$.

Results

Chemogenetic activation of DA neurons in VTA, but not SNc, increases responding for sucrose

Immunohistochemical analysis confirmed DREADD expression (hM3Dq-mCherry) in DA neurons in the VTA and SNc, in VTA:Dq+ and SN:Dq+ rats, respectively (***Fig 1A***). In absence of Cre (Cre- control group), no DREADD expression was observed (data not shown).

Responding for sucrose under a PR schedule of reinforcement was tested following treatment with CNO compared to saline. In the VTA:Dq+ group, CNO treatment significantly increased the number of ALP (***Fig 1B***; Group*Treatment interaction

$F_{3,34}$=11.841, P<0.0005; post hoc CNO vs Sal VTA:Dq+ P<0.0005). Consequently, CNO-treated VTA:Dq+ rats earned more rewards, and reached a higher breakpoint (***Fig 1D+E***; Group*Treatment interaction rewards $F_{3,34}$=11.428, breakpoint $F_{3,34}$=11.913, both P<0.0005; post hoc CNO vs saline VTA:Dq+ both P<0.0005). In contrast, CNO treatment had no effect on ALP in the SN:Dq+ group or in Cre- control animals (***Fig 1B***; post hoc SN:Dq+ P=0.09, VTA:Dq- P=0.952, SN:Dq- P=0.894). CNO also increased the number of inactive lever presses (ILP) in the VTA:Dq+ group, but not SN:Dq+ or Cre- groups (***Fig 1C;*** Group*Treatment interaction $F_{3,34}$=11.011, P<0.0005; post hoc CNO vs Sal VTA:Dq+ P<0.0005; SN:Dq+ P=0.437, VTA:Dq- P=0.712, SN:Dq- P=0.523). Relative to the total number of lever presses, the percentage ILP was increased with CNO treatment (Group*Treatment interaction $F_{3,34}$=3.212, P=0.035; post hoc CNO vs Sal VTA:Dq+ P=0.008; SN:Dq+ P=0.489, VTA:Dq- P=0.393, SN:Dq- P=0.498). Thus, chemogenetic activation of VTA DA neurons significantly increased responding for sucrose under a PR schedule of reinforcement, whilst activation of SNc DA neurons had no effect.

Because of the absence of behavioural effects of CNO treatment in SN:Dq+ rats, only VTA:Dq+ and VTA:Dq- groups were included in subsequent analyses.

Completion of high response ratios following VTA DA neuron activation is characterized by increasing ALP bouts

To determine which changes in behaviour underlie the CNO-induced increase in responding following VTA DA neuron activation, we analysed ALP patterns over the course of the PR session (***Fig 2***). We observed that completion of a higher ratio requirement was characterized by an increased number of ALP bouts (***Fig 2A***). Comparing the highest achieved ratio following CNO treatment versus saline in VTA:Dq+ rats, rats showed on average 40 ALP bouts with CNO treatment, significantly more compared to 15 ALP bouts under saline treatment conditions (***Fig 2B***, t_8=-5.768, P<0.0005). In contrast, the number of lever presses within a bout (***Fig 2C***) or the time between consecutive bouts (***Fig 2E***) were similar across ratios. Comparing these parameters during the final achieved ratio under CNO and saline treatment conditions, there was no difference between treatments (***Fig 2D+F***; ALP per bout t_8=0.208, P=0.84; interval t_8=0.495, P=0.634). Although the time to complete the higher achieved ratio was longer following CNO treatment (***Fig 2G-H***; t_8=-3048, P=0.016), the overall response rate did not differ between CNO and saline treatments (***Fig 2I-J***; t_8=-0.213, P=0.823). These results show that completion of high ratios following chemogenetic activation of VTA DA neurons was specifically characterised by an increase in the number of ALP bouts, rather than longer bouts or a faster rate of responding.

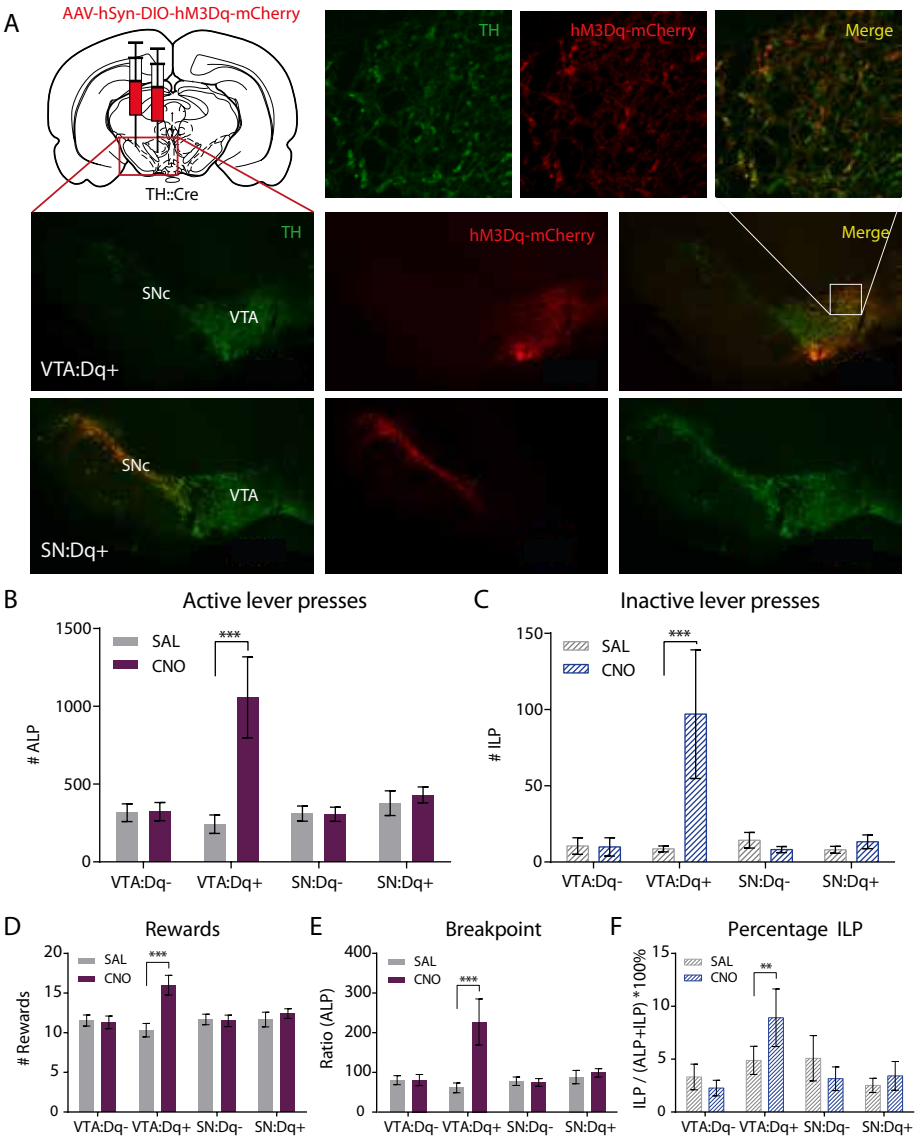
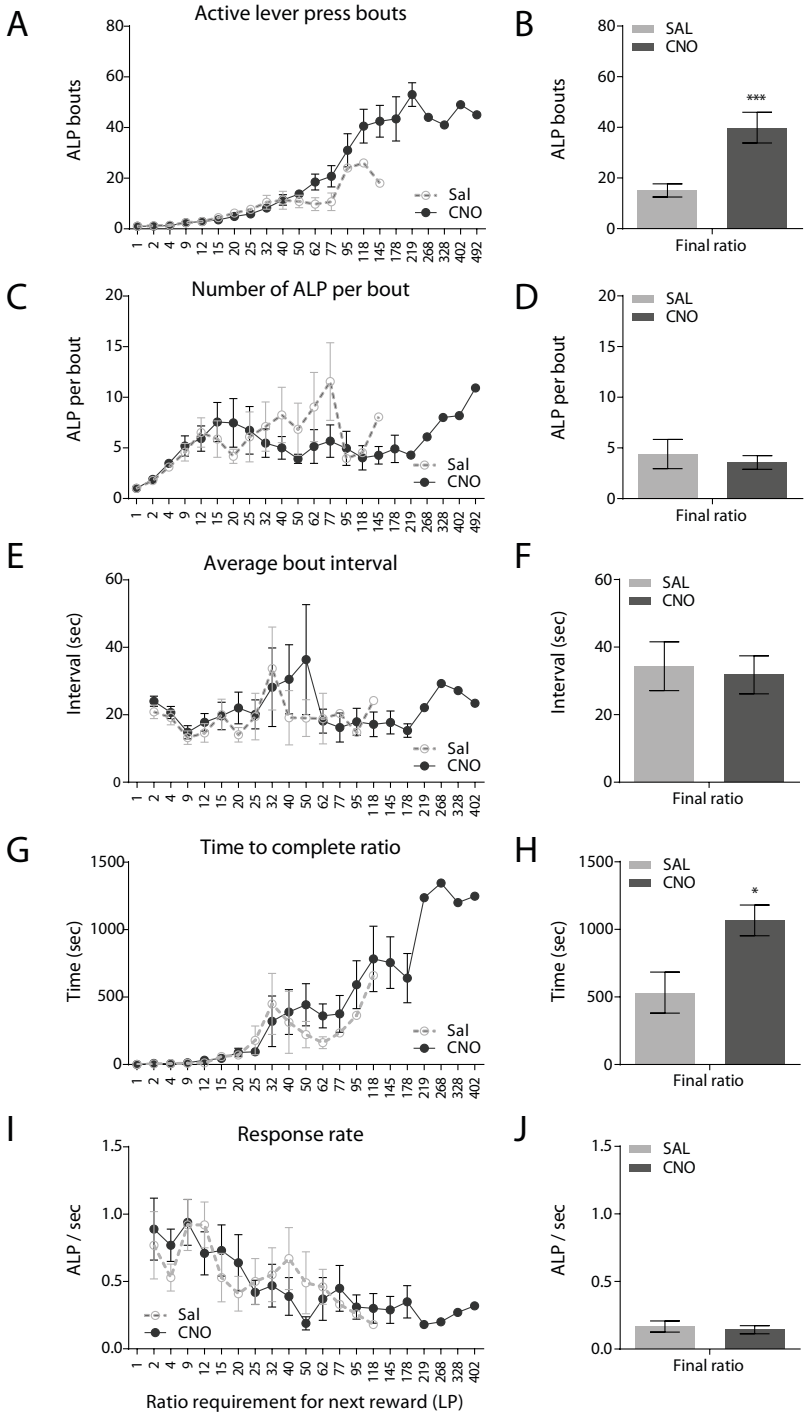


Figure 1. Chemogenetic activation of DA neurons in VTA, but not SNc, increases responding for sucrose under a PR schedule of reinforcement. *A) Expression of the excitatory DREADD (hM3Dq-mCherry) in DA (TH+) neurons in VTA or SNc. B-F) Effects of chemogenetic activation of DA neurons in VTA or SNc on responding for sucrose under PR schedule. In VTA:Dq+ rats, CNO treatment increased ALP (B), ILP (C), rewards (D), breakpoint (E), and percentage ILP (F). CNO treatment did not affect performance in SN:Dq+ rats or Cre- control groups (VTA:Dq- and SN:Dq-). Data are presented as mean ± s.e.m. **P<0.01, ***P<0.001 CNO compared to saline (SAL).*


A
Active lever press bouts
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F
SAL
CNO
Interval (sec)
Final ratio
G
Time to complete ratio
Time (sec)
Sal
CNO
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SAL
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Time (sec)
Final ratio
I
Response rate
ALP / sec
Sal
CNO
Ratio requirement for next reward (LP)
J
SAL
CNO
ALP / sec
Final ratio

Effect of VTA DA neuron activation on motivational behaviour is dependent on effort requirements, but is not affected by sucrose pre-feeding

In order to test which aspects of motivational behaviour were affected by chemogenetic activation of VTA DA neurons, we determined the effects on responding for sucrose following sucrose pre-feeding, absence of reinforcement, low effort requirements, and lever contingency reversal.

To test whether increased responding following VTA DA neuron activation is dependent on the animals' motivational state, we tested the effect of CNO on responding following sucrose pre-feeding. As predicted, pre-feeding diminished responding for sucrose under a PR schedule of reinforcement (***Fig 3A***; main effect of pre-feeding on ALP in saline condition, compared to baseline, $F_{1,19}$=55.986, P<0.0005; no effect on %ILP $F_{1,19}$=0.006, P=0.941, data not shown). CNO treatment increased ALP in the VTA:Dq+ group (***Fig 3A***; Group*Treatment interaction $F_{1,20}$=17.612, post hoc VTA:Dq+ P<0.0005, Cre- P=0.417), but had no effect on %ILP (Group*Treatment interaction $F_{1,20}$=2.390, P=0.138; data not shown). Interestingly, CNO-treated VTA:Dq+ rats performed as many ALP as they did in the PR task without pre-feeding (***Fig 3A***; paired samples t_8=1.124, P=0.26). Thus, enhanced ALP following VTA DA neuron activation was not affected despite sucrose pre-feeding.

As these results suggest that the incentive value of the reward did not affect instrumental responding, we tested responding in the absence of reward. In extinction, VTA DA neuron activation also enhanced instrumental responding. CNO treatment increased ALP in the VTA:Dq+ group (***Fig 3B***; Group*Treatment interaction $F_{1,20}$=11.301, P=0.003; post hoc CNO vs Sal VTA:Dq+ P=0.002, Cre- P=0.396), as well as ILP (Group*Treatment interaction $F_{1,20}$=16.489, P=0.001; VTA:Dq+ Sal: 13.1±2.4 vs CNO: 54±11.2 ILP, P<0.0005, Cre- P=0.654). The percentage of ILP was also increased following CNO treatment (***Fig 3C***; Group*Treatment interaction $F_{1,20}$=8.545, P=0.008; post hoc VTA:Dq+ P=0.001, Cre- P=0.96). Thus, in the absence of reward, VTA DA neuron activation increased instrumental responding.

Figure 2 (previous page). Effects of VTA DA neuron activation on lever press patterns under PR schedule. *A) Number of ALP bouts. B) Number of ALP per bout. C) Interval between ALP bouts. D) Time to complete ratio. E) ALP rate. Left panel shows patterns throughout progressively increasing ratios, right panel shows ALP pattern during highest ratio achieved. Data are presented as mean ± s.e.m. *P<0.05, ***P<0.001 CNO compared to saline (SAL).*

To test whether the increase in instrumental responding was dependent on the effort (i.e., ratio) requirement, we tested the effects of VTA DA neuron activation under a low effort, i.e., FR5, schedule. Furthermore, we assessed whether sucrose pre-feeding affected performance under an FR5 schedule. Similar to the results observed under the PR schedule, sucrose pre-feeding diminished ALP (***Fig 3D***; main effect of Pre-feeding $F_{1,7}$=39.425, P<0.0005). However, CNO treatment did not affect ALP, irrespective of pre-feeding conditions (***Fig 3D***; main effect of Treatment $F_{1,7}$=0.191, P=0.675; Pre-feeding*Treatment interaction $F_{1,7}$=5.302, P=0.055). The percentage ILP was not affected by either pre-feeding or CNO treatment (Pre-feeding $F_{1,7}$=4.240, P=0.078; Treatment $F_{1,7}$=4.865, P=0.063; Pre-feeding*Treatment interaction $F_{1,7}$=1.228, P=0.304; data not shown). The difference between PR and FR5 schedules could not be attributed to differences in task duration. When PR performance was limited to 60 minutes (similar to the FR5 schedule), CNO treatment increased ALP at baseline and following sucrose pre-feeding (ALP baseline Sal: 235±65 vs CNO: 576±161, P=0.003; pre-fed Sal: 63±11 vs CNO: 427±145, P=0.004), whilst there was no difference between baseline and pre-fed ALP following CNO treatment (P=0.265). Thus, at low ratio requirements, chemogenetic activation of VTA DA neurons did not affect instrumental performance, and did not alter sensitivity to reduction in incentive value.

Finally, to test whether responding following VTA DA neuron activation was flexible and goal-directed, we tested performance under a PR schedule following reversal of the active and inactive lever. In this session, CNO-treated VTA:Dq+ rats were compared to CNO-treated Cre- controls (both treated with CNO), in order to circumvent learning effects over multiple sessions. In this reversal session, the VTA:Dq+ group showed increased ALP compared to the Cre- control group (***Fig 3E*** day 1; VTA:Dq+ vs Cre- Z= -2.562, P=0.009), but no difference in ILP (***Fig 3F***; VTA:Dq+ vs Cre- Z=1.095, P=0.195). The VTA:Dq+ rats pressed the (new) active lever more often than the (new) inactive one, whilst Cre- rats showed a preference for the inactive lever (i.e., the former active lever) (***Fig 3F***; %ALP VTA:Dq+ vs Cre- $t_{6.14}$=4.281, P=0.005; VTA:Dq+ %ALP >50% t_4=5.47, P=0.005, Cre- %ALP <50% t_5= -2.65, P=0.045). The preference for the (new) active lever did not persist in VTA:Dq+ rats during a second session, when animals were treated with saline. On the second day, both groups showed no preference for either lever (***Fig 3F*** day 2; %ALP VTA:Dq+ vs Cre- t_9=0.678, P=0.515; %ALP vs 50% VTA:Dq+ t_4=1.493, P=0.21, Cre- t_5=0.125, P=0.905), and there was no difference in the number of lever presses between the groups (***Fig 3E***; VTA:Dq+ vs Cre- ALP Z=-0.274, P=0.829; ILP Z=-1.905, P=0.329). Thus, VTA DA neuron activation increased responding under a PR schedule for sucrose, in a manner that is sensitive to changes in action-outcome contingencies.

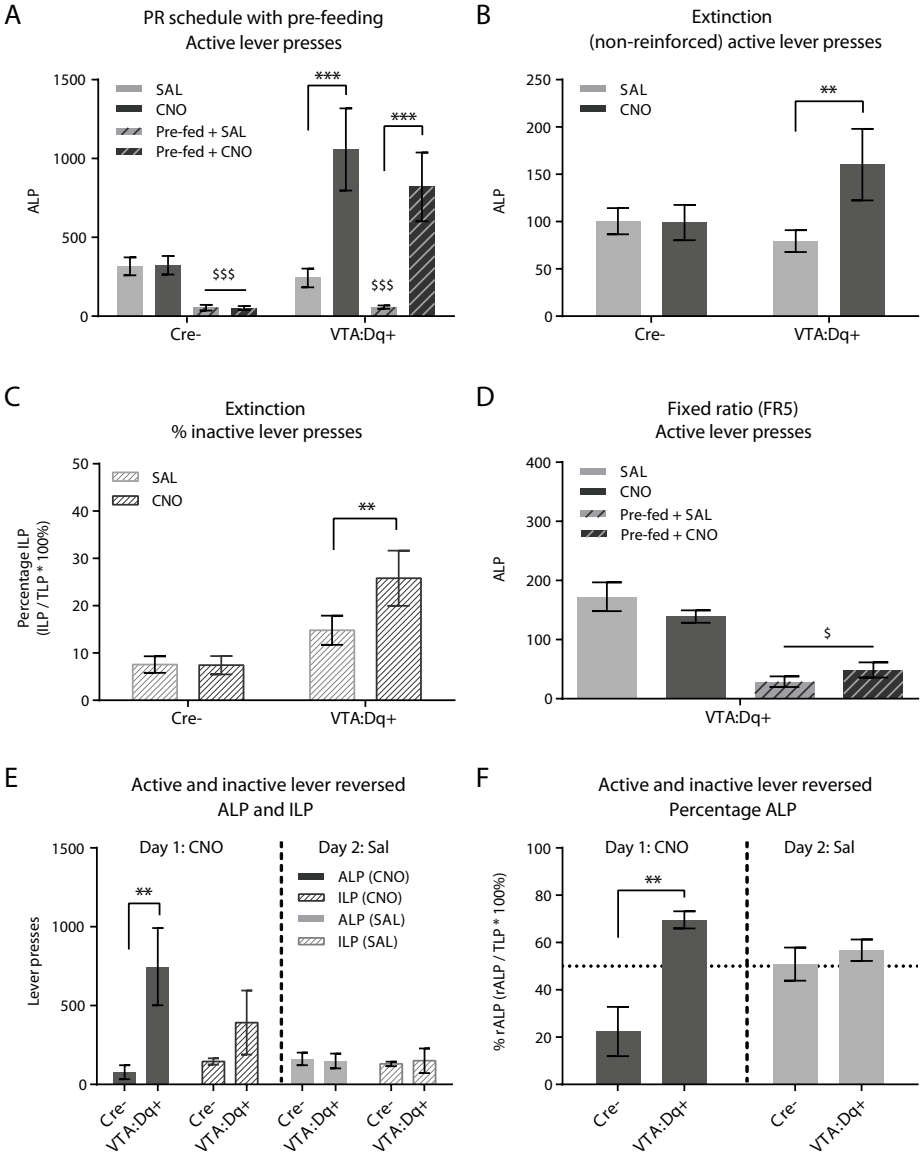

Figure 3. Effects of chemogenetic activation of VTA DA neurons on distinct aspects of motivation. *A) Sucrose pre-feeding decreased ALP under the PR schedule, whilst CNO treatment increased ALP in VTA:Dq+ group. B) CNO treatment increased ALP in extinction in VTA:Dq+ group. C) CNO treatment increased %ILP in extinction in VTA:Dq+ group. D) Under an FR5 schedule, pre-feeding decreased ALP, but CNO treatment did not affect performance in VTA:Dq+ group. E) CNO increased reversed ALP, but not ILP in VTA:Dq+ group compared to Cre- control group.* *(legend continues on next page)*

*F) Percentage of ALP was higher in CNO-treated VTA:Dq+ group compared to Cre- control group. On the second day, following SAL treatment, performance did not differ between groups. Data are presented as mean ± s.e.m. **P<0.01, ***P<0.001 CNO vs Sal; $P<0.05, $$$P<0.001 main effect of pre-feeding.*

Above, we showed that VTA DA neuron activation resulted in the completion of higher ratios under a PR schedule of reinforcement, which was characterized by an increased number of ALP bouts (***Fig 2A-B***). To determine whether this was a robust effect of chemogenetic activation of VTA DA neurons, we analysed ALP patterns under PR schedules following sucrose pre-feeding and following lever reversal. We observed that, after sucrose pre-feeding, rats performed on average three ALP bouts during the highest achieved ratio with saline treatment. Following CNO treatment, this was increased to 21 ALP bouts (***Fig 4A***, saline t_8=-4.817, P=0.001). In the reversal session, CNO-treated VTA:Dq+ rats performed on average 45 ALP bouts during the highest achieved ratio, compared to 11 ALP bouts in the CNO-treated Cre- control group, showing a non-significant trend towards a similar effect (***Fig 4B***; Z=-1.776, P=0.095). Other parameters of responding did not differ between the two groups (all P>0.1). Thus, enhanced responding for sucrose under a PR schedule following VTA DA neuron activation was repeatedly found to be characterized by an increase in ALP bouts.

Discussion

In this study, we determined the effects of chemogenetic DA neuron activation on motivational behaviour. Previously, we have shown that chemogenetic activation of the mesolimbic DA pathway increased the motivation for sucrose in rats (Boender et al, 2014). Here, we expand these findings by unravelling which aspects of motivational behaviour were affected by increased DA neuronal activity. We found that chemogenetic activation of DA neurons in the VTA, but not SNc, robustly increased responding for sucrose under a PR schedule of reinforcement in rats. Furthermore, the completion of high response ratios was characterized by an increased number of ALP bouts, but not increased bout length or response rate. Together, this suggests that VTA DA neuron activation promotes the initiation of reward-seeking actions, resulting in enhanced exertion of effort.

Enhanced incentive motivation following VTA DA neuron activation depends on effort, but not prior access to reward

We found that activation of VTA DA neurons robustly increased instrumental responding. This was observed under a PR schedule of reinforcement, even after pre-feeding, but not under a low effort (FR5) schedule. This indicates that the effects of increased VTA DA neuronal activity on behaviour depend on the level of required effort. This is consistent

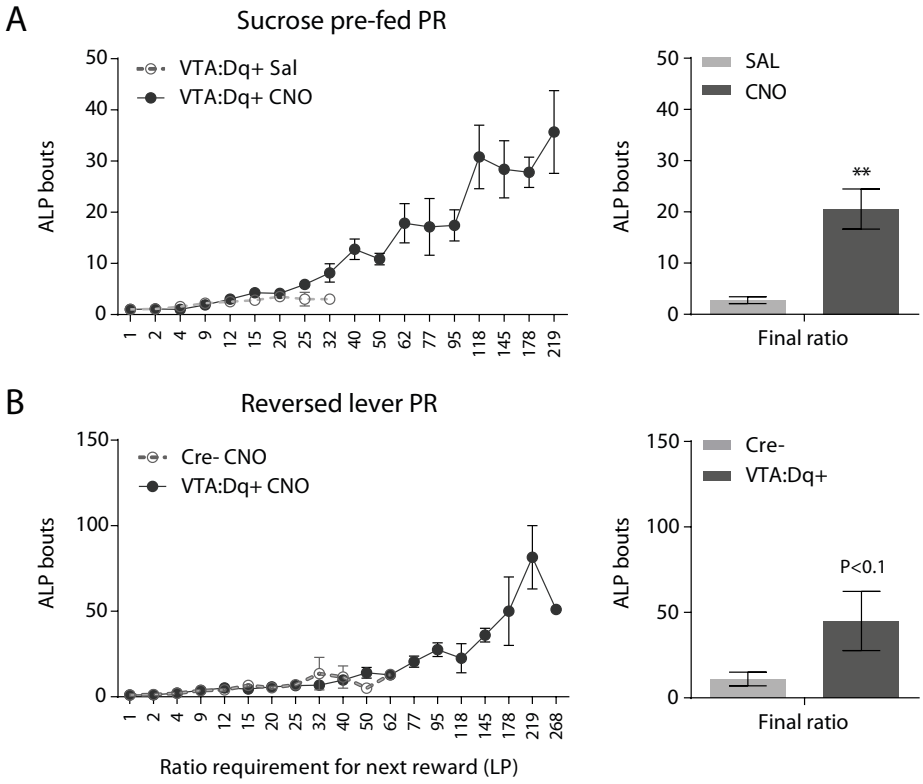

Figure 4. Effects of VTA DA neuron activation on ALP bouts under a PR schedule of reinforcement, following pre-feeding or lever reversal. *A) Number of ALP bouts following sucrose pre-feeding in VTA:Dq+ rats, CNO treatment compared to saline. B) Number of ALP bouts following reversal of lever contingencies. VTA:Dq+ group compared to Cre- group. **P<0.01 CNO compared to Sal.*

with previous studies, showing that NAc DA depletion selectively impaired instrumental performance when ratio requirements were high, whilst leaving performance at low ratio schedules unaffected (Aberman and Salamone, 1999; Ishiwari et al, 2004; Salamone et al, 2001). Based on these findings, it was proposed that mesolimbic DA primarily affects sensitivity to increases in work load (Aberman and Salamone, 1999; Salamone et al, 2001). Our result support this hypothesis, showing that enhanced VTA DA neuronal activity promotes reward-directed behaviour despite high response costs.

Interestingly, we observed that increased responding induced by VTA DA neuron activation was not affected by sucrose pre-feeding. Whilst pre-feeding potently reduced

motivation to lever press for sucrose under saline treatment conditions, this effect was no longer observed following chemogenetic activation of VTA DA neurons. This could suggest that elevated DA signalling counteracted the reduced incentive reward value of sucrose. However, at low ratio requirements, i.e., under an FR5 schedule, VTA DA neuron activation did affect the reduced responding for sucrose following pre-feeding. This indicates that enhanced VTA DA neuron activity did not generally affect the incentive value of the reward, but rather increased activational aspects of motivation, that are particularly taxed under high effort requirements (Berridge, 2007; Salamone and Correa, 2012). Our findings also suggest that the post-ingestive effects of sucrose consumption, which reduces motivation for sucrose under saline treatment conditions, did no longer impact on the willingness to perform effort for the reward when DA neurons were activated. This supports the notion that homeostatic state and mesolimbic DA activity differentially affect motivational behaviour.

Enhanced action initiation drives increased motivational behaviour

Analysis of lever press activity over the course of progressively increasing ratio requirements, revealed that completion of high response ratios following VTA DA neuron activation was associated with an increase in ALP bouts. This effect was consistently observed during responding under a PR schedule, also following sucrose pre-feeding or lever reversal (although the latter effect failed to reach statistical significance). Thus, as ratio requirements increased over the course of the task, animals performed an increased number of ALP bouts, whilst the number of presses within a bout or the interval between bouts remained the same. This suggests that VTA DA neuronal activation drives motivational behaviour by enhancing the initiation of reward-seeking actions, even when these actions are not immediately reinforced. Indeed, a selective effect on the willingness to repeatedly engage in goal-directed behaviour, in the anticipation of a reward, also explains why VTA DA neuronal activation did not affect responding at low ratio requirements, such as under an FR5 schedule.

Recently, it was shown that transient increases in NAc DA levels – representing phasic activity of VTA DA neurons – were associated with action initiations during the performance under a PR schedule in rats (Ko and Wanat, 2016). Although this study also found relationships between DA transients and the number of presses per bout and interval between bouts, our results suggest that these aspects of responding were not directly affected by increased VTA DA neuronal excitability. Together, these findings support a vital role for mesolimbic DA activity in the initiation of reward-anticipated actions (Hamid et al, 2015; Nicola, 2010; Syed et al, 2015), and provide a neural mechanism for altered sensitivity to work load.

VTA DA neuron activation promotes flexible reward-seeking

In addition to more responding on the ALP, VTA DA neuron activation enhanced ILP under a PR schedule of reinforcement. Also in extinction, rats showed an increased in ILP. We hypothesize that this represents enhanced reward-seeking in a situation when the anticipated reward is not delivered. Previously, we showed that chemogenetic activation of VTA to NAc pathway increased locomotor activity (Boender et al, 2014) (see also *Chapter 2*), indicating increased behavioural activation and enhanced responsivity to stimuli (Robbins and Everitt, 2007). However, in the present study, we observed that rats could adaptively adjust their behaviour following VTA DA neuron activation, and switch their lever press preference towards the former inactive lever when action-outcome contingencies were reversed. This suggest that instrumental responding was directed towards obtaining the sucrose reward. This flexible reward-seeking behaviour may reflect a strategy shift towards ILP when ALP are no longer rewarded, e.g., at high ratio requirements, in extinction, or following lever reversal.

No evidence for effects of SNc DA neuron activation on motivational behaviour

Although it has been suggested that nigrostriatal DA may be involved in motivational processes as well (Ikemoto et al, 2015; Palmiter, 2008), we found no effects of chemogenetic activation of SNc DA neurons on responding for sucrose under a PR schedule of reinforcement. To our knowledge, we are the first to directly test the effects of increased activity of SNc DA neurons on incentive motivation. A number of studies has investigated the effects of diminished DA signalling in the dorsal striatum (the target site of SNc DA neurons). These studies found that neither lesions of the dorsal striatum, nor D2-R overexpression, affected motivation to respond for sucrose in rodents (Eagle et al, 1999; Trifilieff et al, 2013). Pharmacological blockade of DA-R in the dorsal striatum has been shown to dose-dependently decrease motivation for cocaine in rats, but the effects on motivational for food were not assessed in that study (Veeneman et al, 2012). Together, these data suggest that SNc DA neuronal activity is not directly involved in the regulation of motivation for palatable food, although future studies are needed to substantiate this.

Relevance to motivational deficits in psychiatry

Mesolimbic DA signalling has been identified as a key regulator underlying motivational deficits in psychiatric disorders (Whitton et al, 2015). Major depressive disorder is characterized by a blunted DA signal in NAc during reward anticipation, which disrupts motivational processes (Pizzagalli et al, 2009). It is hypothesized that this attenuated mesolimbic DA response may be caused by reduced phasic VTA DA neuronal activity

(Whitton et al, 2015). Also, in schizophrenia, aberrant DA neuronal activity has been proposed to account for inappropriate salience processing and motivational deficits (Howes and Kapur, 2009; Maia and Frank, 2016; Winton-Brown et al, 2014). At present, it remains unknown which specific alterations in DA signalling are related to distinct aspects of motivational deficits in psychiatric disorders. Preclinical studies may provide novel insights into the (DAergic) mechanisms underlying motivation, and thereby aid in the development of targeted treatments for disorders in which motivational processes are disrupted. Our findings indicate that enhanced activity of VTA DA neurons primarily affects reward-anticipated action initiation, specifically when a high level of effort is required. Since certain motivational deficits are characterized by impaired goal-directed behaviour and diminished ability to initiate actions in the anticipation of future rewards (Chaudhuri and Behan, 2004; Salamone et al, 2016), enhanced VTA DA neuronal excitability might be a promising approach to relieve these symptoms. Recently, it was shown that enhanced DAergic function could relieve motivational deficits in a rodent model (Soares-Cunha et al, 2016). However, in schizophrenia, enhanced DA neuronal activity is likely to exacerbate psychotic symptoms (Grace, 2015; Howes and Kapur, 2009; Maia and Frank, 2016). Thus, future studies are needed to elucidate which treatment approach is most appropriate for different types of motivational deficits.

Conclusions

In this study, we show that chemogenetic activation of VTA DA neurons robustly increased motivational responding for sucrose in rats. Our results indicate that enhanced activity of VTA DA neurons promotes reward-directed action initiations, and thereby facilitates the exertion of effort. This is in line with previous findings, indicating that mesolimbic DA signalling is necessary for high effort performance and goal-directed actions (Hart et al, 2014; Nunes et al, 2013). In line with a role in activational (rather than directional) aspects of motivation (Salamone et al, 2016), we found that responding at both the active and inactive levers was promoted in the absence of immediate reinforcement, and that enhanced effort exertion was particularly characterised by a repeated initiation of responding. Together, our findings emphasize that mesolimbic DA neuronal activity acts as a mediator that translates incentive motivation into physical actions (Balleine and O'Doherty, 2010). These findings may have implications for the development of novel treatments for motivational deficits in psychiatry.

Author Disclosures

Role of Funding Source

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Contributors

LB, JWdJ, GvdP and RAHA designed the studies. LB, ECW, JHKM, and MCML performed the experimental procedures. LB performed statistical analysis of the results. LB, GvdP, LJMJV and RAHA wrote the manuscript. All authors have approved the final manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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CHAPTER FOUR

Just a little bit (hey baby), just a little bit
- Aretha Franklin, "R.E.S.P.E.C.T."

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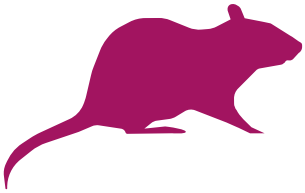
DOES ACTIVATION OF MIDBRAIN DOPAMINE NEURONS PROMOTE OR REDUCE FEEDING?



Abstract

Background: Much debate remains about the role of dopamine (DA) neuronal activity in the control of food intake. Enhanced DA signalling is associated with food intake and enhances motivation for food, whilst pharmacological DA reuptake inhibition reduces appetite and food intake. One unresolved question is whether an increase in DA neuronal activity either stimulates or inhibits feeding. Secondly, the respective roles of DA neurons in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) are incompletely understood.

Methods: In this study, we used designer receptors exclusively activated by designer drugs (DREADD) technology to determine the effects of enhanced DA neuronal activity on feeding behaviour. We chemogenetically activated selective midbrain DA neuronal subpopulations and neuronal pathways, and assessed the effect on feeding microstructure in rats.

Results: Consistent with earlier studies, we observed anorexic effects following pharmacological DA stimulation. Treatment with amphetamine or selective DA reuptake inhibitor GBR 12909 delayed feeding latency, reduced meal size and duration, and suppressed total feeding time and total food intake. We found that enhanced excitability of DA neurons in the VTA reproduced some, but not all, of these effects. Specifically chemogenetic activation of the mesolimbic pathway from VTA towards nucleus accumbens (NAc) resulted in smaller and shorter meals, as well as an increased feeding frequency, whilst total food intake was not affected. Chemogenetic activation of SNc DA neurons or VTA projections towards prefrontal cortex or amygdala did not affect feeding. In addition to disrupting feeding behaviour, chemogenetic activation of VTA DA neurons or VTA to NAc pathway induced locomotor hyperactivity.

Conclusions: Together, our results indicate that enhanced mesolimbic DA neuronal activity affects feeding behaviour. However, rather than a general increase or decrease in food intake, DA neuronal activity seems to mediate the initiation and cessation of feeding.

Introduction

Dopamine (DA) signalling in the brain is necessary for food intake. DA-deficient mice fail to eat, and die of starvation without additional treatments (Zhou and Palmiter, 1995). However, the role of DA in the control of food intake remains poorly understood. Eating disorders, including obesity, have been associated with alterations in the DA system. Obese individuals were found to have decreased DA D2 receptor (D2-R) expression, inversely correlating with body mass index (BMI) (Wang et al, 2001). Furthermore, drugs that block DA receptors (DA-R), such as antipsychotics, induce weight gain and increase risk for obesity (American Diabetes Association et al, 2004; Nielsen et al, 2016). Together, these findings suggest that DA is an important regulator of feeding behaviour. Furthermore, the rewarding properties of food and are mediated via the DA system (Wise, 2006), indicating a crucial role for DA in overconsumption. However, the causal relationship between DA neuronal activity and food intake remains elusive.

One unresolved question is whether activation of DA neurons either stimulates or inhibits feeding. On the one hand, psychostimulant drugs that enhance DA signalling, such as amphetamine and methylphenidate, reduce appetite and food intake (Davis et al, 2012; Foltin and Fischman, 1989; Janhunen et al, 2013; Leibowitz et al, 1986). On the other hand, food intake induces DA release in the striatum, associated with the rewarding properties of food (Brown et al, 2011; Small et al, 2003). Furthermore, mesolimbic DA is crucially involved in the motivation to work for food (Berridge, 2007; Salamone and Correa, 2012; Wise, 2004).

According to the "DA hypothesis", DA neurons in the ventral tegmental area (VTA) are activated in a state of negative energy balance, in order to promote engagement in feeding behaviour (Palmiter, 2007). VTA DA neurons can be activated (either directly or indirectly) via inputs from the lateral hypothalamus (LH), or by peripheral feeding hormones (Meye and Adan, 2014). Feeding hormones, including leptin and ghrelin, affect DA neuronal activity, as well as food intake (DiLeone, 2009; Perello and Dickson, 2015; van Zessen et al, 2012). The behavioural effects of VTA DA neuron activity are likely mediated through mesolimbic DA projections towards to the nucleus accumbens (NAc), which is the main output centre of VTA DA neurons, and has a major role in action selection, approach initiation, and motivational output (Berridge, 2007; Nicola, 2007, 2010; Robbins and Everitt, 2007).

Importantly, in order to determine the role of DA neuronal activity in the control of food intake, it is essential to directly target DA neurons. Pharmacological or genetic manipulations of post-synaptic DA signalling may obscure the physiological effects of

phasic and tonic DA release, and may therefore affect behaviour differently compared to DA neuron activation by endogenous excitatory inputs. As such, it is unclear to what extent the effects of post-synaptic stimulation of DA signalling reflect physiological regulation of feeding (Palmiter, 2007).

A second outstanding issue is the respective role of midbrain DA neurons in the VTA compared to substantia nigra pars compacta (SNc). With regard to DA's role in feeding, the VTA has received most attention (Berridge, 2009; McCutcheon, 2015; Meye and Adan, 2014; Narayanan et al, 2010; van Zessen et al, 2012). However, receptors for feeding hormones are present in both VTA and SNc (Figlewicz et al, 2003), and changes in homeostatic and motivational state were shown to affect VTA and SNc DA neuronal activity (van der Plasse et al, 2015; Rossi et al, 2013). Importantly, studies in DA-deficient mice have shown that selective restoration of DA signalling in the nigrostriatal pathway (from SNc towards dorsal striatum) was sufficient to rescue, and even enhance, normal feeding behaviour, whilst restoring DA in the NAc was not (Hnasko et al, 2006; Szczypka et al, 2001). This indicates that particularly SNc DAergic activity is necessary for feeding behaviour. Together, these findings suggest that DA neurons in the SNc may play an important role in the control of food intake (Narayanan et al, 2010; Palmiter, 2008). However, the direct effects of enhancing DA neuronal activity in VTA or SNc on feeding behaviour remain unknown.

In this study, we took a novel approach to determine whether enhancing endogenous activity of midbrain DA neurons directly affects feeding behaviour. First, we confirmed that pharmacological DA reuptake inhibition suppressed food intake. Then, we tested the effects of enhanced DA neuronal activity in either the VTA or SNc on food intake and feeding microstructure, using designer receptor exclusively activated by designer drugs (DREADD) in TH::Cre rats. To identify which pathways underlay the observed changes in feeding, we selectively activated distinct midbrain neuronal pathways. In addition, we analysed locomotor activity during the feeding episode. Together, this study provides new insights into how enhanced DA neuronal activity influences feeding behaviour.

Materials and Methods

All experiments were carried out in accordance with Dutch and international laws (Wet op de Dierproeven, 1996) and European regulations (Guideline 86/609/EEC), and were approved by the Animal Ethics Committee of Utrecht University. Experiments were performed as described in *Chapter 2.*

Subjects and surgical procedure

TH::Cre rats (Witten et al, 2011) were bred in-house, by crossing heterozygous Cre$^{+/-}$ rats with wild type Long Evans mates. Thirty male rats were injected with Cre-dependent DREADD virus AAV2.5-hSyn-DIO-hM3Dq-mCherry (1 µl bilaterally, 6.4–8.0*E12 virus molecules/ml; UNC Vector Core) into either the VTA or SNc. For each region, 15 rats were injected: 8 Cre+ rats (VTA:Dq+ and SN:Dq+), and 7 Cre- littermates serving as control groups (VTA:Dq- and SN:Dq-). Stereotactic coordinates were adjusted according to the animals' body weight (all coordinates in mm relative to Bregma), and were set at AP -5.2; ML +1.1 (5º angle); DV -7.4 for VTA (rats 7 weeks old, mean body weight 156 gram), and AP -5.4; ML +2.2; DV -7.7 for SNc (adult rats, mean body weight 337 gram).

To allow for chemogenetic activation of selective pathways (Boender et al, 2014), 32 male Wistar rats (Crl:WU, Charles River, Sulzfeld, Germany) were injected bilaterally with the same DREADD virus (1 µl, 1.0*E12 virus molecules/ml) into the VTA (AP -5.4; ML +2.2 [10º angle]; DV -8.9; adult rats, mean body weight 325 gram). In addition, canine adenovirus expressing Cre recombinase (CAV2Cre; 1 µl, 1.25*E12 virus molecules/ml; IGMM, France) was infused into one of three VTA projection sites: NAc (VTA>NAc, n=11, AP +1.2; ML +2.8 [10º angle]; DV -7.5), prefrontal cortex (PFC) (VTA>PFC, n=10, AP +2.7; ML +1.4 [10º angle]; DV -4.9), or amygdala (VTA>Amy, n=11, AP -2.2; ML +5.0 [0º angle]; DV -9.2). Anaesthesia and peri-operative care for both experiments was carried out as described in *Chapter 2*.

Behavioural testing

All behavioural tests were performed in adult male rats, at least four weeks following viral infusion. Rats were housed individually in 16 PhenoTyper© home cages (Noldus IT, Wageningen, The Netherlands), 43x43x90cm, equipped with infrared cameras in the top, and an automated weighing system (Tiesjema et al, 2007). Rats were mildly food restricted during the light phase, in order to ensure similar homeostatic states across multiple testing days. Chow was removed from the cage at 10:00, and returned at 16:00, at the start of the dark phase (Janhunen et al, 2011).

All tests were performed using a counter-balanced within-subjects design. Drugs were administered at 15:30, 30 minutes before access to chow. Subsequent injections were separated by at least 24 hours. Effects of amphetamine and GBR 12909 were tested in the TH::Cre rats, with Cre+ and Cre- animals pooled. Each dose was counterbalanced against a vehicle treatment. Dose-response testing for clozapine-N-oxide (CNO) was performed using a Latin-squared design, with 48 hours in between injections. Food restriction continued during wash-out days.

Drugs

All drugs were administered intra-peritoneally (i.p.) at a volume of 0.1 ml per 100 gram body weight. The selective DREADD ligand CNO (kindly provided by Bryan Roth [University of North Carolina, Chapel Hill NC, USA] and the NIMH Chemical Synthesis and Drug Supply Program; dose 0.03 – 1.0 mg/kg) and amphetamine (*d*-amphetamine sulphate; OPG Utrecht, the Netherlands; dose 0.3 and 1.0 mg/kg) were dissolved in sterile saline (0.9% NaCl). Selective DA reuptake inhibitor GBR 12909 (GBR 12909 dihydrochloride, Sigma-Aldrich; 1.0, 3.0, and 10 mg/kg) was dissolved in MQ water.

Tissue preparation and immunohistochemical analysis

Tissue preparation and immunohistochemistry were performed as previously described in ***Chapter 2***. In brief, rats received a lethal dose of sodium pentobarbital (TH::Cre groups), or isoflurane (CAV2Cre groups), prior to transcardial perfusion with 0.9% NaCl, and 4% paraformaldehyde in phosphate buffered saline (PBS). Sucrose-saturated brains were sliced at 40 µm. Presence of hM3Dq-mCherry and TH were visualized using primary antibodies Rabbit anti-dsRed (Clontech) and Mouse anti-TH (Millipore), and secondary antibodies Goat anti-Rabbit Alexa 568 (Abcam) and Goat anti-mouse Alexa 488 (Abcam), respectively. Fluorescent pictures of mounted brain slices (5x magnification) were taken with a Zeiss Axioscope A1 microscope and Axiovision software to analyse expression patterns. Confocal pictures (20x magnification) were taken with a Olympus Fluoview 1000 microscope and FluoView software. Images were processed with ImageJ.

Data analysis

No animals were excluded from analysis based on DREADD expression. Due to a defective feeder scale, one rat in the VTA>PFC group was excluded from the analysis.

Feeding data were collected every 12 sec, and analysed using a custom-made macro in Visual Basics, Microsoft Excel. Definition for a "meal" was set at at least 0.3 gram (equivalent to 1 kCal), and at least 5 min separated from another meal. Food intake was analysed for the first two hours of food access, as this was shown to produce robust results within animals over days, and drugs were physiologically active during this period. The following parameters were calculated: total food intake (gram), total number of meals, average meal size (total intake / number of meals), total time spent feeding (minutes), and average meal duration (total time spent feeding / number of meals). For the first meal, the latency to start (min), size (g), and meal interval (time between first and second meal) were measured.

Home cage locomotor activity was analysed with EthoVision XT9 and XT11, as described in Chapter 2, during the first two hours of food access. For analysis of activity bouts, continuous activity was recorded, and threshold was set at 3% for ambulatory movement ("active state").

Statistical analyses were performed in SPSS 16.0. Food intake, meal size, and feeding duration were analysed with parametric tests, following a natural log transformation. Non-parametric tests were used for number of meals and latency to start the first meal. Amphetamine and GBR 12909 treatments were compared to vehicle using paired samples t-test or Wilcoxon signed rank test. Effects of CNO on feeding patterns were tested using repeated measures general linear model (RM-GLM), with Group (e.g., VTA:Dq-/VTA:Dq+/SN:Dq-/SN:Dq+) as between-subjects factor and Treatment (e.g., Saline/CNO) as within-subjects factor. Following a significant Treatment effect or Treatment*Group interaction, post hoc comparisons between CNO and saline treatment were carried out per group. For non-parametric testing, Wilcoxon signed rank tests were used. For dose-response testing, main effect of treatment in VTA:Dq+ rats was tested with RM-GLM. Greenhouse-Geisser and Huyn-Feldt corrections were applied to adjust for sphericity (when Mauchley's epsilon<0.7 or >0.7, respectively). Following a significant treatment effect, CNO doses were each compared to saline with LSD post hoc tests. As non-parametric equivalent, Friedman tests followed by Wilcoxon signed rank test were used. Threshold for statistical significance was set at α=0.05.

Results

Pharmacological DA reuptake inhibition suppresses food intake

To affirm the anorectic properties of DA-enhancing psychostimulants in our setup, we tested the effects of amphetamine (DA and noradrenalin reuptake inhibitor and releaser) and GBR 12909 (a selective DA reuptake inhibitor) on feeding microstructure and total food intake.

Both amphetamine (0.3 and 1.0 mg/kg) and GBR 12909 (10 mg/kg) treatment affected multiple aspects of food intake (***Fig 1***). The lower dose of GBR 12909, 3.0 mg/kg, was not sufficient to affect feeding behaviour (all P>0.1). Exemplifying feeding patterns following treatment with amphetamine and GBR 12909 are depicted in ***Figure 1A+B***. Both drugs decreased average meal size (***Fig 1C***; AMPH 0.3 and 1.0 mg/kg P<0.0005; GBR P=0.012). Amphetamine, but not GBR 12909, also significantly reduced average meal duration (***Fig 1D;*** AMPH 0.3 mg/kg P=0.001, 1.0 mg/kg P<0.0005; GBR P=0.071), and first meal size (AMPH: saline 3.40±0.39 vs 0.3 mg/kg 1.95±0.28, P<0.0005; saline 3.30±0.35 vs 1.0 mg/kg 1.27±0.15, P<0.0005; GBR: vehicle 2.13±0.38 vs 10 mg/kg 1.61±0.35, P=0.182).

The meal frequency was significantly increased with 0.3 mg/kg amphetamine (***Fig 1E***; Z= -2.846, P=0.003), but not 1.0 mg/kg (Z=-1.354, P=0.242). In contrast, rats ate fewer meals following GBR 12909 treatment (***Fig 1E***; Z=-2.309, P=0.035).

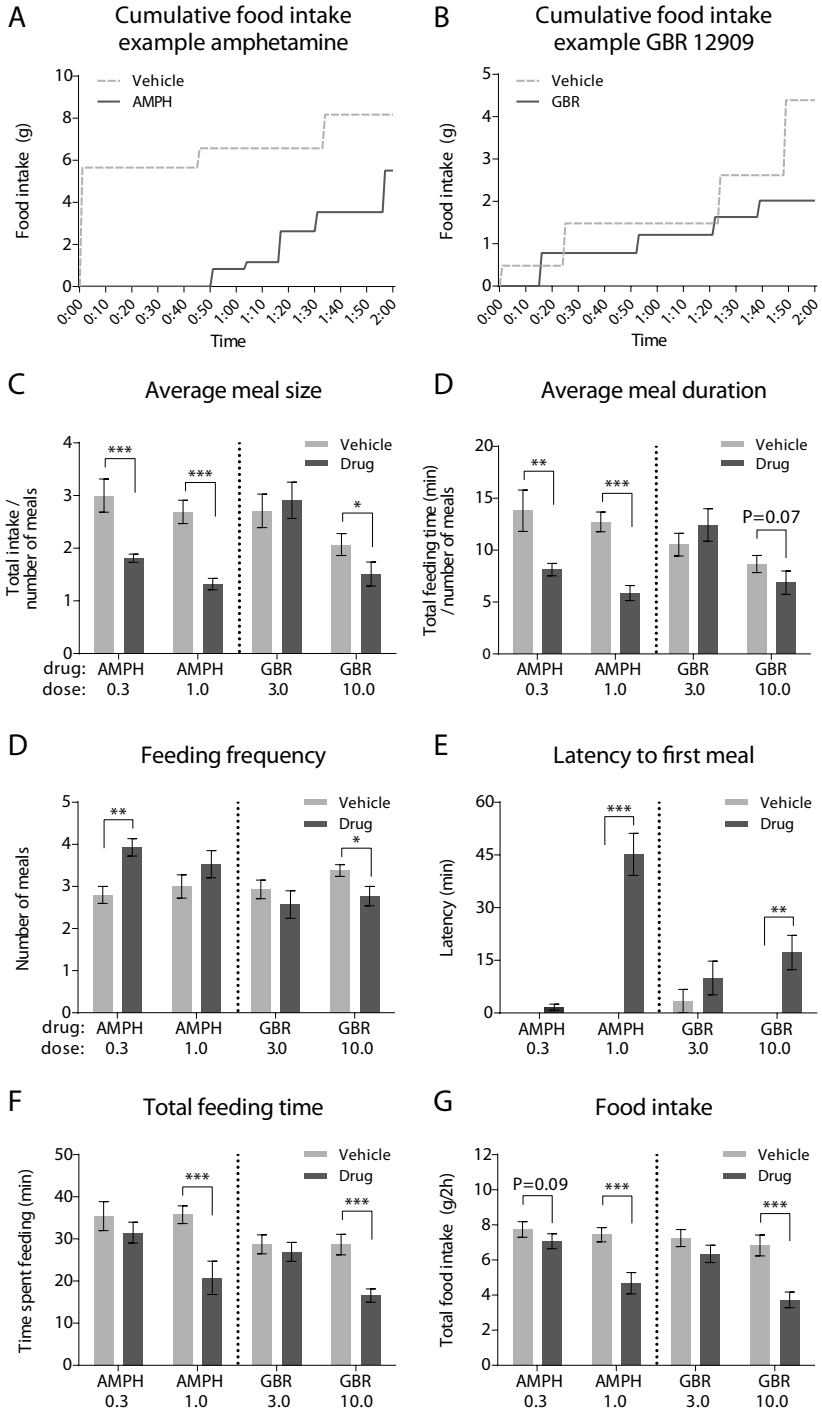
Furthermore, the high doses of amphetamine and GBR 12909 significantly delayed latency to start feeding (***Fig 1F***; AMPH 0.3 mg/kg P=0.25, 1.0 mg/kg P<0.0005; GBR P=0.008), and decreased total feeding time (***Fig 1G***; AMPH 0.3 mg/kg P=0.095, 1.0 mg/kg, P<0.0005; GBR P<0.0005). Interval between first and second meal was not affected (all doses P>0.1, data not shown). Together, these effects resulted in a significant decrease in total food intake following 1.0 mg/kg amphetamine or 10 mg/kg GBR 12909 (***Fig 1H***; both P<0.0005; AMPH 0.3 mg/kg P=0.087).

Chemogenetic activation of DA neurons in VTA, but not SNc, disrupts feeding behaviour

To test whether increasing excitability of DA neurons in either VTA or SNc is sufficient to affect food consumption, feeding behaviour was measured in TH::Cre rats expressing the excitatory DREADD hM3Dq in DA neurons in the VTA (VTA:Dq+, ***Fig 2A***) or SNc (SN:Dq+, ***Fig 2B***). Immunohistochemical analysis confirmed DREADD expression in VTA and SNc, respectively (***Fig 2A-B***), as well as co-localisation with TH (≥95%, see Chapter 2). No expression was observed in Cre-negative control groups (not shown).

A representative example of cumulative food intake is shown in ***Figure 2C***. Analysis of feeding microstructure showed that CNO-induced activation of VTA DA neurons significantly decreased the average meal size (***Fig 2D***; Treatment effect $F_{1,26}$=7.698, P=0.01, and Treatment*Group interaction $F_{3,26}$=7.566, P=0.001; post hoc CNO vs saline VTA:Dq+ group P<0.0005). In contrast, CNO treatment had no effect in the SN:Dq+ group, or in VTA:Dq- and SN:Dq- control groups (all post hoc tests P>0.1). In addition, VTA:Dq+ rats showed a trend towards an increased meal frequency (***Fig 2E***; CNO vs saline VTA:Dq+ Z=-2.121, P=0.063; other groups P>0.1). Combined, these effects

Figure 1. (next page) Effects of pharmacological DA stimulation, by amphetamine or GBR12909, on feeding patterns. *Representative examples of feeding patterns following treatment with amphetamine (1.0 mg/kg, A) or GBR 12909 (10 mg/kg, B). C) Amphetamine and GBR 12909 decreased average meal size. D) Amphetamine decreased average meal duration. E) Low dose of amphetamine increased meal frequency, whilst high dose of GBR 12909 decreased number of meals. The high doses of amphetamine and GBR 12909 increased latency to start feeding (F), decreased total feeding time (G) and decreased total food intake (H). Data are presented as mean ± s.e.m., n=13-15 per group. *P<0.05, **P<0.01, ***P<0.001 drug compared to vehicle.*


A
Cumulative food intake
example amphetamine
Vehicle
AMPH
Food intake (g)
Time
B
Cumulative food intake
example GBR 12909
Vehicle
GBR
Food intake (g)
Time
C
Average meal size
Total intake /
number of meals
Vehicle
Drug
drug: AMPH AMPH GBR GBR
dose: 0.3 1.0 3.0 10.0
D
Average meal duration
Total feeding time (min)
/ number of meals
P=0.07
D
Feeding frequency
Number of meals
E
Latency to first meal
Latency (min)
F
Total feeding time
Time spent feeding (min)
G
Food intake
Total food intake (g/2h)
P=0.09

resulted in a modest yet significant decrease in total food intake (***Fig 2F***; Treatment effect $F_{1,26}$=7.325, P=0.012; Group*Treatment interaction $F_{3,26}$=6.345, P=0.08; post hoc CNO vs saline VTA:Dq+ P=0.001, other groups P>0.1).

All animals typically started feeding immediately (within 1 min) upon access to chow (***Fig 2C, Table 1***). The latency to start the first or second meal was not affected by CNO treatment (***Table 1***). Consistent with a smaller average meal size, CNO reduced the first meal size in VTA:Dq+ rats (***Table 1***; Treatment effect $F_{1,25}$=7.794, P=0.01; Treatment*Group interaction $F_{3,25}$=3.413, P=0.033; post hoc CNO vs saline VTA:Dq+ P<0.0005, other groups P>0.1). Average meal duration and total time spent feeding were not affected (***Table 1***). In summary, chemogenetic activation of VTA DA neurons decreased meal size and total food intake, whilst activation of SNc DA neurons did not affect feeding behaviour.

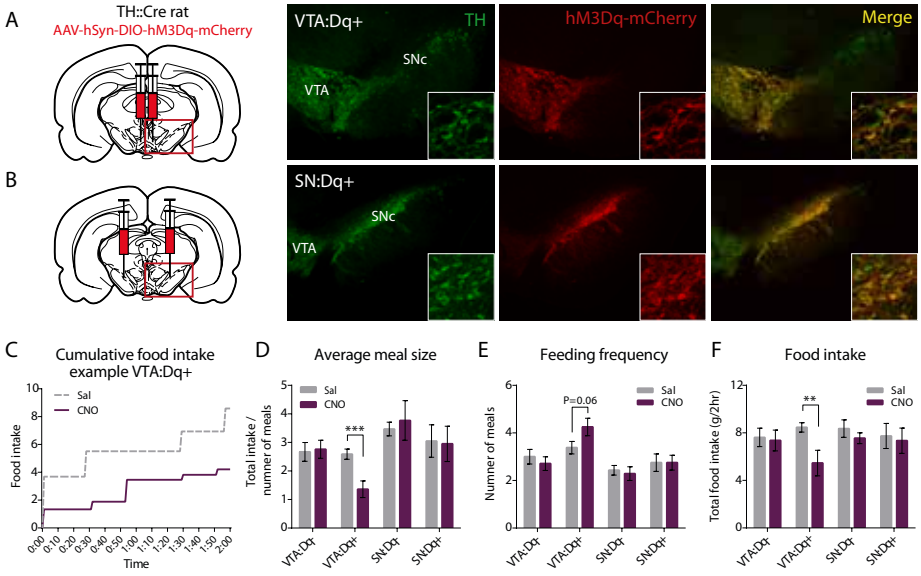


Figure 2. Chemogenetic activation of DA neurons in VTA, but not SNc, affects feeding patterns. *TH::Cre rats were infused with Cre-dependent DREADD virus into either VTA (A) or SNc (B). Right panels show expression of DREADD (hM3Dq-mCherry) in DA (TH-immunoreactive) neurons in VTA (VTA:Dq+) and SNc (SN:Dq+). C) Representative feeding pattern of VTA:Dq+ rat following treatment with saline (Sal) or CNO, showing cumulative intake over time. Each vertical step represents a meal. D) Average meal size was decreased by CNO treatment in VTA:Dq+ group, but not SN:Dq+ group or Cre- control groups (VTA:Dq- and SN:Dq-). E) VTA:Dq+ group showed a trend towards increased meal frequency. F) Total food intake was decreased by CNO in VTA:Dq+ group, but not other groups. Error bars represent mean ± s.e.m. n=7-8 per group. **P<0.01, ***P<0.001 CNO compared to saline.*

Table 1. Additional measures of feeding microstructure, following chemogenetic activation of DA neurons in VTA or SNc. *Data represent mean and s.e.m. following treatment with either saline (SAL) or CNO. n=7-8 per group. ***P<0.0005 CNO compared to saline. Other tests not significant (P>0.1).*

	Latency to start first meal (min)				First meal size (g)			
	SAL	sem.	**CNO**	sem.	**SAL**	sem.	**CNO**	sem
VTA:Dq-	**0.00**	0.00	**3.00**	3.00	**3.39**	0.50	**3.55**	0.74
VTA:Dq+	**0.00**	0.00	**1.15**	1.15	**4.33**	0.44	**2.34*** **	0.76
SN:Dq-	**0.00**	0.00	**0.00**	0.00	**4.65**	0.54	**4.59**	0.69
SN:Dq+	**0.00**	0.00	**0.00**	0.00	**3.73**	0.77	**3.18**	0.88

	First meal interval (min)				Average meal duration (min)			
	SAL	sem.	**CNO**	sem.	**SAL**	sem.	**CNO**	sem
VTA:Dq-	**24.57**	7.74	**34.86**	12.01	**12.38**	2.40	**12.67**	1.38
VTA:Dq+	**25.53**	5.35	**22.35**	6.80	**12.29**	1.24	**10.19**	2.44
SN:Dq-	**29.14**	7.93	**40.30**	4.76	**15.26**	0.97	**16.09**	2.79
SN:Dq+	**28.73**	5.96	**25.63**	4.41	**13.42**	1.87	**13.93**	1.73

	Total feeding time (min)			
	SAL	sem.	**CNO**	sem.
VTA:Dq-	**34.49**	4.61	**33.71**	4.22
VTA:Dq+	**40.83**	5.19	**42.25**	9.45
SN:Dq-	**36.66**	2.95	**32.60**	3.38
SN:Dq+	**33.88**	3.37	**35.60**	2.85

Dose-dependent effects of CNO on meal size and frequency in VTA:Dq+ rats

To investigate the effect of VTA DA neuron activation in more detail, we performed a dose-response test in VTA:Dq+ rats. Similar to the single dose experiment (*Fig 2D*), CNO treatment significantly decreased average meal size (*Fig 3A*, solid line; Treatment effect $F_{4,28}$=13.951, P<0.0005). This effect was present using doses of 0.1 mg/kg CNO and higher (*Fig 3A*; post hoc tests CNO vs saline: 0.1, 0.3 and 1.0 mg/kg all P<0.01), but not the lowest dose of 0.03 mg/kg (*Fig 3A*; P=0.4). The decreased meal size was also reflected in smaller first meals (*Fig 3A*, dotted line; Treatment effect $F_{4,28}$=6.163, P=0.001; post hoc

tests CNO vs saline: 0.03 mg/kg P=0.24; 0.1, 0.3, and 1.0 mg/kg all P<0.05), as well as a shorter average meal duration (***Fig 3B;*** Treatment effect $F_{4,28}$=3.178, P=0.28; post hoc tests CNO vs saline: 0.03 mg/kg P=0.4; 0.1, 0.3, and 1.0 mg/kg all P<0.05).

In addition, CNO treatment increased meal frequency (***Fig 3C***; Chi-square=10.59, P=0.024), with significant effects at 0.1 and 1.0 mg/kg (CNO vs saline: 0.03 mg/kg P=0.8, 0.1 and 1.0 mg/kg P<0.05, 0.3 mg/kg P=0.078). Combined, there was no net effect on total food intake (***Fig 3D***; Treatment effect $F_{4,28}$=1.909, P=0.137). Consistent with the single dose experiment, CNO treatment did not affect latency to start feeding, or total feeding time (both P>0.1, data not shown).

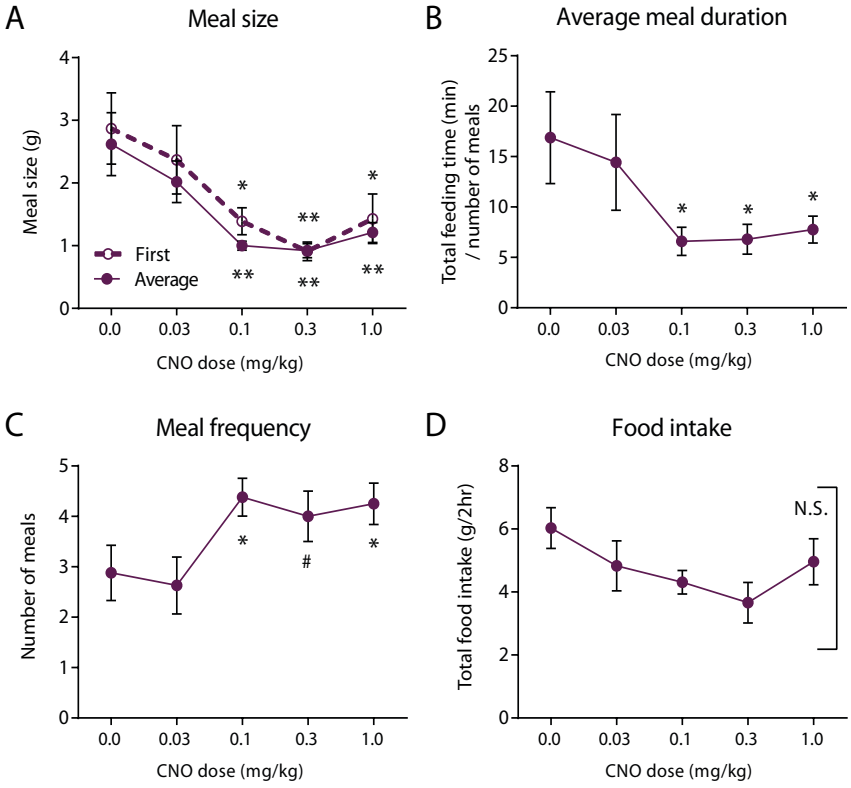


Figure 3. Dose-response curve for effect of CNO on feeding in VTA:Dq+ rats. *Total food intake and feeding microstructure following 0.03 up to 1.0 mg/kg of CNO compared to saline (0 mg/kg). A) Average meal size and first meal size were reduced by 0.1, 0.3, and 1.0 mg/kg CNO. B) Average meal duration was reduced by 0.1, 0.3, and 1.0 mg/kg CNO. C) Meal frequency was increased by 0.1 and 1.0 mg/kg. A) Total food intake was not significantly affected by CNO treatment. Data are presented as mean ± s.e.m. (n=8). #P<0.1, *P<0.05, **P<0.01, CNO compared to saline. N.S. not significant.*

Taken together, VTA DA neuron activation robustly decreased meal size and duration, and increased feeding frequency. These effects were induced by CNO doses of 0.1 mg/kg and higher, whilst 0.03 mg/kg CNO was not sufficient to affect feeding behaviour.

Decreased meal size and increased meal frequency are mediated by VTA pathway towards NAc, but not PFC or amygdala

To determine which neuronal pathway originating from the VTA underlay the DA-induced effects on feeding behaviour, we selectively targeted VTA neurons projecting to NAc (VTA>NAc), PFC (VTA>PFC), or amygdala (VTA>Amy). DREADD expression in the VTA was confirmed in all groups (***Fig 4A***), but was most abundantly observed in the VTA>NAc group, consistent with a major output of VTA neurons towards NAc.

Representative feeding patterns of a VTA>NAc rat are depicted in ***Figure 4B***. In the VTA>NAc group, CNO treatment significantly decreased the average meal size (***Fig 4C***; Treatment effect $F_{1,28}$=6.297, P=0.018; Treatment*Group interaction $F_{2,28}$=13.336, P<0.0005) as well as first meal size (***Table 2;*** Treatment effect $F_{1,28}$=8.614, P=0.007; Treatment*Group interaction $F_{2,28}$=3.628, P=0.04; post hoc CNO vs saline P<0.0005). This effect was not observed in VTA>PFC or VTA>Amy groups (***Table 2***; all post hoc tests P>0.1). Also meal duration was reduced selectively in VTA>NAc rats (***Table 2***; Treatment*Group interaction $F_{2,28}$=10.434, P<0.0005; post hoc CNO vs saline VTA>NAc P<0.0005, VTA>PFC P=0.100, VTA>Amy P=0.808).

In addition, the VTA>NAc group showed an increased meal frequency (***Fig 4D***; 9/11 rats, P=0.012, VTA>PFC and VTA>Amy P>0.1), and shorter interval between the first and second meal (***Table 2***; Treatment effect $F_{1,28}$=5.35, P=0.028; post hoc CNO vs saline VTA>NAc P=0.027, VTA>PFC P=0.907 and VTA>Amy P=0.062). CNO treatment did not affect the initial latency to start feeding or total feeding time (***Table 2***; both P>0.1) in any group. Combined, the effects of smaller meals and increased feeding frequency had no net effect on total food intake (***Fig 4E***; Treatment effect $F_{1,28}$=2.826, P=0.104; Treatment*Group interaction $F_{2,28}$=1.455, P=0.25).

To summarize, chemogenetic activation of VTA>NAc pathway decreased the average size and duration of meals, and increased feeding frequency. In contrast, activation of VTA>PFC or VTA>Amy pathway had no significant effects on feeding patterns.

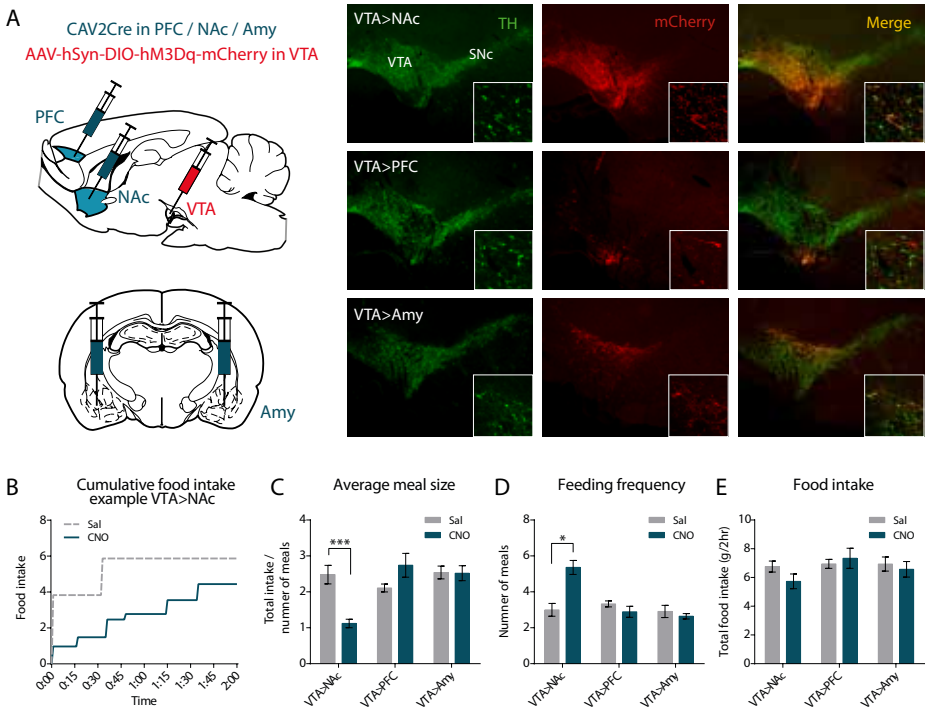


Figure 4. Effects of chemogenetic activation of selective neuronal pathways from VTA to NAc, PFC, or amygdala on feeding patterns. *A) Rats were infused with Cre-dependent DREADD virus in VTA, and retrogradely transported CAV2Cre in either NAc, PFC, or amygdala to induce DREADD expression in selective VTA neuronal pathways. B) Representative example of feeding pattern following saline treatment vs CNO-induced activation of VTA>NAc pathway. C) CNO reduced meal size in VTA>NAc group, selectively. D) CNO increased meal frequency in VTA>NAc group, selectively. E) Food intake was not significantly affected by CNO treatment in any group. Data are presented as mean ± s.e.m. n=9-11 per group. *P<0.05, ***P<0.001 CNO compared to Sal.*

Table 2. Additional measures of feeding microstructure, following chemogenetic activation of VTA neurons projecting to NAc, PFC, or amygdala. *Data represent mean and s.e.m. following treatment with either saline (SAL) or CNO. n=9-11 per group. #P<0.1, *P<0.05, **P<0.01,***P<0.001 CNO compared to saline. Other tests not significant (P>0.1).*

	Latency to start first meal (min)				First meal size (g)			
	SAL	sem	**CNO**	sem	**SAL**	sem	**CNO**	sem
VTA>NAc	**0.00**	0.00	**1.93**	1.93	**3.44**	0.40	**1.52****	0.28
VTA>PFC	**3.09**	3.09	**0.00**	0.00	**3.35**	0.41	**3.74**	0.69
VTA>Amy	**0.00**	0.00	**1.85**	1.85	**3.56**	0.33	**2.86**	0.38

	First meal interval (min)				Average meal duration (min)			
	SAL	sem	**CNO**	sem	**SAL**	sem	**CNO**	sem
VTA>NAc	**28.16**	10.09	**11.05***	1.26	**13.17**	1.81	**6.48*****	1.40
VTA>PFC	**10.78**	1.66	**12.82**	3.53	**10.37**	0.55	**15.36**	1.94
VTA>Amy	**22.35**	4.15	**12.35#**	1.96	**13.17**	0.94	**13.62**	0.92

	Total feeding time (min)			
	SAL	sem	**CNO**	sem
VTA>NAc	**35.20**	2.70	**31.71**	5.08
VTA>PFC	**34.76**	2.91	**41.73**	5.25
VTA>Amy	**35.38**	1.84	**35.33**	2.61

Both chemogenetic and pharmacological DA stimulation increase locomotor activity

Since effects on motor behaviour can critically contribute to altered feeding, we monitored home cage locomotor activity during the feeding episode. Both chemogenetic and pharmacological stimulation of DA signalling were found to significantly increase locomotor activity, although the magnitude of effects differed considerably between manipulations (*Fig 5A-C*). All treatments increased total distance moved (*Fig 5A*), as well as total time spent active (i.e., ambulatory movement; *Fig 5B*), and the frequency of activity bouts (data not shown). Furthermore, chemogenetic activation of VTA DA neurons or VTA>NAc pathway, or treatment amphetamine also enhanced the mean duration of an active bout (*Fig 5C*). CNO treatment had no effect on locomotor behaviour in VTA:Dq- and SN:Dq- control groups, or in VTA>PFC and VTA>Amy groups (all $P>0.1$, data not shown). Thus, both chemogenetic and pharmacological stimulation of DA increased locomotor activity during feeding behaviour.

Interestingly, the doses of drugs that affected feeding were also effective in inducing hyperactivity, indicating that these outcomes may be related. In VTA:Dq+ rats, we found that 0.1, 0.3, and 1.0 mg/kg CNO all induced a maximal hyperactive effect (*Fig 5D*). Whilst the lowest dose, 0.03 mg/kg, was not sufficient to affect feeding behaviour, it caused an intermediate increase in locomotion (*Fig 5D*).

Figure 5E depicts a typical example of feeding and locomotor patterns during the first 10 minutes of food access in a single VTA:Dq+ rat, following treatment with either saline, CNO, or amphetamine. The track visualizations illustrate the time spent in the feeding zone, as well as total locomotor activity. Under saline treatment conditions, rats started feeding directly upon food access, and spent the first 10 minutes mainly in the feeding (and drinking) zone, whilst overall ambulatory activity was quite low (*Fig 5E*). Amphetamine treatment increased locomotor activity, and suppressed the initiation of food intake, represented by diminished time spent in the feeding area (*Fig 5E*). With CNO treatment, VTA:Dq+ rats still rapidly initiated feeding, but showed disrupted feeding behaviour, together with locomotor hyperactivity (*Fig 5E*).

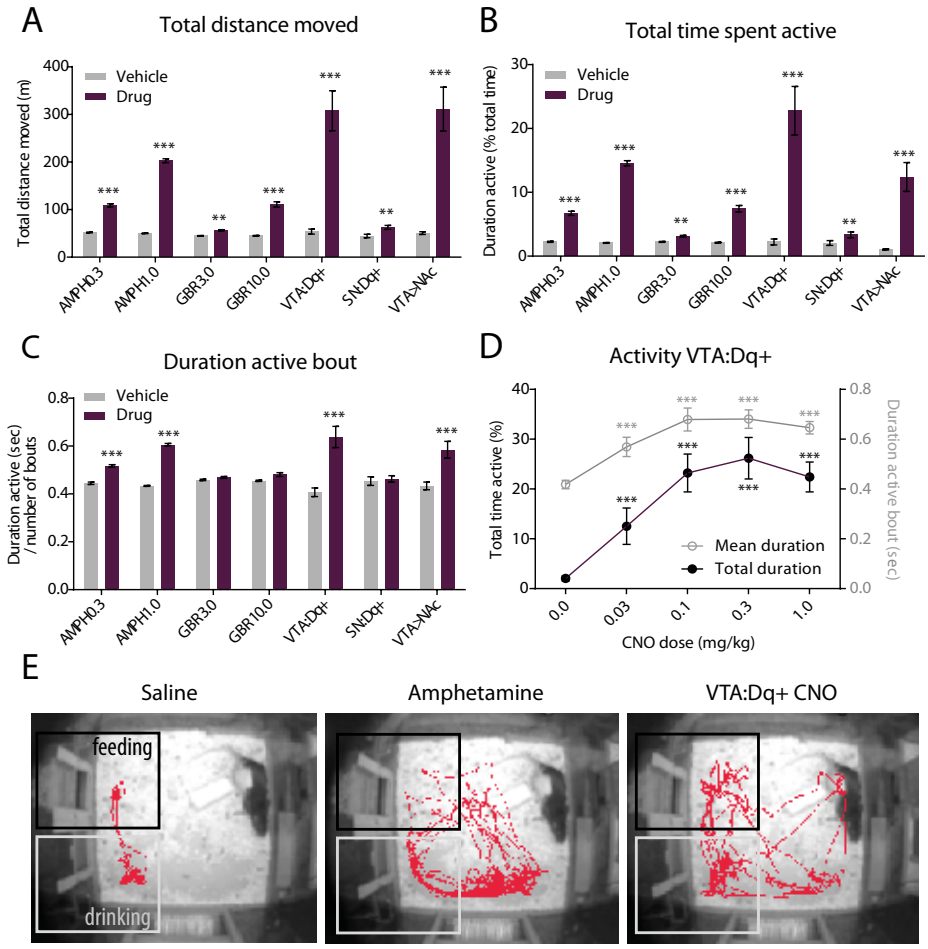


Figure 5. Effect of enhanced DA signalling on locomotor patterns. *A) Total distance moved (m/2hr). B) Time spent active (% of total time). C) Mean duration of an active bout. D) CNO dose-response curve for time spent active and mean duration of an active bout in VTA:Dq+ group. E) Track visualisation during first 10 min of food access. Example of locomotor activity of the same VTA:Dq+ rat (identical background image) following treatment with saline, CNO (0.3 mg/kg) or amphetamine (1.0 mg/kg). Rectangles represent feeding and drinking areas of the home cage. Data are presented as mean ± s.e.m., n=7-15 per group. *P<0.05, **P<0.01, ***P<0.001 drug compared to vehicle.*

Discussion

In this study, we took a novel approach to determine the effects of enhanced DA neuronal activity on feeding behaviour. Using DREADD technology to chemogenetically activate selective midbrain neuronal subpopulations in rats, we found that activating the mesolimbic DA pathway disrupted feeding microstructure, without affecting total intake. Consistent with earlier reports, we observed that treatment with psychostimulant amphetamine or selective DA reuptake inhibitor GBR 12909 had hypophagic effects, including delayed feeding latency and reduced meal size and duration (Foltin and Fischman, 1989; Grinker et al, 1980; van der Hoek and Cooper, 1994; Leibowitz et al, 1986). Chemogenetic activation of DA neurons in the VTA, but not SNc, reproduced some of these effects, resulting in smaller and shorter meals. Activation of selective neuronal pathways revealed that this effect was mediated by VTA neuronal projections towards the NAc, but not PFC or amygdala. The majority of neurons in the mesolimbic pathway towards NAc, approximately 80%, is DAergic (***Chapter 2***, Boender et al., 2014). Together, this suggests that reduced meal size resulted from enhanced mesolimbic DA signalling, complying with earlier studies showing opposite effects, i.e., longer continuation of feeding, following blockade of DA D1- or D2-R in the NAc (Baldo et al, 2002; Janhunen et al, 2013). Interestingly, the smaller meals were accompanied by an increase in feeding frequency. This indicates that the rats were stimulated to engage in food intake, but ongoing feeding activities were prematurely aborted. Combined, these effects resulted in a modest or non-significant decrease in total food intake. Thus, based on these findings, we propose that enhanced mesolimbic DA neuronal activity does not directly stimulate or inhibit food intake, but rather promotes the initiation and cessation of feeding behaviours.

Role of mesolimbic DA neuronal activity in control of food intake: behavioural activation and permissive feeding

Previously, it was shown that NAc DA depletions or DA-R antagonists affected food intake through effects on locomotor behaviour, including approach behaviour, food handling, or behavioural switching (Baldo et al, 2002; Koob et al, 1978; Salamone et al, 1990). Here, we found that chemogenetic activation of VTA DA neurons or VTA>NAc pathway induced locomotor hyperactivity, in agreement with earlier chemogenetic and pharmacological findings (Boender et al, 2014; Creese and Iversen, 1974; Delfs et al, 1990; Wang et al, 2013). In contrast, activation of SNc DA neurons only modestly increased locomotor activity, and did not affect feeding. Importantly, dose-response testing showed that a low dose of CNO (0.03 mg/kg) was sufficient to sub-maximally increase locomotor activity, but not to disrupt feeding behaviour. Higher doses of CNO induced a maximal hyperactive phenotype, and all had similar effects on meal size and

frequency. This suggests that the effects of enhanced DA neuronal activity on feeding behaviour may be secondary to increased behavioural activity, which is reflected in increased initiation as well as cessation of feeding bouts.

Amphetamine and GBR 12909 both suppressed food intake by delaying the latency to start feeding, which may indicate reduced appetite or diminished interest in food. In contrast, latency to start feeding was not affected by chemogenetic activation of VTA DA neurons or VTA to NAc pathway. The smaller and shorter meals following enhanced mesolimbic DA activity may suggest that animals were satiated more quickly (Adan et al, 2008). However, the meal interval was either reduced or not affected, opposing an effect on satiety. Furthermore, the increase in meal frequency suggests that animals were compensating for a reduced meal size, indicating that they were still motivated to eat. Taken together, we conclude that it is unlikely that chemogenetic activation of VTA DA neurons directly affects hunger or satiety.

Our results suggest that elevated DA signalling in the NAc is sufficient to reduce meal size, but not to suppress total amount of food taken over time. Striatal DA release, induced by mesolimbic DA neuron activation, increases excitability of striatal D1-R expressing medium spiny neurons (MSNs), and reduces excitability of D2-MSNs. Together, this promotes behavioural activation (Friend and Kravitz, 2014; Kravitz et al, 2010). In addition, stimulation of D1-MSNs projecting to the LH has been found to terminate food intake, and inactivation of these neurons is necessary to "permit" feeding behaviour (Krause et al, 2010; O'Connor et al, 2015). Thus, the finding that meal initiation was not inhibited by chemogenetic DA neuron activation, suggests that the post-synaptic effects were not sufficient to continuously activate LH-projecting D1-MSNs.

In contrast, psychostimulant drugs enhance post-synaptic DA signalling, through reuptake inhibition (GBR 12909 and amphetamine) and evoked DA release (amphetamine). Our results suggests that these effects might be sufficient to prevent inactivation of D1-MSNs, thereby prohibiting the engagement in food intake. Alternatively, these drugs might exert their anorexic actions directly at the LH, rather than striatum (Leibowitz et al, 1986). Ideally, future studies should combine pharmacological and/or chemogenetic stimulation of DA with recordings from identified D1- or D2-MSNs in NAc, in order to directly link post-synaptic DAergic effects to feeding behaviour. Together, our findings highlight the importance of the mode of DA manipulation, and confirm that DA reuptake inhibition may have significantly different effects compared to enhanced DA neuronal activity.

Thus, we show that enhanced activity of VTA DA of VTA>NAc neurons facilitates behavioural activation, which is reflected in locomotor hyperactivity, as well as enhanced initiation and cessation of food intake. In line with optimal foraging theory, mesolimbic DA neurons may be activated in a state of negative energy balance in order to promote food-seeking behaviour (Ranaldi, 2014). However, whereas this may be beneficial for the pursuit of reward, our results indicate that enhanced mesolimbic DA neuronal activity disrupts feeding behaviour when food is readily available.

Conclusions

In this study, we showed that enhancing endogenous activity of mesolimbic DA neurons is sufficient to affect feeding behaviour. Chemogenetic activation of VTA DA neurons or VTA to NAc pathway caused rats to eat smaller, but more meals, which was accompanied by locomotor hyperactivity. Importantly, whilst DA-enhancing psychostimulant drugs suppressed food intake by postponing the initiation of feeding behaviour, chemogenetic DA neuron activation did not. This indicates that enhanced mesolimbic DA neuronal activity facilitates both the initiation and cessation of feeding behaviour, without directly affecting appetite.

These findings provide new insights into how DA neuronal activity and striatal DA function are involved in the control of food intake, and may have implications for the role of DA in overeating. Indeed, weight gain induced by antipsychotic drug treatment may be attributed to DAergic effects on meal size, as well as locomotor activity (Davoodi et al, 2009; Lee and Clifton, 2002; van der Zwaal et al, 2010). However, DA signalling may differentially affect food intake in lean and obese subjects (Grinker et al, 1980), and future studies are needed in order to translate these findings to the clinic.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

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CHAPTER FIVE

Curiosity becomes a heavy load, too heavy to hold
- Arctic Monkeys, "Do me a favour"

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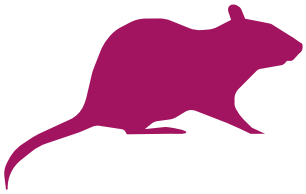
CHEMOGENETIC ACTIVATION OF MIDBRAIN DOPAMINE NEURONS AFFECTS ATTENTION, BUT NOT IMPULSIVITY, IN THE FIVE-CHOICE SERIAL REACTION TIME-TASK IN RATS



Abstract

Attentional impairments and exaggerated impulsivity are key features of psychiatric disorders, such as attention-deficit/hyperactivity disorder, schizophrenia, and addiction. These deficits in attentional performance and impulsive behaviours have been associated with aberrant dopamine (DA) signalling, but it remains unknown whether these deficits result from enhanced DA neuronal activity in the midbrain. Here, we took a novel approach by testing the impact of chemogenetically activating DA neurons in the ventral tegmental area (VTA) or substantia nigra pars compacta (SNc) on attention and impulsivity in the five-choice serial reaction time task (5-CSRTT) in rats. We found that activation of DA neurons in both the VTA and SNc impaired attention by increasing trial omissions. In addition, SNc DA neuron activation decreased attentional accuracy. Surprisingly, enhanced DA neuron activity did not affect impulsive action in this task. These results show that enhanced midbrain DA neuronal activity induces deficits in attentional performance, but not impulsivity. Furthermore, DA neurons in the VTA and SNc play different roles in regulating attention. These findings contribute to our understanding of the neural substrates underlying attention deficits and impulsivity, and provide valuable insights to improve treatment of these symptoms.

Introduction

Attentional deficits and exaggerated impulsivity are core features of several psychiatric disorders, including attention-deficit/hyperactivity disorder, schizophrenia, and addiction (DSM-5, 2013). The brain dopamine (DA) system is known to be involved in the psychopathology of these disorders (Everitt and Robbins, 2005; Franken et al, 2005; Howes and Kapur, 2009; Tripp and Wickens, 2009; Volkow and Baler, 2015), as well as in the regulation of attention and impulsivity (Bari and Robbins, 2013; Cools, 2011; Nieoullon, 2002; Pattij and Vanderschuren, 2008). However, the relationship between DA signalling, attention and impulse control remains incompletely understood. Previous research has mainly focused on DA signalling in target areas such as the striatum and prefrontal cortex (PFC). DA signalling in these areas depends on DA neuronal activity in the midbrain, but it is unknown if abnormal activity of DA neurons underlies attentional deficits and/or impulsivity.

Here, we took a novel approach by testing the impact of chemogenetic activation of midbrain DA neurons on attentional performance and impulsive behaviour in rats. We used designer receptors exclusively activated by designer drugs (DREADD) (Armbruster et al, 2007) in TH::Cre transgenic rats, in order to selectively increase DA neuronal activity in either the ventral tegmental area (VTA) or substantia nigra pars compacta (SNc). Effects on attention and impulsivity were tested in the five-choice serial reaction time task (5-CSRTT) (Carli et al, 1983). In this task, rats are trained to respond to a visual stimulus, which is briefly and pseudo-randomly presented in one of five nose-poke holes. Impulsivity in the 5-CSRTT is measured by assessing premature responses, that are made before the stimulus onset. Elevated DA signalling in the striatum, especially the nucleus accumbens (NAc) and dorsomedial striatum (DMS), has been associated with premature responding (Agnoli et al, 2013; Baarendse and Vanderschuren, 2012; Economidou et al, 2012; Van Gaalen et al, 2006; Moreno et al, 2013; Pattij et al, 2007; Pezze et al, 2007). Attentional performance is tested by assessing response accuracy and trial omissions, which are mainly affected by DAergic manipulations in PFC and dorsal striatum (Agnoli et al, 2013; Agnoli and Carli, 2011; Baunez and Robbins, 1999; Economidou et al, 2012; Granon et al, 2000; Rogers et al, 2001; Winstanley et al, 2010). These findings suggest a role for both the mesocorticolimbic and nigrostriatal DA pathway – ascending from DA neurons in VTA and SNc, respectively (Bjorklund and Dunnett, 2007; Moore and Bloom, 1978) – in the regulation of attention and impulsivity. In this study, we tested if enhanced midbrain DA neuronal activity is sufficient to induce attentional deficits and/or impulsive actions in rats. Since pharmacological stimulation of DA signalling, via DA receptor (DA-R) agonists or DA reuptake inhibition, has repeatedly been shown to promote impulsive actions (Agnoli et al, 2013; Baarendse and Vanderschuren, 2012; Economidou et al,

2012; Van Gaalen et al, 2006; Moreno et al, 2013; Pattij et al, 2007; Pezze et al, 2007), we hypothesized that chemogenetic activation of midbrain DA neurons would increase premature responding. Given the major role of the NAc and DMS in impulsivity (Dalley et al, 2011; Eagle and Baunez, 2010), we predicted that particularly VTA DA neuron activation would enhance impulsive actions, whilst SNc DA neuron activation would have a more modest effect. Attentional performance in the 5-CSRTT has been shown to either be improved or impaired following administration of DA-R agonists and DA reuptake inhibition (Agnoli et al, 2013; Baarendse and Vanderschuren, 2012; Besson et al, 2010; Economidou et al, 2012; Granon et al, 2000; Pezze et al, 2007; Winstanley et al, 2010). Therefore, we hypothesized that activation of DA neurons in either VTA or SNc would affect attentional accuracy and trial omissions, although the directionality of the effects was not readily predictable.

Materials and methods

Animals

TH::Cre$^{+/-}$ rats were bred in-house, by crossing heterozygous TH::Cre$^{+/-}$ rats with wild type Long Evans mates. In total, 42 male rats were used, divided into three groups: 1) VTA:Dq+, n=12 Cre$^{+/-}$ rats, with DREADD in the VTA; 2) SN:Dq+, n=16 Cre$^{+/-}$ rats with DREADD in the SNc; and 3) Cre-, n=14, a control group of Cre$^{-/-}$ littermates, which received DREADD virus in the VTA, but do not express Cre and thus do not express DREADD. To reduce the number of animals used in this study, we have included a single Cre-negative control group, infused with Cre-dependent DREADD virus in the VTA. Earlier (pilot) studies have shown that infusion of DREADD virus into the SNc in Cre-negative rats did not result in any DREADD expression, nor did we observe any behavioural effects of CNO administration. Rats were socially housed in Macrolon type III or IV cages, in a temperature and humidity controlled room. A wood block was provided for cage enrichment. Rats were housed under a reversed 12 hour day-night cycle (lights on at 19:00), allowing behavioural training and testing during the animals' active phase. Rats had ab libitum access to water. Throughout the experiment, rats were food restricted to maintain 90% of their free-feeding weight. All experiments were approved by the Animal Ethics Committee of Utrecht University and were conducted in accordance with Dutch laws (Herziene Wet op Dierproeven, Art 10.a.2, 2014) and European regulations (Guidelines 86/609/EEC and 2010/63/EU).

Surgery

All animals were injected bilaterally with 1µl of AAV-DIO-hSyn-hM3Dq-mCherry (6.4–8.0*E12 molecules/ml; UNC Vector Core), using a stereotactic apparatus. Surgery was performed in two cohorts, one before operant training (n=22, 7 weeks old, ca. 200

gram), and one after training (n=20, 27 weeks old, ca. 450 gram). For the first cohort, coordinates for VTA were set at AP -5.4, ML +1.3 (5° angle), DV -8.0 mm; and SNc at AP -5.2, ML +2.0, DV -7.2. The second cohort received VTA injections at AP -5.8, ML +1.3 (5° angle), DV -8.4; and SNc at AP -5.4, ML +2.2, DV -7.7. All coordinates are in mm relative to Bregma. Virus was infused at a rate of 0.2 µl/min for 5 min, and the needle was left in place for 10 min after infusion. Surgery was performed under anaesthesia, induced by intramuscular fentanyl/fluanisone (0.315 mg/kg fentanyl, 10 mg/kg fluanisone; Hypnorm, Janssen Pharmaceutica). Local anaesthesia was provided by xylocaine, sprayed on the skull (Lidocaine 100 mg/ml, AstraZeneca BV). Carprofen (5.0 mg/kg, subcutaneously, Carporal, AST Farma BV) was given for pain relief, at the day of surgery and the two following days. Rats were housed individually for one week under DM-II conditions, to recover from surgery. There were at least eight weeks in between surgery and behavioural testing.

Five-choice serial reaction time task

Behavioural training and testing on the 5-CSRTT were performed as previously published (Baarendse and Vanderschuren, 2012), with slight adjustments. All training and testing took place in operant conditioning chambers (30.5*24*21 cm, Med-Associates, USA), placed in sound attenuated boxes, and controlled by MED-PC version IV (Med-Associates). Chambers were equipped with five evenly spaced nose-poke holes in a curved wall, each with a yellow light-emitting diode stimulus light and an infrared detector, and a food magazine for pellet delivery in the opposite wall. Following habituation to the setup, rats were trained to respond to an illuminated nose-poke hole, which was rewarded with delivery of a sucrose pellet (45 mg, TestDiet, USA) in the food magazine. Over six different training stages, rats learned to perform this task under baseline conditions of 5 sec inter-trial interval (ITI), 1 sec stimulus duration (SD) and 5 sec limited hold.

All sessions started with the delivery of a free sucrose pellet into the food magazine, after which the rat could initiate a trial by nose-poking into the food magazine. Each trial started with a 5 sec ITI, before a stimulus light was presented in one of the five nose-poke holes, in a pseudorandom order. When a correct response was made, i.e., a nose-poke in the illuminated hole within the limited hold period, a sucrose pellet was delivered. A response in one of the other four holes was recorded as an incorrect response, a failure to respond within the limited hold period as an omission, and a nose-poke made during the ITI as a premature response. Incorrect responses, omissions, and premature responses were punished with a 5 sec time-out, during which stimulus lights were switched off and the house light was switched on. Repeated responses in one of the nose-poke holes were recorded as perseverative responses, but had no programmed

consequences. Latencies to respond (premature, correct, incorrect, and food magazine entry) were recorded as well. The next trial started either when the animal entered the food magazine to collect the reward, or automatically after a time-out. Sessions lasted for 100 trials or 30 minutes, whichever occurred first. For analysis, omissions were defined as the percentage of omitted trials relative to total number of trials (omissions / [correct+incorrect+omissions] *100%). The number of omissions following either a correct response or a time-out were calculated from trial-by-trial data, and analysed as percentage of total omissions, to correct for any changes in total number of omitted trials. Accuracy was defined as percentage of correct responses relative to total responses (correct / [correct+incorrect] * 100%). Other parameters were analysed as absolute values.

Pharmacological testing started after approximately three months, when the rats showed stable baseline performance levels. Task performance was tested 30 minutes after administration of selective DREADD ligand clozapine-n-oxide (CNO) or saline, using a within-subject, counter-balanced design. Challenge sessions were separated by at least two baseline training sessions (5 sec ITI, 1 sec SD). Dose-response testing for CNO was conducted using a Latin Square design, with at least one wash-out baseline day between test sessions.

Drugs

CNO (kindly provided by Bryan Roth (University of North Carolina, Chapel Hill NC, USA) and the NIMH Chemical Synthesis and Drug Supply Program) and *d*-amphetamine sulphate (OPG Utrecht, the Netherlands) were dissolved in sterile 0.9% saline and kept at 4 °C in between testing. All injections were given intraperitoneally, at 1 ml per kg bodyweight. CNO was given at a dose of 0.3 mg/kg, unless stated otherwise. This dose was chosen based on previous work, in which we showed that 0.3 mg/kg yielded significant behavioural effects (i.e., increased locomotor activity) in rats expressing hM3Dq in DA neurons in either VTA or SNc (Boender et al., 2014; ***Chapter 2***).

Tissue preparation and immunohistochemistry

Euthanasia, tissue preparation, and immunohistochemical analysis were performed as previously described (Boender et al, 2014). Rats received a lethal dose of sodium pentobarbital (0.1 ml/100g bodyweight; Euthanimal, Alfasan BV, The Netherlands), and were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). Brain slices (40 µm) were stained for tyrosine hydroxylase (TH) and hM3Dq-mCherry with Mouse anti-TH (EMD Millipore, 1:500) and Rabbit anti-dsRed (Clontech, 1:500), respectively. Overnight incubation (at 4 °C) with

these primary antibodies was followed by 2 hour incubation with secondary antibodies Goat anti-Rabbit 568 (1:500) and Goat anti-Mouse 488 (Molecular Probes, 1:1000). All antibodies were dissolved in PBS containing 0.05% Tween. Slices were mounted with FluorSave (EMD Millipore).

Quantification of DREADD expression

Fluorescent pictures were taken using a Zeiss Axio Scope A1 microscope and AxioVision software, with identical settings for all animals. Intensity of hM3Dq-mCherry fluorescence in midbrain and striatum was analysed using ImageJ. Fluorescence intensity (0-255, arbitrary units) was measured at selected sub-regions in the midbrain (VTA and dorsal part of SNc (SNcd) -5.2 to -6.0 mm from Bregma; medial part of SNc (SNm) -5.6 to -6.0 mm from Bregma) and striatum (NAc Core, NAc Shell, DMS, dorsolateral striatum (DLS), 2.0 to 1.0 mm from Bregma). mCherry fluorescence was not detectable in other DAergic target regions, including the PFC. Background fluorescence (measured at peri-acquaductal grey for midbrain, and insular cortex for striatum) was subtracted from the measurements, and average fluorescence intensity was calculated per animal (two to eight measurements per region).

Data analysis

Fluorescence levels per region were compared between groups using Newman-Keuls test, followed by post hoc Mann Whitney U tests (P-value corrected for multiple comparisons). In total, 6-10 rats were included for the Cre- control group, and 13-15 rats for VTA:Dq+ and SN:Dq+ groups.

No animals were excluded from analysis based on DREADD expression. For behavioural analysis, rats were included based on baseline performance criteria (≤20% omissions and ≥85% accuracy). One rat in the SN:Dq+ group did not meet these criteria and was excluded from all analyses. Furthermore, animals were excluded from analysis of specific tests when baseline performance prior to drug administration did not meet the selection criteria. In total, 12-15 animals per group were included for each behavioural analysis. Effects of CNO compared to saline were tested using repeated measures general linear model (RM-GLM). When RM-GLM indicated a significant interaction between experimental Group (VTA:Dq+/SN:Dq+/Cre-) and Treatment (CNO/saline), post hoc pairwise comparisons were performed per group. Exploratory studies for the effects of amphetamine or a 7s ITI were analysed by paired samples t-tests. Effects of challenges compared to baseline conditions were tested with RM-GLM, using only data from saline test days, and post hoc tests were performed following either a significant main effect of Challenge, or Challenge*Group interaction. Dose-response analysis of CNO

was tested with RM-GLM. Based on Mauchly's test of sphericity, degrees of freedom were adjusted according to Huyn-Feldt correction (when Huyn-Feldt epsilon ≥0.7) or Greenhouse-Geisser (when Huyn-Feldt epsilon <0.7). Statistical significance was set at α=0.05, and Bonferroni corrections were made for multiple comparisons. Statistical tests were performed using SPSS 16.0 and graphs were made using GraphPad Prism 6 and Adobe Illustrator.

Results

DREADD expression

All rats were injected with Cre-dependent DREADD virus (AAV-hSyn-DIO-hM3Dq-mCherry) in either the VTA or SNc (***Fig 1A***). Immunohistochemical analysis confirmed DREADD expression in the VTA in VTA:Dq+ rats, and in SNc in SN:Dq+ rats. (***Fig 1A***). To quantify expression levels in midbrain as well as striatum, mCherry fluorescence intensity was measured in specific sub-regions (***Fig 1A-B***). This revealed that the VTA:Dq+ group primarily expressed DREADD in the VTA and SNm (fluorescence levels both P<0.0015 compared to Cre-), and also showed modest expression in SNcd (P=0.015 compared to Cre-) (F***ig 1A***). This was reflected in the striatum by DREADD expression primarily in NAc Core and Shell, as well as DMS, and to a lesser extent DLS (***Fig 1B***; all P<0.01 compared to Cre-). In contrast, the SN:Dq+ group primarily showed DREADD expression in SNm and SNcd, as well as modest expression in VTA (all P<0.0015 compared to Cre-). In striatum, this was reflected by expression primarily in DLS, as well as DMS and NAc Core (***Fig 1B***). Compared to the VTA:Dq+ group, the SN:Dq+ group showed significantly higher expression levels in SNcd and DLS, but lower expression in NAc Shell (***Fig 1A-B***). Thus, VTA:Dq+ rats mainly showed DREADD expression in the mesolimbic pathway, including projections to NAc and DMS, whilst SN:Dq+ rats mainly showed expression in the nigrostriatal pathway towards dorsal striatum.

Rats operated in the second cohort showed higher intensity of DREADD expression compared to the first cohort, which is likely due to the use of a different virus batch. Since behavioural effects of CNO did not differ between cohorts, results were pooled for statistical analysis.

Effects of DA neuron activation on attention

Chemogenetic activation of DA neurons impaired attentional performance in the 5-CSRTT under baseline conditions. In both VTA:Dq+ and SN:Dq+ rats, omissions were increased after CNO treatment (***Fig 2A***; Group*Treatment interaction $F_{2,37}$=5.4, P=0.009; post hoc VTA:Dq+ and SN:Dq+, both P<0.0005). Furthermore, CNO decreased accuracy in the SN:Dq+ group (***Fig 2B***; Group*Treatment interaction $F_{2,37}$=10.432, P<0.0005; post hoc

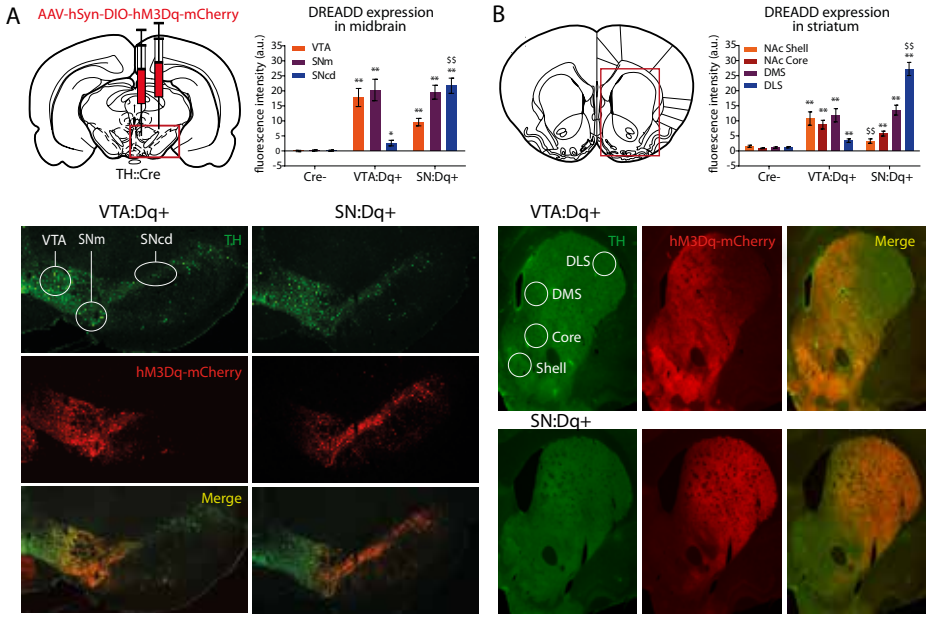


Figure 1. DREADD expression in DA neurons in VTA or SNc. *DREADD expression in midbrain (A) and striatal projection areas (B). All rats received bilateral injection with Cre-dependent DREADD virus into VTA or SNc (A, top left). Red rectangle represents area depicted in lower panels, showing representative examples of expression of tyrosine hydroxylase (TH) and DREADD (hM3Dq-mCherry) in midbrain and striatum of TH::Cre rats expressing DREADD in the VTA (VTA:Dq+) or SNc (SN:Dq+). Top right panels show quantified fluorescence levels in selected sub-regions in midbrain and striatum. Error bars represent mean ± s.e.m. *P<0.05, **P<0.01 difference compared to Cre- control group. $$ P<0.01 difference between VTA:Dq+ and SN:Dq+ groups.*

SN:Dq+ P<0.0005). These attentional deficits were also reflected in a decreased number of correct responses in both VTA:Dq+ and SN:Dq+ groups (***Table 1***, Group*Treatment interaction $F_{2,37}$=6.08, P=0.005, post hoc VTA:Dq+ and SN:Dq+, both P<0.0005), and an increased number of incorrect responses in the SN:Dq+ group (***Table 1***, Group*Treatment interaction $F_{2,37}$=4.998, P=0.012, post hoc SN:Dq+ P=0.003). Perseverative responses were not affected by CNO treatment (***Table 1***, Group*Treatment interaction $F_{2,37}$=1.818, P=0.177).

In addition, CNO treatment affected the rats' response latencies, with differential effects in the VTA:Dq+ and SN:Dq+ group. CNO reduced the reward collection latency in VTA:Dq+ rats, whilst this was increased in SN:Dq+ rats (***Table 1***, Group*Treatment

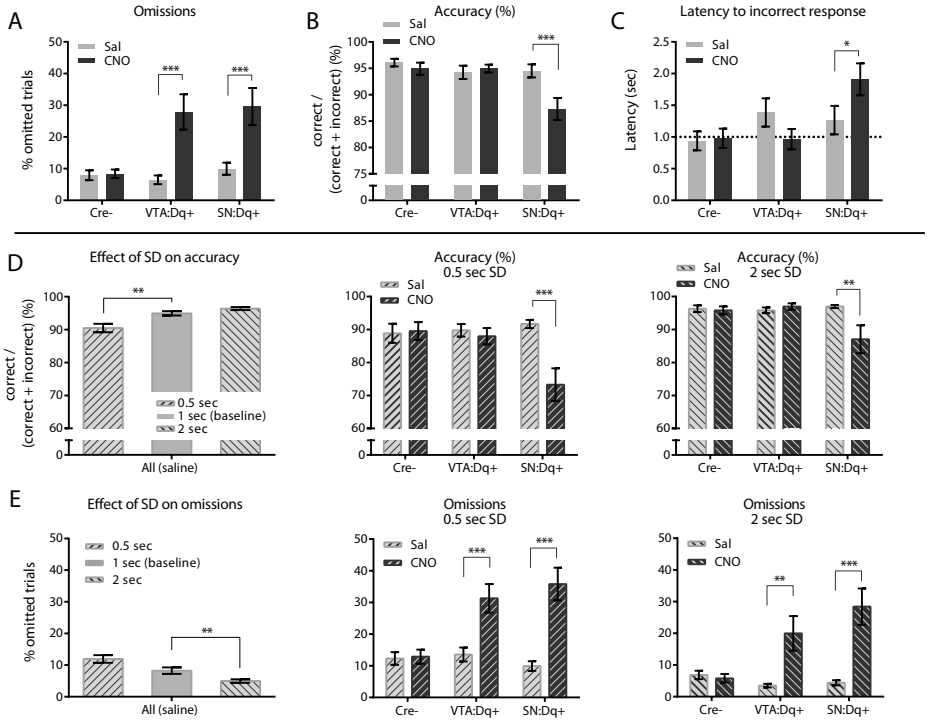


Figure 2. Chemogenetic activation of VTA or SNc DA neurons impairs attentional performance. *A) CNO increased number of omissions in VTA:Dq+ and SN:Dq+ group. B) CNO decreased accuracy in SN:Dq+ group. C) CNO increased latency to make an incorrect response in SN:Dq+ group to >1.0 sec (dotted line, represents stimulus duration, SD). D) Shorter SD decreased accuracy compared to baseline (left panel). CNO decreased accuracy in SN:Dq+ group, also with 0.5 or 2 sec SD. E) Longer SD decreased omissions compared to baseline. CNO increased number of omissions in VTA:Dq+ and SN:Dq+ groups, also with 0.5 or 2 sec SD. Error bars represent mean ± s.e.m. *P<0.05, **P<0.01, ***P<0.001.*

interaction $F_{2,37}$=10.186, P<0.0005; post hoc VTA:Dq+ P=0.009, SN:Dq+ P=0.001). SN:Dq+ rats also showed a delayed latency to make correct or incorrect responses, whilst this was unaffected in VTA:Dq+ rats (***Table 1*** and ***Fig 2C***, Group*Treatment interaction correct latency $F_{2,37}$=12.022, P<0.0005, post hoc SN:Dq+ P<0.0005, VTA:Dq+ P=0.147; incorrect latency $F_{2,37}$=3.654, P=0.036, post hoc SN:Dq+ P=0.025, VTA:Dq+ P=0.188).

To test whether the attentional deficits caused by CNO were dependent on attentional load, we tested the animals with a shorter (0.5 sec) and longer SD (2 sec). Indeed, SD affected attentional performance: a shorter SD decreased accuracy (***Fig 2D***; effect of SD $F_{1.57,32.28}$=19.603, P<0.0005; post hoc tests 0.5s SD vs baseline P=0.001; 2s SD

Table 1. Effects of chemogenetic activation of VTA or SNc DA neurons on performance in 5-CSRTT. *Figures represent mean ± s.e.m. *P<0.05, **P<0.01, ***P<0.001 CNO compared to saline (SAL).*

	Correct (#)				Incorrect (#)			
	SAL	sem	**CNO**	sem	**SAL**	sem.	**CNO**	sem
Cre-	**89.4**	2.1	**87.5**	4.7	**3.6**	1.0	**4.6**	1.2
VTA:Dq+	**88.8**	2.1	**68.7*** **	4.7	**5.5**	1.0	**3.8**	1.2
SN:Dq+	**86.1**	2.0	**62.3*** **	4.5	**4.8**	1.0	**8.6** **	1.2
	Perseverative responses (#)				**Latency correct (sec)**			
	SAL	sem	**CNO**	sem	**SAL**	sem.	**CNO**	sem
Cre-	**24.6**	7.8	**25.9**	6.6	**0.57**	0.03	**0.57**	0.03
VTA:Dq+	**23.0**	7.8	**14.8**	6.6	**0.55**	0.03	**0.59**	0.03
SN:Dq+	**36.2**	7.5	**24.4**	6.3	**0.56**	0.03	**0.75*** **	0.03
	Latency incorrect (sec)				**Latency collect reward (sec)**			
	SAL	sem	**CNO**	sem	**SAL**	sem.	**CNO**	sem
Cre-	**0.94**	0.21	**0.98**	0.20	**1.67**	0.13	**1.68**	0.13
VTA:Dq+	**1.39**	0.21	**0.97**	0.20	**1.64**	0.13	**1.40** **	0.13
SN:Dq+	**1.27**	0.20	**1.91***	0.19	**1.88**	0.13	**2.18** **	0.13

vs baseline P=0.069), whilst a longer SD decreased omissions (***Fig 2E***; effect of SD $F_{1.81,61.6}$=14.819, P<0.0005; post hoc tests 0.5s SD vs baseline P=0.066; 2s SD vs baseline P=0.009). However, the effect of CNO in SN:Dq+ or VTA:Dq+ rats was not affected by these changes in SD. Similar to baseline conditions, CNO decreased accuracy in the SN:Dq+ group (***Fig 2D***; 0.5s SD $F_{2,39}$=10.103, P<0.0005, post hoc SN:Dq+ P<0.0005; 2s SD $F_{2,35}$=4.935, P=0.013, post hoc SN:Dq+ P=0.001), and increased omissions in both VTA:Dq+ and SN:Dq+ groups (***Fig 2E***; 0.5s SD $F_{2,29}$=7.559, P=0.002, post hoc VTA:Dq+ and SN:Dq+, both P<0.0005; 2s SD $F_{2,35}$=7.343, P=0.002, post hoc VTA:Dq+ P=0.002, SN:Dq+ P<0.0005). With saline treatment, one third (33 ± 3.6%) of omissions occurred following a time-out – as opposed to 67% following a correct response. Although the percentage omissions made after a time-out was somewhat higher in VTA:Dq+ and SN:Dq+ groups following CNO treatment (49 ± 7.2% and 56 ± 5.0%, respectively), this difference did not reach statistical significance (Group*Treatment interaction $F_{2,36}$=2.138, P=0.133).

In summary, chemogenetic activation of DA neurons in either VTA or SNc significantly impaired attention, which was not dependent on attentional load. In VTA:Dq+ rats, CNO treatment increased omissions, whilst in SN:Dq+ rats, CNO treatment increased omissions and decreased accuracy. Furthermore, activation of VTA DA neurons decreased latency to collect rewards, whilst SNc DA neuron activation increased latency to collect reward, and to make correct and incorrect responses.

Effects of DA neuron activation on impulsivity

Under baseline conditions, CNO treatment did not affect the number of premature responses (***Fig 3A***; Group*Treatment interaction $F_{2,37}$=2.351, P=0.109), nor the latency to make a premature response (***Fig 3B***; Group*Treatment interaction $F_{2,37}$=1.050, P=0.360).

As a positive control for enhancement of impulsivity, we tested the effects of systemic amphetamine (AMPH) administration, or testing with a longer ITI of 7 seconds (7s ITI). Both manipulations have been reported to increase premature responding in the 5-CSRTT (Baarendse and Vanderschuren, 2012; Cole and Robbins, 1989; Dalley et al, 2007; Van Gaalen et al, 2006). We found that, as expected, both AMPH and a longer ITI increased the number of premature responses (***Fig 3C***; 0.3 mg/kg AMPH vs Sal t_{11}= -2.434, P=0.033; 1.0 mg/kg AMPH vs Sal t_{11}=-2.603, P=0.025; 7s ITI vs 5s ITI t_{12}=-4.951, P<0.0005; negative control Sal vs Sal t_{10}=-1.33, P=0.213).

Next, we tested whether CNO would affect impulsive behaviour under conditions of increased impulsivity, i.e., during a 7s ITI, or variable long ITIs, varying from 5 to 15 sec (vITI). We found that longer ITIs profoundly increased the number of premature responses under saline conditions (***Fig 3D***; main effect of ITI F2,70=82.431, P<0.0005; post hoc comparisons all P<0.0005). With longer ITIs, also the latency to make a premature response was increased (***Fig 3E***; main effect of ITI F1.7;58.3=140.446, P<0.0005; post hoc comparisons all P<0.0005). However, similar to baseline conditions, treatment with CNO did not affect the number of premature responses (***Fig 3D***; Group*Treatment interaction 7s ITI: F2,37=0.045, P=0.956; vITI: F2,39=0.9, P=0.415). CNO did significantly decrease the latency to make a premature response, in the VTA:Dq+ group both under 7s ITI and vITI conditions, and in the SN:Dq+ group under the vITI condition (***Fig 3E***; Group*Treatment interaction 7s ITI: F2,37=3.778, P=0.032, post hoc VTA:Dq+ P=0.003; vITI: F2,39=4.872, P=0.013, post hoc VTA:Dq+ P=0.003, SN:Dq+ P=0.025).

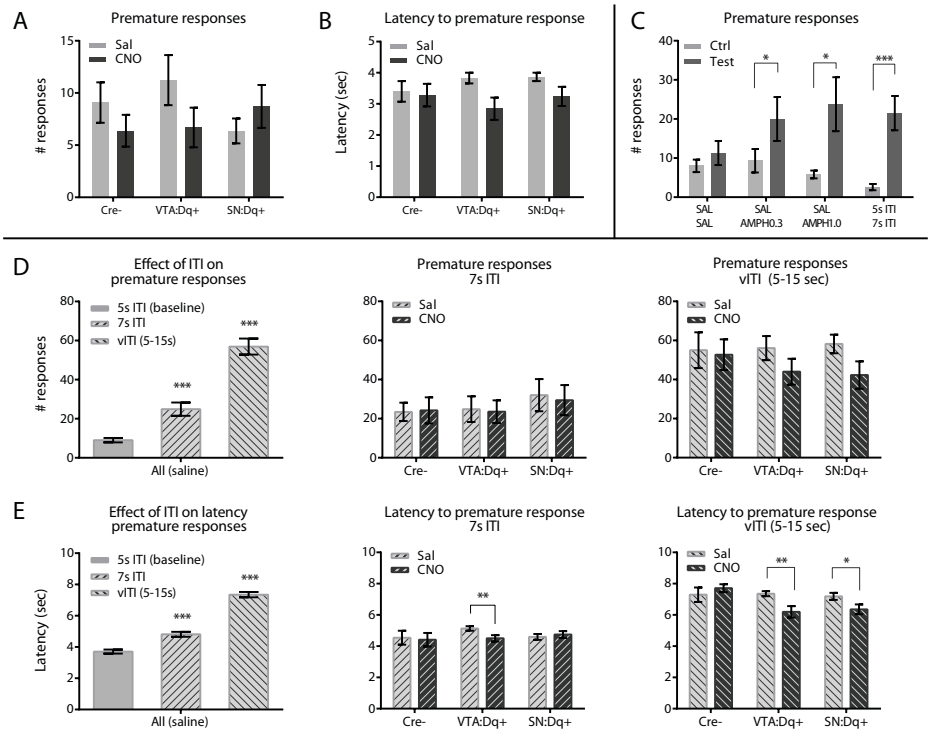


Figure 3. Chemogenetic activation of VTA or SNc DA neurons does not affect premature responses. *No significant effect of CNO vs saline on number of premature responses (A) or latency to make premature response (B). C) Systemic injections of amphetamine (0.3 or 1.0 mg/kg), or prolonged inter-trial interval of 7 sec (7s ITI) increased number of premature responses. D) Total number of premature responses was increased by challenges with longer ITI (7 sec ITI or variable long ITI compared to baseline), but was not affected by CNO. E) Latency to make premature responses was increased by challenges with longer ITI (7 sec ITI or variable long ITI compared to baseline), and decreased by CNO in VTA:Dq+ and SN:Dq+ group. Error bars represent mean ± s.e.m. *P<0.05, **P<0.01, ***P<0.001.*

In summary, chemogenetic activation of DA neurons in VTA or SNc did not affect the number of premature responses – either under baseline conditions, or during challenges with prolonged ITIs. DA neuron activation did reduce the latency to make a premature response, both in VTA:Dq+ and SN:Dq+ rats, especially during prolonged ITIs.

Dose-dependent effects of CNO on attention

To determine the dose-dependency of the effects of CNO, we tested five different doses, under baseline conditions (5 sec ITI, 1 sec SD). Consistent with our earlier results, we found no significant effect of CNO on premature responding (***Fig 4A***; Group*Treatment interaction $F_{7\cdot5,135\cdot3}$=1.239, P=0.284). CNO dose-dependently increased omissions in

both VTA:Dq+ and SN:Dq+ groups (***Fig 4B***; Group*Treatment interaction $F_{6.0,107.8}$=9.551, P<0.0005). In the SN:Dq+ group, CNO increased omissions at 0.03 mg/kg and higher (CNO vs saline P=0.001; 0.1 and 0.3 mg/kg, both P<0.001), whilst in the VTA:Dq+ group only the highest dose (0.3 mg/kg) significantly increased omissions (CNO vs saline P=0.004). In SN:Dq+ rats, doses of 0.03 mg/kg and higher also decreased accuracy (***Fig 4C***; Group*Treatment interaction $F_{4.6,82.7}$=5.515, P<0.0005; SN:Dq+ 0.03, 0.1 and 0.3 mg/kg CNO vs saline, all P<0.01), whilst response latency was increased at 0.1 and 0.3 mg/kg CNO (***Fig 4D***; Group*Treatment interaction $F_{4.2,74.8}$=5.643, P<0.0005; SN:Dq+ 0.1 and 0.3 mg/kg CNO vs saline, both P<0.001). No significant effects were found on the latency to collect the reward (***Fig 4E***; Group*Treatment interaction $F_{3.4,61.1}$=1.778, P=0.154) or the latency to make a premature response (***Fig 4F***; Group*Treatment interaction $F_{8,144}$=1.311, P=0.242). Thus, low doses of CNO (0.03 mg/kg and higher) were sufficient to impair attention in SN:Dq+ rats, whilst only the highest dose tested (0.3 mg/kg) significantly affected attention in VTA:Dq+ rats.

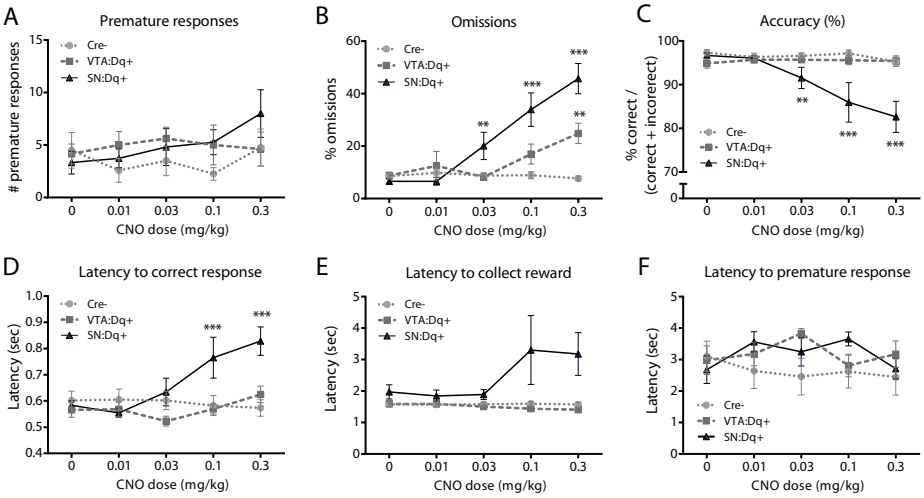


Figure 4. Dose-dependent effects of CNO on 5-CSRTT performance. *A) Premature responses were not affected by CNO. B) Omissions were increased at 0.03 mg/kg CNO and higher doses in SN:Dq+ group, and at 0.3 mg/kg CNO in VTA:Dq+ group. C) Accuracy was decreased at 0.03 mg/kg CNO and higher doses in SN:Dq+ group, and unaffected in VTA:Dq+ group. D) Latency to make a correct response was increased at 0.1 and 0.3 mg/kg CNO in SN:Dq+ group, and unaffected in VTA:Dq+ group. E-F) Latency to collect the reward and latency to make a premature response were not significantly affected by CNO. Error bars represent mean ± s.e.m. **P<0.01, ***P<0.001 CNO compared to saline (0 mg/kg CNO).*

Discussion

In this study, we show that chemogenetic activation of VTA and SNc DA neurons impairs attentional performance in the 5-CSRTT. In contrast, we found no evidence for increased impulsivity as a result of enhanced midbrain DA neuron activity. In addition, we show that DA neuronal activation in the VTA compared to SNc results in a distinct behavioural profile with respect to attentional accuracy and response latency. Although increasing DA neuron activity in both regions increased omissions, SNc DA neuronal activation decreased accuracy, and delayed correct response, incorrect response and reward collection latency, whilst VTA DA neuronal activation did not affect accuracy, correct response latency and incorrect response latency, and reduced reward collection latency. Thus, the attentional impairments in these groups were mediated through different mechanisms.

VTA DA neuronal activation impairs sustained attention and enhances behavioural responsivity

We found that chemogenetic activation of DA neurons in the VTA significantly increased omissions in the 5-CSRTT. This effect persisted during tests with a variable attentional load as a result of shorter or longer SDs. Furthermore, VTA DA neuron activation decreased the latency to collect rewards and to make premature responses, whilst the attentional accuracy was unaffected. These results are in line with a role for mesolimbic DA in the speed and probability of responding, but not in attentional accuracy (Cole and Robbins, 1989). In the present study, we have focused on the role of midbrain DA neuronal activity, whereas the majority of previous studies has investigated DA signalling in striatal and cortical target areas. Based on our immunohistochemical results, we presume that VTA DA neuron activation primarily resulted in enhanced DA signalling in the ventral and dorsomedial parts of the striatum. We expect a relatively small contribution of altered mesocortical DA signalling to the observed behavioural effects, since DA levels have been shown to be significantly lower in PFC compared to striatum (10-20 fold difference) (Ihalainen et al, 1999; Moghaddam et al, 1990), and we were not able to detect DREADD expression in the PFC. However, future studies should clarify the neurochemical effects of chemogenetic DA neuron activation in target regions of VTA DA neurons. Previous pharmacological studies have shown that infusions of DA-R antagonists or partial agonists into NAc or DMS increased omissions (Besson et al, 2010; Pezze et al, 2007), whilst local DA stimulation in NAc Core and Shell sub-regions did not affect attention in the 5-CSRTT (Economidou et al, 2012; Moreno et al, 2013). In addition, enhanced DA signalling in the medial PFC (mPFC), through reuptake inhibition or D1-R agonism, has been shown to decrease omissions (Agnoli and Carli, 2011; Economidou et al, 2012; Granon et al, 2000). These results are, to some extent, in

contrast with our observation that enhanced activity of mesocorticolimbic DA neurons increased the number of omitted trials. However, systemic administration of a D1- or D2-R agonist was shown to have the same effect (Winstanley et al, 2010). Importantly, in order to fully understand the contribution of DA signalling to omissions in the 5-CSRTT, it is essential to consider the broader behavioural phenotype and its underlying mechanisms. For example, treatment with selective D1- or D2-R antagonists may impair stimulus discrimination, resulting in more omissions as well as decreased accuracy (Pezze et al, 2007). In contrast, enhanced VTA DA neuronal activity may affect other functions, such as behavioural activation (see below), thereby affecting omissions and response latency, whilst leaving accuracy intact. Future studies are needed to further distinguish the neurochemical and functional mechanisms that determine the effects of DA signalling on attentional behaviour.

DA signalling in the NAc has been implicated in approach initiation, especially in anticipation of a reward (Hamid et al, 2015; Ko and Wanat, 2016; Nicola, 2010; Syed et al, 2015). We found that VTA DA neuronal activation reduced the rats' latency to retrieve rewards, and to make premature responses. In contrast, the latency to make correct or incorrect responses was unaffected. This suggests that, at least under some conditions, enhanced VTA DA neuronal activity promotes rapid action initiation. A fast response initiation may be interpreted as enhanced behavioural activation and/or increased motivation, processes that both involve mesolimbic DA (Nicola, 2010; Robbins and Everitt, 2007; Salamone and Correa, 2012; Wise, 2004). Previously, we have shown that chemogenetic activation of VTA mesolimbic neurons, or local D2-R knock-down, increased motivation to work for sucrose in rats (Boender et al, 2014; de Jong et al, 2015). However, in the present study, we showed that VTA DA neuron activation increased omissions, and did not affect latency to make a correct nose-poke, which contradicts a putative increase in motivation. Therefore, we propose that the behavioural profile, including more omissions and faster responses, may reflect effects on behavioural activation rather than motivation. Indeed, chemogenetic activation of midbrain DA neurons or VTA to NAc pathway has been shown to increase locomotor activity (Boender et al, 2014; Wang et al, 2013), consistent with a role for increased mesolimbic DA activity in locomotor hyperactivity (Ikemoto, 2002; Kelly et al, 1975). As a result, increased VTA DA neuronal activity likely interferes with the animal's ability to focus attention on a specific stimulus during a restricted time period, thus impairing sustained attention. Enhanced responsivity to cues may have a dual effect on task performance in the 5-CSRTT. On the one hand, it may facilitate the initiation of certain actions, resulting in faster responses. On the other hand, locomotor hyperactivity and responsivity to environmental stimuli that are not relevant for task performance would

distract the animal's attention away from the cue lights, and result in more omissions.

SNc DA neuronal activation impairs attention and delays responsivity

Chemogenetic activation of SNc DA neuronal activity caused significant attentional deficits in the 5-CSRTT, as apparent from increased omissions and reduced accuracy. In addition, response latencies were increased, i.e., the rats were slower to make nose-poke responses and to collect the reward. Interestingly, similar results have been observed following lesions of the DMS or DLS, or dorsal striatal DA depletion (Baunez and Robbins, 1999; Rogers et al, 2001). These findings indicate that the nigrostriatal DA pathway is crucially involved in attentional processes, and that disruption of this pathway, either by inhibition or stimulation, may severely affect behavioural performance.

The dorsal striatum is part of a neural circuitry involving frontal cortex and basal ganglia, that regulates divided attention and executive function (Alexander et al, 1986; Dalley et al, 2008; Wimmer et al, 2015). Within this circuitry, the dorsal striatum serves an important role in sensorimotor integration and response control (Nieoullon, 2002; Rogers et al, 2001). The DMS receives projections from the PFC, including medial PFC and anterior cingulate cortex (ACC) (Donoghue and Herkenham, 1986; McGeorge and Faull, 1989). Importantly, lesions or chemogenetic inhibition of these cortical regions have been shown to impair attentional accuracy and response latencies in the 5-CSRTT, similar to the effects of nigrostriatal DA dysfunction (Chudasama et al, 2003; Koike et al, 2015; Muir et al, 1996). In addition, glutamatergic and DAergic signalling in this cortico-striatal circuitry have been shown to interact in the regulation of attentional performance (Agnoli et al, 2013; Agnoli and Carli, 2011). Our results complement these findings by showing that enhanced DA neuronal activity in the SNc also results in impaired attention and response control, comparable to dorsal striatal or prefrontal dysfunction. Previously, it was shown that a selective D1-R agonist or antagonist infused into mPFC could improve or impair attentional accuracy in rats, respectively, depending on low or high baseline performance (Granon et al, 2000). Taken together, these results suggest that there is an optimal level of DA signalling required for accurate task performance. Here, we show a novel finding, indicating that also enhanced DA signalling in the nigrostriatal pathway can be detrimental for attentional performance. We found that SNc DA neuronal activation consistently increased omissions, impaired accuracy, and delayed response latency, irrespective of attentional load. An increase in omissions and response latencies could indicate a reduced motivational state or impaired motor function. However, activation of SNc DA neurons with a low dose of CNO, that did not affect response latencies, was sufficient to impair attention. Furthermore, optogenetic activation of SNc DA neurons has been shown to promote voluntary movements (Barter

et al, 2015). Together, this indicates that enhanced SNc DA neuronal activity is sufficient to impair attentional performance, without detrimental effects on motor function or motivation. Based on behavioural deficits in patients with Parkinson's disease, it has been suggested that nigrostriatal dysfunction affects the selection of an appropriate motor response, in particular when there is uncertainty about the stimulus and its associated response (Nieoullon, 2002). Again, our data suggest that enhanced nigrostriatal DA activity results in a similar phenotype: the animals appear to have difficulty selecting the appropriate response to an unpredictable stimulus, which may result in a slower response, selection of an incorrect motor response, or an omission. We suggest that enhanced SNc DA neuronal activity disrupts DA signalling in dorsal striatal regions, and thereby interferes with sensorimotor integration and the control of appropriate responses to environmental stimuli. However, relatively little is known about the effects of increased nigrostriatal DA activity, and future studies need to elucidate which anatomical and neurochemical aspects are causally related to the observed attentional impairments.

Midbrain DA neuronal activation does not induce impulsivity

Chemogenetic activation of DA neurons in either the VTA or SNc did not affect impulsivity in the 5-CSRTT. Previous studies have implicated DA in the control of impulsive actions, particularly DA signalling in the (ventral and dorsomedial) striatum (Agnoli et al, 2013; Cole and Robbins, 1989; Economidou et al, 2012; Moreno et al, 2013; Pattij et al, 2007; Pezze et al, 2007). Therefore, we hypothesized that enhancing midbrain DA neuronal activity, in particular of VTA DA neurons that project to NAc or DMS, would increase impulsivity. The discrepancy of our findings with previous reports may be related to several factors, including anatomical sub-region, magnitude of manipulation, and individual differences in impulsivity (Economidou et al, 2012; Moreno et al, 2013; Pezze et al, 2007; Winstanley et al, 2010). Importantly, the effects of CNO depend on endogenous neuronal activity. As mentioned above, neurochemical studies have indicated that phasic DA release in the NAc – related to VTA DA neuron burst firing – is particularly associated with action initiation in anticipation of a reward (Hamid et al, 2015; Roitman et al, 2004; Syed et al, 2015). This typically occurs in response to a cue that is associated with a reward, such as a light stimulus. When post-synaptic DA signalling in the NAc is pharmacologically elevated, e.g. by amphetamine or DA-R agonists, this may mimic the neurochemical situation of "action initiation in anticipation of a reward", and thus the animal is driven to make a response. During the ITI, this would result in a premature response. Indeed, consistent with previous findings (Baarendse and Vanderschuren, 2012; Cole and Robbins, 1989; Van Gaalen et al, 2006), we observed that systemic administration of amphetamine (inducing DA release at synaptic terminals) increased the number of

premature responses, and previous studies showed that that infusion of a DA-R agonist or a reuptake inhibitor into the NAc or DMS does the same (Agnoli et al, 2013; Moreno et al, 2013; Pezze et al, 2007). CNO, however, is expected to preferentially enhance DA activity when DA neurons are already endogenously active. During the ITI, when the animals are waiting and no particular cues are presented that predict reward availability, there is presumably low baseline DA neuronal activity (Hamid et al, 2015; Syed et al, 2015), although there is evidence for "ramping" activity in anticipation of the stimulus in VTA DA target areas (Donnelly et al, 2015). When the waiting period is longer than expected (prolonged ITI), this may drive the animal to make a response prior to stimulus onset. We found that, under these high-impulsive conditions, CNO reduced the latency to perform premature responses in both VTA:Dq+ and SN:Dq+ groups, but the total number of premature responses remained unaffected. Our results therefore indicate that enhancing endogenous DA neuron activity in either VTA or SNc may promote action initiation vigour, but is in itself not sufficient to induce impulsive actions.

Summary and conclusion

In this study, we determined the effects of enhanced DA neuronal activity in VTA and SNc on attentional performance and impulsive behaviour in rats. We found that VTA DA neuronal activation promotes the vigour of goal-directed actions, but also impairs sustained attention. These effects likely result from enhanced behavioural responsivity. On the other hand, SNc DA neuronal activation impaired sustained attention and accuracy, and slowed responsiveness in the task. This may be attributed to disrupted sensorimotor integration, resulting in impaired execution of a stimulus-induced response. Thus, DA neurons in the VTA or SNc support distinct aspects of cognitive performance. Our findings contribute to the understanding of the neural substrates underlying attention deficits and impulsivity, and provide valuable insights to improve treatment of these symptoms in the clinic.

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CHAPTER SIX

I got energy, got a lotta energy
- Adapted from Drake, "Energy"

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SUMMARY AND GENERAL DISCUSSION
INSIGHTS INTO THE ROLE OF MIDBRAIN DOPAMINE NEURONAL ACTIVITY IN BEHAVIOUR, AND IMPLICATIONS FOR PSYCHIATRY

In this thesis, we aimed to further elucidate the role of dopamine (DA) in the regulation of behaviour. We determined the effects of chemogenetic activation of DA neurons on a variety of behavioural domains relevant to psychiatric disorders. Using designer receptors exclusively activated by designer drugs (DREADD) technology in rats, we tested the impact of DA neuron activation on locomotor activity (*Chapter 2*), motivational behaviour (*Chapter 3*), feeding patterns (*Chapter 4*), and impulsivity and attention (*Chapter 5*). Here, I will briefly summarize our findings, and combine them in order to provide a comprehensive image of the behavioural consequences of chemogenetic DA neuron activation in the ventral tegmental area (VTA) or substantia nigra pars compacta (SNc). Furthermore, I discuss how these findings translate to neuropsychiatric disorders, with a focus on schizophrenia and ADHD. Finally, I present a careful yet hopeful glimpse into the future, providing a perspective on the potential of DREADD technology in the development of novel treatments.

Highlights

- Chemogenetic activation of midbrain DA neurons, using DREADD technology, profoundly affected behaviour in rats

- Activation of VTA DA neurons, or of the mesolimbic pathway from VTA to ventral striatum, induced locomotor hyperactivity, increased motivational behaviour, disrupted feeding patterns, and impaired sustained attention

- Activation of SNc DA neurons modestly increased locomotor activity, and significantly impaired attention, but did not affect motivational behaviour or feeding patterns

- We propose that enhanced mesolimbic DA neuronal activity promotes behavioural activation, resulting in a hyperactive and distracted phenotype, whilst increased SNc DA neuron activity disrupts selection of appropriate motor actions, resulting in attentional deficits

- These findings have implications for understanding the neural substrates underlying psychiatry-related behavioural domains, and for the development of novel, target-specific treatments in psychiatry

Summary of findings

In *Chapter 2*, we showed that chemogenetic activation of DA neurons in either the VTA or SNc was sufficient to increase locomotor activity in rats. Whilst SNc DA neuron activation modestly enhanced locomotion, VTA DA neuron activation resulted in a pronounced hyperactive phenotype. In a follow-up experiment, we combined DREADD with canine adeno-virus expressing Cre recombinase (CAV2Cre) to activate selective pathways, and found that specifically the projection from VTA towards nucleus accumbens (NAc) was crucial for inducing locomotor hyperactivity.

The behavioural effects in this study clearly demonstrated the pharmacodynamics of selective DREADD ligand clozapine-N-oxide (CNO) in the presence of the excitatory DREADD hM3Dq. Within 30 minutes of peripheral administration, CNO increased locomotor activity in rats expressing hM3Dq in VTA DA neurons, and the greatest behavioural effect was reached 60-90 minutes after CNO injection. Using our default dose of 0.3 mg/kg, CNO-induced locomotor hyperactivity lasted for several hours. A dose-response study showed similar effects for 0.1 and 1.0 mg/kg, whilst the lowest dose of 0.03 mg/kg resulted in an intermediate hyperactive phenotype. These results are an experimental confirmation of the hypothesis that low doses of the designer drug (CNO) will yield maximal effects, due to abundant presence of designer receptors (hM3Dq) (Roth, 2016).

In *Chapter 3*, we showed that chemogenetic activation of DA neurons in the VTA, but not SNc, increased motivational behaviour, as indicated by enhanced responding for sucrose under a progressive ratio (PR) schedule of reinforcement. We found that VTA DA neuron activation specifically promoted the repeated initiation of reward-seeking actions, resulting in elevated effort exertion. These findings provide valuable insights into how DA neuronal activity regulates selective aspects of motivational behaviour, and may thereby aid the development of treatments for certain motivational deficits found in psychiatric disorders, such as major depressive disorder.

In *Chapter 4*, we investigated the effects of DA neuron activation on food intake. As shown in *Chapter 3*, VTA DA neuron activation increases motivation for food, but much debate remains whether enhanced DA neuronal activity actually stimulates or inhibits free food consumption. We found that activation of DA neurons in the VTA, but not SNc, significantly disrupted feeding behaviour. This effect was mediated by mesolimbic projections towards the NAc, and was characterized by smaller and shorter meals, along with an increased feeding frequency. In contrast to the anorexic effects of psychostimulant drugs (that enhance DA signalling via reuptake inhibition), activation of

mesolimbic DA neurons did not suppress the initiation to engage in feeding or total food intake. Together, these results indicate that enhanced mesolimbic DA neuronal activity promotes the initiation as well as the cessation of feeding behaviour, without directly affecting appetite. We propose that these effects may be secondary to an increase in locomotor activity.

In ***Chapter 5***, we tested the effects of chemogenetic activation of DA neurons in either VTA or SNc on performance in a sustained attention task. Activation of DA neurons in both populations significantly impaired attentional performance in the 5-choice serial reaction time task (5-CSRTT), reflected by an increase in omitted trials. In addition, SNc DA neuron activation impaired attentional accuracy and delayed response latency. In contrast, VTA DA neuron activation did not affect attentional accuracy, and reduced response latency. We hypothesized that activation of DA neurons would increase impulsivity, based on previous pharmacological studies. Surprisingly, however, impulsive actions were not affected. Together, our results show that enhanced DA neuronal activity in either the VTA or SNc is sufficient to impair attention, but not to induce impulsive behaviour. Furthermore, we show that VTA and SNc DA neuronal populations are involved in dissociative aspects of attentional performance.

Taken together, we found dissociative behavioural effects following chemogenetic activation of DA neurons in the VTA compared to SNc, summarized in ***Table 1***. In short, DA neuronal activation in the VTA: i) induced locomotor hyperactivity; ii) increased motivation to work for palatable food; iii) disrupted feeding patterns; and iv) impaired sustained attention. These effects were typically replicated by selective activation of VTA neurons projecting towards the ventral striatum (including NAc). In contrast, chemogenetic activation of SNc DA neurons: i) modestly enhanced locomotor activity; ii) did not affect motivation to work for palatable food and iii) did not affect feeding patterns; but iv) significantly impaired attention and response accuracy. These findings highlight the differential behavioural functions of midbrain DA neurons in VTA compared to SNc.

Table 1. Behavioural effects of chemogenetic DA neuron activation. *Summary of findings presented in this thesis. Arrows represent a significant increase (↑) or decrease (↓) of chemogenetic activation or pharmacological DA stimulation. = represents no significant difference between drug and vehicle. For locomotor activity, the magnitude of effect is depicted by the number of arrows. A dot is placed when results are not tested or not presented in this thesis. DMS = dorsomedial striatum, PFC = prefrontal cortex, Amy = amygdala, DARI = dopamine reuptake inhibitor. B refers to Boender et al., 2014.*

Chemogenetic activation vs *Behavioural effect*	Locomotor activity *(Chapter 2,,4)*	Motivation for sucrose *(Chapter 3)*	Feeding behaviour (intake / meal size / frequency) *(Chapter 4)*	Impulsivity *(Chapter 5)*	Sustained attention *(Chapter 5)*	Attentional accuracy *(Chapter 5)*
VTA DA neurons	↑↑↑	↑	↓ / ↓ / ↑	=	↓	=
SNc DA neurons	↑	=	=	=	↓	↓
VTA>NAc pathway	↑↑↑	↑(B)	= / ↓ / ↑	.	.	.
VTA>DMS pathway	↑	.	.	.	.	.
VTA>PFC pathway	=	.	= / = / =		.	.
VTA>Amy pathway	=	.	= / = / =	.	.	.
Amphetamine	↑↑	.	↓ / ↓ / ↑	↑	.	.
GBR 12909 (DARI)	↑↑	.	↓ / ↓ / ↓	.	.	.

Insights into the role of DA neuronal activity in regulating behaviour

"Dopamine Go" – consistent effects of enhanced mesolimbic DA activity on behavioural activation

Throughout our studies, we consistently observed effects indicating that chemogenetic activation of VTA DA neurons induced (excessive) behavioural activation. Following activation of VTA DA neurons, rats showed: i) locomotor hyperactivity (***Chapter 2***); ii) enhanced initiation of reward-seeking actions, resulting in increased effort exertion under a PR schedule (***Chapter 3***); iii) enhanced initiation of feeding, as well as premature cessation of ongoing feeding activities (***Chapter 4***); and iv) faster responses in the 5-CSRTT, along with a disability to sustain attention towards an unpredictable stimulus (***Chapter 5***). Together, these results suggest that the animals were unable to sit still, or focus longitudinally on a single activity or stimulus. Thus, enhanced mesolimbic DA activity seemed to provide a "Go" signal, promoting responsivity to stimuli, and the initiation of novel actions.

Mesolimbic DA signalling is crucially involved in locomotor hyperactivity, responsivity to (unpredicted) environmental cues, and behavioural switching (Ikemoto, 2002; Nicola, 2007; Robbins and Everitt, 2007). The majority of VTA DA neurons projects towards the NAc (Bjorklund and Dunnett, 2007), suggesting that our findings mainly reflect results of enhanced DA signalling in the NAc. Indeed, we found that the behavioural effects were typically replicated by selective chemogenetic activation of the mesolimbic pathway from VTA towards ventral striatum (which, in turn, primarily consists of DA neurons, see ***Chapter 2*** and Boender et al, 2014). Locomotor hyperactivity was selectively induced by activation of projections towards NAc, but not towards dorsomedial striatum (DMS) (***Chapter 2***). VTA>NAc pathway activation disrupted feeding patterns, whilst activation of VTA neurons projecting towards prefrontal cortex (PFC) or amygdala did not (***Chapter 4***). Previously, we showed that chemogenetic activation of VTA>NAc pathway increased motivation for palatable food (Boender et al, 2014), similar to VTA DA neuron activation (***Chapter 3***). We hypothesize that also the impaired sustained attention and reduced response latency observed in the 5-CSRTT following VTA DA neuron activation were driven by NAc DA signalling (***Chapter 5***). Since enhanced DA signalling in the NAc promotes behavioural activation and responsivity to environmental stimuli (du Hoffmann and Nicola, 2014; Robbins and Everitt, 2007), it is likely to underlie enhanced response vigour as well as increased distractibility. Importantly, sub-maximal activation of VTA DA neurons expressing hM3Dq with a low dose of CNO was sufficient to increase locomotor activity (***Chapters 2 and 4***), but not to disrupt feeding behaviour (***Chapter 4***), or impair

attention (***Chapter 5***). This may indicate that a greater DAergic response was necessary to affect these latter behaviours. However, the nature of the observed effects – i.e., enhanced behavioural switching and responsivity to stimuli – suggests that they were driven by a general increase in behavioural activation, which was maximal when VTA DA neurons were activated by higher doses of CNO.

Interestingly, we found that enhancing endogenous activity of VTA DA neurons was not sufficient to induce impulsive action (***Chapter 5***), or to suppress food intake (***Chapter 4***). This was somewhat surprising, since psychostimulant drugs that enhance DA signalling (such as amphetamine) do have these effects, as we confirmed in our studies. As discussed in ***Chapters 4 and 5***, we propose that these dissociative effects may be attributed to differences in DA dynamics following chemogenetic DA neuron activation compared to pharmacological DA stimulation. Psychostimulants increase post-synaptic DA activity by blocking reuptake and/or evoking pre-synaptic DA release. In contrast, chemogenetic activation enhances endogenous neuronal activity. These different mechanisms may have dissociative effects on the level and duration of synaptic DA signalling, which may underlie the observed discrepancy in behavioural effects. In addition, systemic administration of drugs affects DA functioning throughout the brain, compared to anatomically restricted effects following chemogenetic activation of selected neuronal populations or pathways. Although further experiments are needed to evaluate the neurochemical effects of chemogenetic DA neuron activation and their relationship to behavioural outcomes, our findings highlight that these effects may be crucially different compared to pharmacological DA stimulation.

In summary, we found that chemogenetic activation of VTA DA neurons has a profound effect on a broad spectrum of behavioural outputs. The overall behavioural profile suggests that the majority of effects could be explained by an increase in behavioural activation, resulting from enhanced mesolimbic DA activity. Whilst the effects were often comparable to those of pharmacological DA stimulation, we also observed significant differences. Although our approach has yielded robust behavioural outcomes following activation of VTA DA neurons, or the VTA>NAc pathway, increased behavioural activation may also obscure other putative functions of mesolimbic DA. I propose that, for future studies, it will be important to consider behavioural effects of low doses of CNO, in order to avoid non-selective, confounding, effects of locomotor activity. Alternatively, one should investigate functions that do not depend on behavioural activity. This may include sensory gating, measured by electro-encephalography, or functional brain responses, assessed with neuro-imaging techniques.

SNc DA neuron activation interferes with cortico-striatal regulation of motor patterns

As clinical studies have implied a role for nigrostriatal DA signalling in the pathophysiology of, a.o., schizophrenia and ADHD (Ernst et al, 1999; Fusar-Poli and Meyer-Lindenberg, 2013; Heckers and Konradi, 2013; Ludolph et al, 2008), we also examined the behavioural consequences of chemogenetic activation of DA neurons in the SNc. However, compared to VTA, enhanced DA neuronal activity in the SNc resulted in relatively modest effects in the majority of our experiments, despite equal levels of DREADD expression. Nigrostriatal DA neuron activation mildly increased locomotor activity (***Chapter 2***), and did not affect food motivation or consumption (***Chapters 3 and 4***). Although this might suggest that VTA DA neurons are perhaps more susceptible to chemogenetic activation, we found that in vitro DA neurons expressing hM3Dq in either VTA or SNc showed a very similar response to CNO (***Chapter 2***). Somewhat surprisingly, following these initial inconspicuous effects, we found that chemogenetic activation of SNc DA neurons profoundly affected performance in the 5-CSRTT (***Chapter 5***). SNc DA neuron activation increased trial omissions, reduced attentional accuracy and delayed response latencies, indicating significant attentional impairments.

The precise role of nigrostriatal DA signalling in regulating reward-related behaviours and cognitive functioning remains poorly understood. A current hypothesis is that nigrostriatal DA is mainly involved in action selection based on predictive and habitual stimulus-response associations (Balleine and O'Doherty, 2010; Nicola, 2007). Nigrostriatal DA is necessary for voluntary movements (Hnasko et al, 2006; Szczypka et al, 2001; Zhou and Palmiter, 1995), which may complicate the interpretation of (non-selective) motoric effects following lesions or DA depletion, particularly in dorsolateral striatum (Baunez and Robbins, 1999; Eagle et al, 1999). Electrophysiological recordings from midbrain DA neurons in behaving animals have shown that these cells display reward prediction signals (Schultz et al, 1997). In addition, neuronal firing patterns in the nigrostriatal circuit were found to particularly encode the onset and cessation of actions (Jin et al, 2014; Jin and Costa, 2010). Although some have proposed that nigrostriatal DA is crucially involved in motivational processes (Ikemoto et al, 2015; Palmiter, 2008), others have argued that SNc DA neuronal activity is best explained by the encoding of motor actions (Barter et al, 2015). Our findings indicate that enhanced activity of SNc DA neurons does not directly affect motivation for food or food intake (***Chapters 3 and 4***). However, we propose a vital role for nigrostriatal DA in higher-level functioning (in addition to locomotor control), involving sensorimotor integration and appropriate action selection in response to unpredictable reward-associated stimuli (***Chapter 5***). Congruently with behavioural deficits observed in Parkinson's disease, SNc DA neurons

seem to be particularly involved in coordination of multi-faceted, sequential actions, in response to variable environmental events (Nicola, 2007; Nieoullon, 2002).

Contribution to fundamental understanding of neurocircuitry of behaviour

What have these studies contributed to the field of (translational) neuroscience? Our findings that enhanced mesolimbic DA activity affects locomotor activity and incentive motivation may be not particularly surprising. So what has been the added value of the approach we used here to study the role of DA in behaviour?

Firstly, one of the reasons that chemogenetic and optogenetic approaches have gained such great popularity over the last few years is the ability to directly control neuronal activity in freely moving animals (Deisseroth, 2015; Roth, 2016). This allows us to investigate the behavioural functions of selective neuronal subpopulations and neuro-circuitries. To some extent, chemogenetic and optogenetic studies have confirmed what was already hypothesized based on prior studies, using lesions, DA depletion, electrical stimulation, or pharmacological manipulations. Nonetheless, it is crucial to affirm that alterations in neuronal activity affect behaviour similarly compared to post-synaptic manipulations. In the studies presented here, we found that enhanced mesolimbic DA neuronal activity induced locomotor hyperactivity and enhanced incentive motivation, as was hypothesized based on the literature (e.g., Canales and Iversen, 1998; Salamone and Correa, 2012) (***Chapters 2 and 3***). However, we also observed significantly different behavioural effects following chemogenetic and pharmacological approaches. For example, whilst treatment with DA-enhancing drug amphetamine induced impulsive behaviour and suppressed the initiation of food intake, these effects were not observed following chemogenetic activation of DA neurons (***Chapters 4 and 5***).

Secondly, whilst the majority of previous research has focused on the necessity of DA signalling for certain behavioural functions, we have explored whether or not enhanced midbrain DAergic activity is sufficient to drive and affect behaviour. For example, a major line of evidence implicating mesolimbic DA function in motivational behaviour is derived from rodent studies using DA depletions or DA-R antagonists (e.g., Nunes et al, 2013; Salamone et al, 2009). Our findings contribute to this evidence, by showing that enhanced mesolimbic DA neuronal activity promotes motivational responding for palatable food (***Chapter 3***). On the other hand, nigrostriatal DA signalling was found to be necessary for normal feeding behaviour (Hnasko et al, 2006; Szczypka et al, 2001), whereas we found that increased SNc DA neuron activity was not sufficient to affect food consumption (***Chapter 4***). Finally, our observation that enhanced SNc DA neuron

activity produced similar attentional deficits compared to diminished nigrostriatal DA function (*Chapter 5*), underlines the inverted U-shaped curve between DA levels and cognitive function (Cools and D'Esposito, 2011). Together, these findings highlight the complexity of DA's function in modulating behaviour and cognition.

Thirdly, the studies in this thesis specifically address the functional dissociation between DA neurons in the VTA compared to SNc. As the majority of experimental studies investigates either the VTA or SNc, or makes no distinction between the two, this complicates a direct comparison between these major midbrain DA neuron populations. Previously, pharmacological studies have provided a well-characterised functional anatomy in the striatum, e.g., regarding psychostimulant-induced effects on locomotor activity (Canales and Iversen, 1998; Ikemoto, 2002), or hedonic reactions to palatable food (Peciña and Berridge, 2005; Richard et al, 2013). With our studies, we hope to initiate a similar approach towards behavioural functionality within the DAergic midbrain. Anatomical studies have already provided an extensive overview of similarities and differences in connectivity (Haber, 2014; Ogawa et al, 2013), which may be used to guide targeted investigations of selective inputs and outputs.

Limitations and future directions

The research presented in this thesis contains several limitations, which may challenge the interpretation of our results. Our aim was to chemogenetically control specific neuronal subsets, in order to elucidate their role in the regulation of behaviour. Using Cre-dependent DREADD viruses combined with either TH::Cre rats or CAV2Cre, we were able to selectively target DAergic (TH-positive) cells, or neurons projecting to a certain area. Ideally, however, we would target a neuronal population based on both cell-type and projection-specificity. Thus, rather than activating all VTA DA neurons (including projections to NAc, PFC, and more) or activating all VTA neurons projecting to NAc (including DA and non-DA cells), it would be preferable to selectively control VTA neurons that are both DAergic and project to NAc. Technically, it is possible to locally infuse CNO using a cannula to obtain projection specificity (Mahler et al, 2014; Stachniak et al, 2014). However, this approach compromises the advantages of DREADD that it is both relatively non-invasive, and suitable for long-term experimental studies (typically, repeated cannulated intra-cerebral infusions may cause damage to brain tissue, and the cannula may get clogged over time). Furthermore, the neurochemical and (thus) behavioural effects of terminal stimulation may differ compared to cell soma stimulation. Interestingly, novel advanced viral techniques have been developed that allow for cell-type and projection specificity, using a combination of Cre and Flp ("flip") recombinase (Fenno et al, 2014). Infusion of a Cre-dependent CAV-Flp in the NAc will

induce Flp expression selectively in Cre-positive cells projecting to this area (e.g., DA cells in a TH::Cre rat). Subsequent expression of a Flp-dependent DREADD will then induce DREADD expression selectively in DA neurons projecting to the NAc.

Furthermore, we found that infusing one microliter of virus into the brain was sufficient to induce expression of DREADD or Cre in relatively large areas, enabling activation of the entire VTA or SNc. However, this also resulted in some overlap between the transfected areas in rats that received DREADD virus infused into the VTA (VTA:Dq+) or SNc (SN:Dq+) (see ***Chapters 2 and 5*** for quantification). Similarly, infusion of CAV2Cre into the NAc often resulted in DREADD expression at terminals in the DMS. This suggests that relatively few virus particles, which may enter the DMS via the needle injection tract, were sufficient to induce Cre expression (***Chapter 2***). Smaller volumes, lower titres, and/or a different serotype of the infused viruses should contribute to a more restricted anatomical specificity (see also Burnett and Krashes, 2016).

Although we found clear and robust behavioural effects of chemogenetic DA neuronal activation, it remains challenging to interpret the role of DA neuronal activity in behaviour based on these results. One important limitation is that we currently do not know how chemogenetic activation affects DA neuronal activity in vivo. In vitro, we observed that CNO potently increased neuronal activity of DA neurons expressing hM3Dq. However, in an intact animal, these neurons are under the control of numerous inhibitory and excitatory inputs, regulating tonic and phasic firing activity (Chen and Lodge, 2013). Tonic and phasic firing patterns may have significantly different effects on behavioural functions – i.e., it has been proposed that tonic firing is involved in regulating motivational state, whilst phasic firing is involved in reward prediction (Wise, 2013). As such, in order to fully understand how enhanced DA neuronal excitability affects certain behaviours, it is crucial to measure DA neuronal activity and/or downstream DA signalling during these behaviours. This may established by direct electrophysiological recordings of DA neuronal activity, or by measuring downstream effects of tonic and phasic activity, using microdialysis or fast-scan cyclic voltammetry, respectively. In addition, novel techniques including calcium imaging or photometry may yield valuable information on the neuronal and downstream effects of optogenetic and chemogenetic manipulations of neural circuits (Gunaydin et al, 2014; Steculorum et al, 2016). In order to improve translation of preclinical research to the clinic, it will be highly informative to investigate the effects of chemogenetic DA neuron activation using imaging techniques that are also used in humans, including functional magnetic resonance imaging or positron emission tomography (Lohani et al, 2016).

Finally, in order to get a complete picture of the role of DA neuronal activity in the regulation of behaviour, we would have to determine the effects of enhanced as well as inhibited neuronal excitability. This could be achieved using the inhibitory DREADD, hM4Di, which is also activated by CNO (Rogan and Roth, 2011). Furthermore, a novel inhibitory DREADD, the kappa-opioid receptor DREADD (KORD), has been developed with a different ligand, Salvinorin B (Marchant et al, 2015; Vardy et al, 2015). Thus, the combination of hM3Dq with KORD allows for excitation and inhibition of (DA) neurons within the same animal. Although future studies are needed to optimize this novel DREADD (in particular the solubility of the ligand needs to be improved for behavioural studies (Vardy et al, 2015)), it still provides promising opportunities for the bidirectional chemogenetic control of neuronal subpopulations and circuitries.

Implications for the role of DA neuronal activity in psychiatric disorders

The goal of our studies was to advance understanding of the role of DA neuronal activity in regulating behavioural domains that are disturbed in psychiatric disorders. By determining the direct impact of chemogenetic DA neuron activation on specific behavioural outcomes, we may infer whether or not enhanced DA neuronal activity may contribute to specific symptoms. Furthermore, our findings have implications for the development of novel treatments for psychiatric disorders. In the previous chapters, we have discussed the role of increased DA neuronal activity in specific behavioural domains, including locomotion, motivation, and attention, which may be relevant to multiple disorders. Here, I briefly review our studies with respect to two specific psychiatric disorders that have been associated with aberrant DA neuronal activity: schizophrenia and ADHD.

Relevance of DA neuron activation to aberrant salience in schizophrenia

In healthy animals (including humans), midbrain DA neurons increase firing in response to salient stimuli that require the animal to shift their attention, or adjust their behaviour. Accumulating evidence supports that (psychosis in) schizophrenia is related to increased spontaneous activity of DA neurons, which results in aberrant salience processing (Grace, 2015; Howes and Kapur, 2009; Maia and Frank, 2016). Although schizophrenia (or psychosis) cannot be fully modelled in rodents, we can model a specific patho-physiological substrate, and determine the direct effects on behavioural functioning. Here, we used DREADD technology to increase excitability of midbrain DA neurons, which may be a promising method to model aberrant salience. Behaviourally, the attribution of significance to irrelevant stimuli may be manifested as exaggerated responsivity to external stimuli, and impaired selection of appropriate actions (mediated by striatal

DA signalling). Our findings suggest that chemogenetic activation of (mesolimbic) VTA DA neurons resulted in enhanced behavioural responsivity, indicated by locomotor hyperactivity (***Chapter 2***), behavioural switching (***Chapter 4***), and impaired sustained attention (***Chapter 5***). In addition, enhanced responding at both the active and inactive lever in a motivational task (***Chapter 3***) may reflect increased responsivity to both relevant and irrelevant cues, and a suboptimal allocation of effort. The latter may be relevant to motivational deficits in schizophrenia (Gard et al, 2014; Whitton et al, 2015), although future studies are needed to further clarify which aspects of motivational behaviour are disturbed in this disorder. Chemogenetic activation of DA neurons in the SNc modestly increased locomotor activity, and impaired sustained attention, as well as attentional accuracy (***Chapters 2, 4, and 5***), indicating deficits in appropriate action selection. Through (direct or indirect) connections with the PFC, enhanced SNc DA activity may adversely affect various executive functions, including working memory and cognitive flexibility (Cools and D'Esposito, 2011). Together, these results suggest that enhanced activity of DA neurons in both VTA and SNc may contribute to aberrant salience processing in schizophrenia, although the two populations likely mediate different functional deficits.

Involvement of both VTA and SNc DA neurons in the pathophysiology of schizophrenia is in line with findings from neuro-imaging studies showing that pre-synaptic DA synthesis capacity is elevated throughout the striatum (Fusar-Poli and Meyer-Lindenberg, 2013). Interestingly, the associative striatum has been consistently identified as striatal subdivision showing enhanced (18)F-DOPA binding associated with psychosis (Demjaha et al, 2012; Egerton et al, 2013; Howes et al, 2011; Reith et al, 1994). This area is equivalent to DMS in rodents, which is innervated by DA neurons located in the VTA and medial part of the SNc (Bjorklund and Dunnett, 2007). Indeed, both VTA:Dq+ and SN:Dq+ groups showed DREADD expression in the DMS in our studies (***Chapters 2 and 5***). We found that selective activation of the midbrain pathway towards DMS modestly increased locomotor activity (***Chapter 2***). Furthermore, we hypothesize that the attentional deficits induced by SNc DA neuron activation primarily result from disrupted DA signalling in this area (***Chapter 5***), although future studies are needed to confirm this. Together, these findings indicate that midbrain DA neuronal projections towards the dorsomedial / associative striatum may be an interesting target for the investigation of aberrant salience processing in both preclinical and clinical studies.

Relevance of DA neuron activation to ADHD: effects on hyperactivity and attention, but not impulsivity

In this thesis, we have evaluated the role of DA neuronal activity in several behavioural domains that are relevant to ADHD, including hyperactivity, impulsivity and attention. Chemogenetic activation of DA neurons in the VTA resulted in locomotor hyperactivity (*Chapter 2*), along with increased initiation of instrumental actions (*Chapter 3*), and enhanced behavioural switching during food intake (*Chapter 4*). This led us to hypothesize that enhanced excitability of DA neurons in the VTA (but not SNc) interferes with an animal's ability to sit still, and to maintain focus on a single activity or stimulus, reminiscent of an "ADHD-like" phenotype. In order to test this hypothesis, we determined the effects of chemogenetic VTA DA neuron activation on performance in the 5-CSRTT. We found that, indeed, enhancing VTA DA neuron activity significantly impaired sustained attention, as was reflected by an increase in omitted trials (*Chapter 5*). Our dose-response studies showed that a low dose of CNO, which resulted in an intermediate increase in locomotion (*Chapter 2*), was not sufficient to affect sustained attention or response vigour (latency). This suggests that impaired attention was related to locomotor hyperactivity in these animals, possibly reflecting excessive behavioural activation or responsivity to (irrelevant) stimuli (*Chapter 5*).

Surprisingly, the hyperactive and distracted phenotype induced by VTA DA neuron activation was not accompanied by increased impulsivity (*Chapter 5*). Chemogenetic activation of VTA DA neurons reduced the latency of premature actions – consistent with a role for mesolimbic DA in approach initiation and action vigour (Hamid et al, 2015; Ko and Wanat, 2016; Nicola, 2010). However, the number of premature responses was not affected, even when impulsive behaviour was evoked by prolonging the inter-trial interval, suggesting that impulse control was not affected. In contrast, treatment with amphetamine increased impulsivity, consistent with findings from literature (Baarendse and Vanderschuren, 2012; Cole and Robbins, 1989; Van Gaalen et al, 2006). This suggests that post-synaptic actions of DA signalling, perhaps mediated by DAT functioning, may be necessary to induce impulsive actions. Together, our results indicate that hyperactive and distracted features of ADHD may be related to increased activity of mesolimbic DA neurons, whilst impulsive actions are not.

Limitations and future directions

As mentioned in *Chapter 1*, there any many factors that make it particularly challenging to decipher the neurobiological substrates underlying psychiatric disorders. Even more challenges arise when we aim to translate these substrates across species. Psychiatric disorders encompass multiple symptoms and multiple (neuro)biological substrates, such

as dopaminergic, glutamatergic, and inflammatory dysfunction in schizophrenia (Howes et al, 2015; Kahn and Sommer, 2015). In ADHD, therapeutic efficacy of noradrenaline reuptake inhibition suggests a noradrenergic component in its pathophysiology, in addition to altered DA signalling (Walker et al, 2015). Furthermore, psychiatric disorders often involve developmental deficits, as well as adverse cognitive schemas (Howes and Murray, 2013). Thus, the acute manipulation of a single neuronal substrate in a rodent model, such as midbrain DA neuronal activity, is not expected to mimic a complete neuropsychiatric state. Although we can investigate the neuronal circuitry involved in specific behavioural domains, not all clinical symptoms may be modelled in non-human animals, particularly those symptoms involving a self-reflective component. For all translational research, it remains vital to study readouts that can be (similarly) assessed in humans and animal models (Kas et al, 2011). A valuable addition to behavioural studies may lie in behaviourally-independent measures such as sensory gating, assessed by electro-encephalography, or functional neuro-imaging (Ahnaou et al, 2016; Lohani et al, 2016; Witten et al, 2015).

Nonetheless, translational neuroscience offers promising possibilities for further elucidation of neurobiological substrates involved in psychiatric disorders. For example, a developmental rodent model for schizophrenia (based on a pre-natal disruption of methylation processes) has yielded interesting hypotheses concerning the neurobiological deficits that give rise to the disorder (Grace, 2015). In this model, an increase in DA neuronal activity is observed, which is proposed to be caused by aberrant activity of the ventral subiculum of the hippocampus, which in turn is affected by impaired cortical activity (Grace, 2016). This hypothesis provides an interesting framework that allows for further evaluating of specific components of this neural circuitry in the pathophysiology of schizophrenia and other disorders.

Finally, the translation of neuro-circuitries from rodents to humans warrants a comparison between anatomical substrates. Both in rats and humans, the DAergic midbrain is divided into VTA and SNc, projecting to the ventral and dorsal parts of the striatum in a topographical manner (Haber and Knutson, 2010; Swanson, 1982). However, the distribution of neurons and their projections is somewhat different. In the rat, VTA is located medially in the midbrain, and limbic pathways projects to the ventromedial striatum, including nucleus accumbens, whilst mesocortical projections innervate the PFC. The SNc is located laterally in the midbrain, and projects to dorsal striatum (caudate putamen), which comprises a substantial part of the rodent brain, and extends more caudally compared to NAc (Paxinos and Watson, 1997). In the primate brain, this is largely similar. However, the VTA is relatively small, and the SNc projects to

striatum as well as prefrontal cortex (Bjorklund and Dunnett, 2007; Haber and Knutson, 2010). This suggests that VTA and SNc subpopulations may serve different functions in the rodent and human brain, indicating that comparisons between species should be made with care.

Using DREADD technology to advance treatment of psychiatric disorders

As we have shown in this thesis, chemogenetic activation of DA neurons using DREADD technology significantly affects behaviour. Furthermore, the precise behavioural effects were highly dependent on which specific neuronal subpopulation was targeted. These findings suggest that DA neuronal subpopulations may be an interesting target for goal-directed treatments of psychiatric symptoms. However, our results also indicate that enhancing DA neuronal activity may have broad functional effects. As such, an in-depth understanding of the relationship between neuronal activity and specific aspects of behavioural functions is necessary in order to develop effective target-specific therapies. Preclinical approaches, including chemogenetic control of neuronal subpopulations in freely moving animals, may provide important insights to the neuronal control of normal and abnormal behaviour. In addition, a valid translation to the clinic is vital to predict which interventions will be most effective, and it is crucial to understand the pathophysiological state that is targeted. For example, as we propose in ***Chapter 3***, enhancing excitability of VTA DA neurons may be beneficial for deficits in reward-anticipated action initiations. However, whilst this could be advantageous for motivational deficits in major depressive disorder, activation of these cells will likely exacerbate psychotic symptoms in schizophrenia (Grace, 2016; Maia and Frank, 2016; Whitton et al, 2015).

A major advantage of the use of DREADD technology in the investigation of neurocircuitry underlying behaviour is the potential to identify G-protein coupled receptors that may be targeted for the treatment of certain deficits (Lee et al, 2014). Alternatively, DREADD in itself may be used as a therapeutic treatment. Chemogenetics has certain valuable advantages to this aim: it can be used to target highly specific neuronal cell types, and allows for a direct, yet transient, increase or inhibition of neuronal excitability. Adeno-associated viruses are a promising tool for clinical gene therapy (Hocquemiller et al, 2016), and animal studies have so far not reported any effects of DREADD expression in the absence of CNO (Roth 2016). However, additional safety and efficacy studies are warranted in order to allow a clinical application of chemogenetics. One issue that is currently known to hamper the use of DREADD in humans is the finding that the ligand CNO, an inactive metabolite of clozapine, may be metabolized back into clozapine, an active atypical antipsychotic drug. This has been observed in humans and guinea

pigs (Jann et al, 1994), but not rodents (Roth, 2016). However, future prospects are optimistic. The lab that developed DREADD technology is currently working on a novel ligand ("compound 21"), suitable for use in humans (Chen et al, 2015). Furthermore, the first steps have been initiated towards use of chemogenetics in the treatment of epilepsy (Avaliani et al, 2016; Kätzel et al, 2014), and therapeutic efficacy has been opted for eating disorders, including obesity, as well as diabetes (Guettier et al, 2009; Krashes et al, 2011; Urban and Roth, 2015). Thus, although we are currently in a very premature state of development, it is exciting to consider the powerful therapeutic potential of DREADD technology in a clinical setting.

Concluding remarks

In this thesis, we have shown that DREADD technology is a powerful tool to investigate the neuronal circuitry underlying behaviour. We found that selective chemogenetic activation of DA neuronal subpopulations in rats affected various behavioural domains, including locomotor activity, motivational behaviour, feeding patterns, and attention. These findings do not only improve our fundamental understanding of how DA neuronal activity contributes to regulating behaviour, but also have implications for psychiatry and the development of novel treatments. An interesting prospect is that DREADD technology may be used in the clinic, in order to directly and remotely control aberrant neuronal activity.

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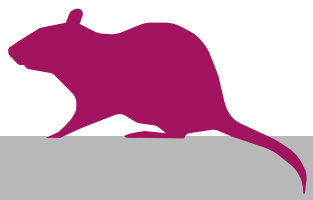
APPENDIX



Keep that list of who to thank in mind
- Vampire Weekend, "Obvious bicycle"

NEDERLANDSE SAMENVATTING

Gedragseffecten van chemogenetische activatie van dopamine-neuronen

Introductie: het gebruik van DREADD technologie om de rol van dopamine in psychiatrische stoornissen beter te begrijpen

Psychiatrische stoornissen hebben een grote impact op de maatschappij. De kans dat iemand gedurende zijn/haar leven te maken krijgt met een mentale stoornis is in de Verenigde Staten geschat op 50%, waarbij de prevalentie voor verschillende stoornissen varieert van 1% voor schizofrenie tot 17% voor depressie. Ondanks vele nieuwe inzichten uit fundamenteel en klinisch onderzoek gedurende de afgelopen decennia, is het lastig gebleken op basis hiervan nieuwe doelgerichte behandelingen te ontwikkelen. Momenteel kan een groot deel van de patiënten niet effectief worden behandeld met de beschikbare medicaties. Bovendien begrijpen we nog steeds niet goed welke neurobiologische processen precies ten grondslag liggen aan specifieke symptomen of ziektebeelden bij psychiatrische aandoeningen.

Vele psychiatrische stoornissen, onder andere schizofrenie, ADHD, verslaving en depressie, zijn gerelateerd aan veranderingen in het dopamine-systeem in het brein. Het is echter nog onduidelijk welke veranderingen in dopaminerge activiteit precies bijdragen aan deze, en andere, ziektebeelden. Onderzoek bij mensen met schizofrenie of ADHD wijst erop dat een verstoorde activiteit van dopamine-cellen mogelijk een belangrijke factor is die bijdraagt aan verscheidene symptomen, waaronder psychose en verstoord beloningsgedrag.

Dopamine-neuronen (hersencellen) bevinden zich in de middenhersenen, en zijn verantwoordelijk voor de verspreiding van dopamine door het gehele brein. Ze zijn betrokken bij vele belangrijke functies, waaronder beloningsgedrag, het aansturen van beweging en cognitieve functies. Dopamine-neuronen in de middenhersenen worden onderverdeeld in twee groepen: het ventraal tegmentaal gebied (VTA) en de substantia nigra pars compacta (SN). Vanuit de VTA lopen dopaminerge projecities naar onder andere het ventrale deel van het striatum en naar de prefrontale cortex (hersenschors). Met name de projectie van de VTA naar de nucleus accumbens in het ventraal striatum is een essentieel onderdeel van het belongingssysteem. De SN stuurt dopaminerge projecties naar het dorsale deel van het striatum en speelt een belangrijke rol bij het aansturen van bewegingen. Bij de ziekte van Parkinson zijn het voornamelijk dopamine-cellen in de SN die afsterven, wat leidt tot motorische problemen.

Dopamine in het brein is essentieel voor beweging, maar ook eetgedrag, motivatie en aandacht. Er is echter nog veel onbekend over de precieze rol van de dopamine-neuronen bij het aansturen van gedrag. Zo is nog onduidelijk welke groepen neuronen (zoals de VTA of SN) en welke projecties (bijvoorbeeld naar nucleus accumbens of prefrontale cortex) precies betrokken zijn bij specifieke typen gedrag. Bovendien is niet bekend in welke mate een verandering in neuronale activiteit daadwerkelijk leidt tot een verandering in gedrag.

Dit proefschrift

In dit proefschrift hebben we onderzocht welke veranderingen in gedrag optreden wanneer dopamine-neuronen tijdelijk extra actief worden. Hiermee willen we meer inzicht krijgen in hoe activiteit van dopamine-cellen gedrag beïnvloedt, en hoe abnormale dopaminerge activiteit kan bijdragen aan bepaalde psychiatrsche symptomen. Hiervoor hebben we ons gericht op zogenoemde gedragsdomeinen, die vóórkomen bij zowel mensen als dieren, en die verstoord zijn bij psychiatrische aandoeningen. Voorbeelden hiervan zijn hyperactiviteit, motivatie en aandacht.

Om tijdelijk dopamine-neuronen extra actief te maken, hebben we gebruik gemaakt van een recent ontwikkelde techniek, genaamd DREADD (*designer receptors exclusively activated by designer drugs*) of chemogenetica. Deze techniek maakt gebruik van onschadelijke virussen waarmee selectief in het brein een *designer-receptor* tot expressie kan worden gebracht, bijvoorbeeld specifiek in dopamine-cellen. Deze receptor heeft op zichzelf geen effect, en kan alleen geactiveerd worden door een *designer-drug*. Deze stof, genaamd CNO, kan extern worden toegediend en zorgt er vervolgens voor dat de (dopamine) cellen tijdelijk extra actief worden. In onze experimenten hebben we de activerende designer-receptor (hM3Dq) tot expressie gebracht in dopamine-neuronen in de VTA of SN bij ratten. Vervolgens hebben we onderzocht hoe toediening van CNO, en dus activatie van dopamine-neuronen, invloed heeft op het gedrag van de ratten. We hebben hierbij gekeken naar fundamenteel gedrag, zoals locomotie (voortbeweging) en eetgedrag, maar ook naar hogere functies, zoals motivatie en aandacht.

Samenvatting van bevindingen en inzichten in de rol van dopamine in de regulatie van gedrag

Onze bevindingen, gepresenteerd in ***hoofdstuk 2 t/m 5*** en samengevat in ***hoofdstuk 6***, laten zien dat het activeren van dopamine-neuronen inderdaad grote effecten kan hebben op gedrag. Bovendien vonden we duidelijke verschillen tussen de gedragseffecten van dopamine-neuronale activatie in de VTA vergeleken met de SN.

Kort samengevat zorgde chemogenetische activatie van dopamine-neuronen in de VTA ervoor dat de dieren extra actief werden. Dit was duidelijk zichtbaar in de verhoogde locomotoractiviteit die de ratten lieten zien na toediening van de designer-drug CNO (***hoofdstuk 2***). Bovendien werden de dieren extra gemotiveerd als dopamine-neuronen in de VTA werden geactiveerd (***hoofdstuk 3***). Ze waren bereid meer moeite te doen (vaker op een pedaaltje te drukken) om een suikerbeloning te verdienen. Waar de ratten normaalgesproken stoppen als ze te vaak moeten drukken voor een beloning, begonnen ze na behandeling met CNO steeds opnieuw met pedaaldrukken, zelfs als ze van tevoren al suiker hadden gegeten. Deze resultaten bevestigen dat dopamine een belangrijke rol speelt in de motivatie voor voedsel. Het is echter onduidelijk in hoeverre voedselinname zelf wordt gereguleerd door dopaminerge activiteit. In ***hoofdstuk 4*** laten we zien dat het eetpatroon van de ratten veranderde na activatie van dopamine-neuronen in de VTA. De dieren aten kleinere maaltijden en begonnen vaker aan een nieuwe maaltijd, maar aten in totaal niet meer dan in de controlesituatie. Ze wisselden dus vaker van gedrag, waren hyperactief, en leken sneller afgeleid. Om te testen of activatie van dopamine-neuronen daadwerkelijk invloed had op aandacht, trainden we de ratten op een cognitieve taak, waarmee zowel aandacht als impulsiviteit kan worden onderzocht (***hoofdstuk 5***). Hieruit bleek dat activatie van VTA dopamine-neuronen inderdaad de aandacht van de ratten verslechterde. Hoewel we verwacht hadden dat de dieren, naast hyperactief en snel afgeleid, ook impulsiever zouden worden door verhoogde dopamine-activiteit, bleek dat niet het geval.

Het merendeel van de dopamine-neuronen in de VTA projecteert naar de nucleus accumbens, in het ventraal striatum. Dopamine in de nucleus accumbens speelt een cruciale rol bij locomotor hyperactiviteit, het reageren op (onverwachte) stimuli in de omgeving en het schakelen tussen gedrag. Niet geheel onverwachts vonden wij dat wanneer we specifiek de projectie van VTA naar nucleus accumbens activeerden, dit vrijwel dezelfde effecten had op gedrag als wanneer we alle dopamine-neuronen in de VTA activeerden (ongeacht de projectie) (***hoofdstuk 2 en 4***). Activatie van projecties vanuit VTA naar andere hersengebieden, zoals het dorsomediale striatum of de prefrontale cortex, had geen of minimale effecten op het onderzochte gedrag (***hoofdstuk 2 en 4***).

Gezamenlijk duiden onze resultaten erop dat verhoogde activiteit van dopamine-neuronen in de VTA leidt tot het activeren van gedrag, en dat deze effecten worden gemedieerd door verhoogde dopamine-activiteit in de nucleus accumbens. Wij veronderstellen dat door (chemogenetische) activatie van dopamine-neuronen in de VTA een "Go"-signaal wordt afgegeven, waardoor de dieren worden aangezet tot actie. Normaalgesproken worden dopamine-neuronen actief wanneer een dier in aanraking

komt met een onverwachte stimulus of beloning, of met een stimulus die gekoppeld is aan een beloning. Door, via DREADD, de dopamine-neuronen extra gevoelig te maken voor stimulatie, lijken de dieren extra sterk te reageren op dergelijke stimuli en extra actief te worden. Afhankelijk van de context kan dat leiden tot locomotor hyperactiviteit, een toename in pedaaldrukken, het afbreken van een maaltijd, of verslechterde aandacht.

Vergeleken met de VTA, had chemogenetische activatie van dopamine-neuronen in de SN een veel minder uitgesproken effect, in het merendeel van onze studies. De locomotoractiviteit nam iets toe, maar de ratten werden niet hyperactief (***hoofdstuk 2***). Bovendien had activatie van dopamine-neuronen in de SN geen effect op motivatie voor suiker (***hoofdstuk 3***) en ook niet op eetgedrag (***hoofdstuk 4***). Klinische studies hebben aangetoond dat het dorsaal striatum, dat dopaminerge projecties ontvangt vanuit de SN, betrokken lijkt te zijn bij o.a. schizofrenie en ADHD. Aan de hand daarvan verwachtten wij dat activatie van dopamine-neuronen in de SN effect zou hebben op functies die zijn verstoord bij deze stoornissen, waaronder aandacht en impulsiviteit. Toch waren we enigszins verbaasd te zien dat SN dopamine-neuronale activatie het uitvoeren van de cognitieve aandachtstaak erg verslechterde (***hoofdstuk 5***). In deze taak worden de dieren getraind om te reageren op een lampje dat kortdurend oplicht in een opening in de wand. Er zijn vijf van deze openingen, en het lampje brandt willekeurig in één van de vijf. Als de ratten een respons maken in de goede opening, krijgen ze een suikerbeloning. Wanneer dopamine-neuronen in de SN werden geactiveerd door CNO, verslechterde dit de prestatie van de dieren drastisch. De ratten werden trager in het maken van een respons, en maakten vaak een verkeerde of helemaal geen respons. Hierin verschilt het effect van activatie van dopamine-neuronen in de SN ten opzichte van de VTA, waarbij de ratten juist sneller reageerden, en niet meer fouten maakten.

Er is nog veel onbekend over hoe dopamine-neuronen in de SN precies bijdragen aan het reguleren van gedrag en cognitieve functies. Wanneer de functie van deze cellen wordt uitgeschakeld, wordt de motoriek van de dieren aangetast (zoals, in mindere mate, bij de ziekte van Parkinson). Hierdoor kunnen ze niet meer goed bewegen, eten of drinken, wat het lastig maakt gedrag te testen. In onze experimenten hebben we onderzocht hoe gedrag wordt beïnvloed wanneer de SN dopamine-neuronen juist extra actief worden. We bevonden dat dit een subtiel effect had op locomotoractiviteit (***hoofdstuk 2***) en geen effect op eetgedrag (***hoofdstuk 4***) of motivatie voor voedsel (***hoofdstuk 3***). Daarentegen was er een sterk effect op aandacht (***hoofdstuk 5***). Hoewel het nog onduidelijk is welke neuronale circuits hier precies bij betrokken zijn, lijkt dopamine in het dorsaal striatum een belangrijke rol te vervullen bij het integreren van sensorische en motorische informatie en het selecteren van de juiste actie in respons op omgevingsstimuli.

Wanneer dopaminerge activiteit in dit gebied verstoord wordt, bijvoorbeeld doordat SN dopamine-neuronen extra actief worden, wordt het lastiger de juiste actie te selecteren. Dit heeft vooral gevolgen voor gedrag wanneer het gaat om het uitvoeren van complex gedrag, waarbij meerdere handelingen worden vereist, en waarbij de omgeving variabel is.

Onze resultaten hebben op verschillende vlakken bijgedragen aan de neurowetenschappen en het begrip van de rol van dopamine-neuronen in het reguleren van gedrag. Ten eerste hebben we laten zien dat het activeren van dopamine-cellen met DREADD in sommige gevallen vergelijkbare gevolgen heeft als het farmacologisch stimuleren van dopaminerge activiteit, maar dat dat niet altijd op gaat. Zo gingen ratten na toediening van de psychostimulante drug amfetamine minder eten, en werden ze impulsiever in de cognitieve taak, terwijl dat niet het geval was wanneer dopamine-neuronen werden geactiveerd. Bovendien benadrukken onze resultaten dat de relatie tussen dopaminerge activiteit en gedrag erg complex is, waarbij een verhoogde activiteit niet altijd het tegenovergestelde effect heeft als een verlaagde activiteit. Tot slot hebben we door deze studies meer inzicht verkregen in de overeenkomsten en verschillen tussen de rol van de twee groepen dopamine-neuronen in de VTA en SN met betrekking tot het reguleren van locomotoractiviteit, motivatie, eetgedrag en aandacht.

Implicaties voor de rol van dopamine-neuronale activiteit bij psychiatrische aandoeningen

Het doel van dit proefschrift was om beter te begrijpen hoe activiteit van dopamine-neuronen bijdraagt aan de regulatie van gedragsdomeinen die verstoord zijn bij psychiatrische aandoeningen. In ***hoofdstuk 2 t/m 5*** bediscussiëren we deze gedragsdomeinen afzonderlijk, aangezien elk domein relevant is voor een andere set aandoeningen. Als afsluiting bespreken we onze bevindingen in relatie tot twee specifieke stoornissen die geassocieerd zijn met abnormale dopamine-neuronale activiteit: schizofrenie en ADHD.

Eén van de huidige en meest onderschreven hypotheses over de rol van dopamine in schizofrenie, is dat er sprake is van abnormale activiteit van dopamine-neuronen. Normaalgesproken wordt dopamine-neuronale activiteit uitgelokt door een belangrijke gebeurtenis in de omgeving, die aandacht vereist en waar wellicht gedrag op moet worden aangepast. Wanneer dopamine-neuronen spontaan actief worden (bijvoorbeeld doordat ze niet goed geremd worden door andere neuronen), verstoort dit de verwerking van wat wel en niet belangrijk is en hoe je daarop moet reageren. Gedragsmatig kan zich dat uiten als overdreven reacties op (irrelevante) stimuli, of het kiezen van een

verkeerde handeling. In onze studies vonden we dat chemogenetische activatie van dopamine-neuronen leidde tot hyperactiviteit, vaker wisselen van gedrag, verslechterde aandacht en het kiezen van verkeerde handelingen – zoals drukken op het inactieve pedaaltje in de motivatietaak (***hoofdstuk 3)*** of een respons in de verkeerde opening in de aandachtstaak (***hoofdstuk 5)***. Deze resultaten duiden erop dat verhoogde activiteit van dopamine-neuronen kan bijdragen aan een verstoorde verwerking van relevante en irrelevante stimuli in schizofrenie, en dat zowel de VTA als SN hierbij betrokken zijn.

Verschillende gedragsdomeinen die we hier onderzocht hebben zijn relevant voor ADHD, zoals hyperactiviteit, aandacht en impulsiviteit. We bevonden dat chemogenetische activatie van dopamine-neuronen – in de VTA, maar niet SN – leidde tot locomotor hyperactiviteit (***hoofdstuk 2***), verhoogde initiatie van instrumenteel gedrag (***hoofdstuk 3***) en verhoogd wisselen van gedrag tijdens voedselinname (***hoofdstuk 4***). Het leek er dus op dat de ratten niet goed stil konden blijven zitten en zich niet goed konden concentreren op één bepaalde activiteit – vergelijkbaar met kinderen met ADHD. Vervolgens testten we of de ratten daadwerkelijk sneller waren afgeleid, en of ze ook impulsiever werden door verhoogde dopamine-neuronale activiteit. In ***hoofdstuk 5*** laten we zien dat ratten inderdaad slechter presteerden op de aandachtstaak. Hoewel ze niet meer fouten maakten, sloegen ze een groot deel van de taak over, een teken dat de dieren waren afgeleid en niet op het goede moment op een kortdurende stimulus konden letten. Enigszins verrassend werden de dieren niet impulsiever wanneer dopamine-neuronen geactiveerd werden. Gezamenlijk duiden deze resultaten er op dat verhoogde activiteit van dopamine-neuronen in de VTA wellicht een belangrijke rol speelt bij de hyperactiviteits- en aandachtsgerelateerde symptomen van ADHD, maar niet bij impulsief handelen.

Conclusie en blik op de toekomst

In dit proefschrift laten we zien dat we door het gebruik van DREADD technologie in een diermodel meer inzicht kunnen krijgen in de neuronale circuits die betrokken zijn bij het reguleren van gedrag. In onze studies vonden we dat chemogenetische activatie van verschillende groepen dopamine-neuronen effect had op verscheidene gedragsdomeinen, waaronder locomotoractiviteit, motivatie, eetgedrag en aandacht. Deze bevindingen dragen bij aan ons begrip van hoe activiteit van dopamine-neuronen gedrag reguleert, en hebben daardoor implicaties voor de psychiatrie en voor het ontwikkelen van nieuwe behandelingen. Een interessant vooruitzicht is dat DREADD technologie wellicht ook toegepast kan worden in de kliniek. Hoewel deze ontwikkeling nog in de kinderschoenen staat, zou het een veelbelovende aanpak kunnen zijn om doelgericht abnormale hersenactiviteit te behandelen.

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wij samen zouden gaan meemaken! Van dansen in de ACU tot in jouw huiskamer, van bikram yoga sessies in "ons wijkje" in Utrecht tot wilde bessen eten en slangen ontwijken in Zweden; het waren stuk voor stuk geniale ervaringen, en ik weet zeker dat er nog veel meer bij gaan komen! Behalve supergezellig ben jij ook een ware levensfilosoof en weet je altijd op het goede moment het juiste advies te geven. Er zijn geen regels! **Manila**, I am so glad we met! You are such a nice, lively and caring person, and it is great to share our love for good food (and drinks ;)) and our frustrations about experiments and being tiny :P Even though our reciprocal Italian-Dutch lessons did not last very long, I have learned a great deal from you. I think every nuchtere Noord-Nederlander (for this once I will consider Groningers en Frysians as the same group :P) should have someone like you in their lives, for a good dose of Italian drama and never-ending warm-heartedness ^_^ I really do hope that one day we will all share our lives on a specific Italian island!!

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Ela, Arjen, Natasha, Remco, Wouter, Perry, Frank, Gijs, Rick, Lindy, Diane, and Tessa: thank you for being such great roommates! **Ela**, I could always ask you anything, and I loved our conversations about everything within and outside of science. It was so nice to see you again in Sweden, and I hope we will keep meeting every once a while. **Remco**, ook al heb je ons verlaten :P het was altijd erg gezellig met jou op de kamer! Bedankt voor de mooie verhalen, of het nou ging over repetitive behaviour, feestjes in Bloemendaal, of een fietstocht around the world. **Wouter, Perry, Frank, Gijs en Rick**, de genetica-invasie ;) Jullie lieten zien dat artsen ook maar mensen zijn, en eigenlijk hele gezellige,

leuke en grappige mensen! **Gijs**, het was fijn om van alles en nog wat te kunnen delen, van rare tics tot M&M's en PhD-frustraties. Jij en **Rick** zijn een gouden combi – mocht het in de wetenschap of kliniek niets worden, dan beveel ik een carrière als komisch duo van harte aan ;) **Tessa**, ook al moesten we altijd over Gijs heen praten, ik ben heel blij dat je bij ons bent aangeschoven! Ik vond het altijd erg fijn om te kunnen kletsen over werkgerelateerde issues, maar ook over huizenzoektochten en willekeurige gezellige onderwerpen. Heel veel plezier in Austerlitz en succes met je onderzoek!

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dingen die je doet voor de opleiding en de afdeling! **Roger²**, bedankt voor alle hulp voor en achter de barrière! Het was altijd fijn om 's ochtends te merken dat meer mensen zo blij waren met de MiCaffé en goede koffie nodig hadden om op gang tekomen ;) Ook **Kim, Kristel, Sophietje, Sara, Angela, Femke, Rixt, Marijke, Marcia, Maaike, Suzanne, Jiska** en alle andere mensen op het lab: ook al zagen of spraken we elkaar niet vaak, het is zo fijn om gezellige en inspirerende mensen om je heen te hebben, bedankt daarvoor!

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Tessa, jij verdient een aparte alinea ;) Binnen het sextet ben jij mijn duo. Altijd in voor een Hendricksje, een biosje, of een tripje naar de UK. En je staat altijd direct klaar als ik je nodig heb! Bedankt voor je eigenwijsheid, je bewondering, je doorzettingsvermogen, je hilariteit, je begrip. Ik mag dan soms roepen dat ik overal en nergens heen ga, maar jij bent nog niet zomaar van me af :P

Mijn lieve vriendinnetje **Charlotte**, Lottie! Jij bent de reden dat ik nu “PG87” genoemd word :P (’n paarteh, ’n paarteh, ’n pa ’n pa ’n pa…) Van jou moest ik na m’n master in Utrecht blijven, dus ja, dan doe ik dat ;) Van neuropsychologische analyses van het leven en de maatschappij tot eindeloos dansen en mid-winter tripjes naar Malaga, wij hebben al zoveel toffe dingen meegemaakt, en daar gaan sowieso nog veel meer bijkomen! Ik heb zoveel respect voor hoe jij de afgelopen jaren hebt aangepakt. Alles leuk en aardig zo’n PhD, maar jij weet pas echt wat tegenslag en doorzettingsvermogen betekent, en hoe belangrijk vriendschappen zijn om de moed erin te houden. Dus laten we zo veel als het kan doorgaan met afspreken, samen eten, dansen, praten, zeiken en lachen :) Because we… are… your friends!

Laiverds, we go wayyy back! Ik ben echt superblij dat we elkaar al die jaren zijn blijven zien, soms wat minder, maar liever wat meer :) **Julia, Marije en Annemiek**, jullie zijn echte schatten, met het hart én de hersenen op de goede plek. Jullie zijn mijn rolmodellen voor hoe je een goed, bescheiden en simpelweg awesome persoon bent. Of het nou pool- en sneakavondjes zijn in Groningen, uitgebreide ontbijtjes in Utrecht, of bezoekjes aan Oxford/Hawaii, ik ga ervanuit dat we elkaar nog heel lang en heel vaak blijven zien!

Malou en Michelle, mijn heilige Cambridge trinity! Wat waren dat een geniale acht maanden. Volgens mij hebben wij elkaar goed aangestoken om te gaan promoveren. Uiteindelijk niet in de UK, maar dat was alleen maar een goede aanleiding om regelmatig briljante reisjes te maken, naar Edinburgh, Brussel, Parijs, Leiden, en de gehele US west coast (volgende keer ga je gewoon mee Michelle!).

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Paps, volgens mij ben jij degene geweest die de wetenschapper in mij heeft aangewakkerd. Samen de breinbrekers van Leon Tolboom oplossen, en later discussies voeren over statistiek in de achtertuin. Jij nam altijd de tijd om mij uit te leggen hoe alles werkt. Met jouw nieuwsgierigheid, precisie en analytische vaardigheden was je zelf ongetwijfeld ook een uitstekend onderzoeker geweest :) Misschien een idee voor als het pensioen toch gaat vervelen? ;) **Mams**, jij hebt altijd benadrukt hoe belangrijk het is om goed voor jezelf te zorgen en lieve mensen om je heen te hebben, en dat je nooit met mate moet genieten ;) Jullie hebben me altijd ondersteund in alles wat ik koos te doen. Wij Grunnegers gebruiken doorgaans niet zoveel woorden, maar bij dezen wil ik jullie allebei heel erg bedanken voor alle vrijheid en zorgzaamheid, en de vanzelfsprekendheid daarvan.

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En lieve, lieve **omi**. Om met jouw woorden te spreken: ga je nu al weg? Wat ongezellig. Want gezellig was het altijd! Boggle aan de keukentafel, tekenen in de tuin, naar het museum in Utrecht, of samen op de achterbank op weg naar Locarno. Van de eerste tot de laatste dag dat ik je heb gekend, heb je iedereen begroet met een gulle glimlach en genoten van de mooie dingen in het leven. Jij was en blijft onze oma knoedeltje. Boffen wij even.

LIST OF PUBLICATIONS

Linde Boekhoudt, Azar Omrani, Mieneke C.M. Luijendijk, Inge G. Wolterink-Donselaar, Ellen C. Wijbrans, Geoffrey van der Plasse, Roger A.H. Adan (2016). Chemogenetic activation of dopamine neurons in the ventral tegmental area, but not substantia nigra, induces hyperactivity in rats. *European Neuropsychopharmacology, 26(11), 1784-1793. doi: 10.1016/j.euroneuro.2016.09.003*

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Linde Boekhoudt, Ellen C. Wijbrans, Jodie H.K. Man, Mieneke C.M. Luijendijk, Johannes W. de Jong, Geoffrey van der Plasse, Louk J.M.J. Vanderschuren, Roger A.H. Adan. Enhancing excitability of dopamine neurons promotes motivational behaviour through increased action initiation. *Manuscript in preparation*

Linde Boekhoudt, Johannes W. de Jong, Anne E. de Leeuw, Theresia J.M. Roelofs, Mieneke C.M. Luijendijk, Inge G. Wolterink-Donselaar, Geoffrey van der Plasse, Roger A.H. Adan. Does activation of midbrain dopamine neurons promote or reduce feeding? *Manuscript in preparation*

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Theresia J.M. Roelofs, Geralda A.F. van Tilborg, Jeroen P.H. Verharen, Willem M. Otte, **Linde Boekhoudt**, Johannes W. de Jong, Mieneke C.M. Luijendijk, Rick M. Dijkhuizen, Roger A.H. Adan. A novel approach to map induced activation of neuronal networks using chemogenetics and functional neuroimaging. *Manuscript submitted*

ABOUT THE AUTHOR

Linde Boekhoudt was born on 8 March 1987 in Groningen. She completed her A-levels at Belcampo, Röling-college, in Groningen in 2005. The following year she started her Bachelor's in Biology at Utrecht University. Here, she focused on neurobiology and animal behaviour, and also obtained a Minor in Cognition at the department of Psychology. Following her Bachelor's degree in 2009, Linde started the Master's programme Neuroscience & Cognition at Utrecht University. During her Master's she further pursued her interest in behavioural pharmacology and translational neuroscience, and participated in multiple projects investigating the neurobiology of behaviour in both rodents and humans. Her first internship was based at Utrecht University and Radboud University Nijmegen, where she studied the effects of a genetic knock-out of the dopamine D1 receptor on decision-making behaviour in rats. For her second internship, she went to the University of Cambridge (UK), to study the effects of pharmacological blockade of dopamine or opioid receptors on gambling behaviour in healthy volunteers. In 2012, she obtained her Master's degree (cum laude), and started her PhD project in the group of Roger Adan at the University Medical Center Utrecht. During the past 4.5 years, she investigated the behavioural effects of chemogenetic activation of dopamine neurons, the results of which are presented in this thesis.

Bij twijfel, altijd links

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