Title: Nucleus accumbens medium spiny neurons subtypes signal both reward and aversion

Running Title: D1- and D2-MSNs encode reward and aversion

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Abstract

Deficits in decoding rewarding (and aversive) signals are present in several neuropsychiatric conditions such as depression and addiction, emphasising the importance of studying the underlying neural circuits in detail. One of the key regions of the reward circuit is the nucleus accumbens (NAc). The classical view on the field postulates that NAc dopamine receptor D1-expressing medium spiny neurons (D1-MSNs) convey reward signals, while dopamine receptor D2-expressing MSNs (D2-MSNs) encode aversion.

Here, we show that both MSN subpopulations can drive reward and aversion, depending on their neuronal stimulation pattern. Brief D1- or D2-MSN optogenetic stimulation elicited positive reinforcement and enhanced cocaine conditioning. Conversely, prolonged activation induced aversion, and in the case of D2-MSNs, decreased cocaine conditioning. Brief stimulation was associated with increased VTA dopaminergic tone either directly (for D1-MSNs) or indirectly via ventral pallidum (VP) (for D1- and D2-MSNs). Importantly, prolonged stimulation of either MSN subpopulation induced remarkably distinct electrophysiological effects in these target regions.

We further show that blocking κ-opioid receptors in the VTA (but not in VP) abolishes the behavioral effects induced by D1-MSN prolonged stimulation. In turn, blocking δ-opioid receptors in the VP (but not in VTA) blocks the behavioral effects elicited by D2-MSN prolonged stimulation.

Our findings demonstrate that D1- and D2-MSNs can bi-directionally control reward and aversion, explaining the existence of controversial studies in the field, and highlights that the proposed striatal functional opposition needs to be reconsidered.

Introduction

Daily, individuals assign emotional/motivational valence to otherwise neutral stimuli, by determining whether they are positive/rewarding and should be approached, or are negative/aversive and should be avoided. One crucial brain circuit in this process is the mesolimbic reward pathway, which comprises dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc). This circuit has been implicated in the recognition of rewards in the environment, which elicit approach and consumatory behavior and attribute motivational value to objects that signal its delivery/presence[1–4]. Besides this traditional role of VTA-NAc projections in rewarded behaviors, these also mediate/signal aversive stimuli, which is crucial to avoid threats and ensure survival[5–7]. Importantly, dysfunction of the reward circuit has been implicated in several neuropsychiatric disorders, being addiction one of the most studied[8].

Several studies suggest that the VTA is composed of anatomically and functionally heterogeneous dopamine neuronal subpopulations with different axonal projections, which may explain its role in both reward and aversion[5,6,9]. Similarly, VTA neurons also receive different inputs; for example, activation of laterodorsal tegmentum inputs to the VTA elicit reward, whereas from the lateral habenula induce aversion[6].

One of the core regions decoding rewarding/aversive signals from the VTA is the NAc[10]. These signals (mostly dopaminergic) act through two distinct neuronal populations of GABAergic medium spiny neurons (MSNs), segregated into those expressing dopamine receptor D1 (D1-MSNs) or dopamine receptor D2 (D2-MSNs). D1-MSNs project directly to output nuclei of the basal ganglia, namely the VTA (direct pathway), but can also project indirectly through the ventral pallidum (VP) (indirect pathway). D2-MSNs project exclusively indirectly to output nuclei of the basal ganglia through the VP[11,12].

The canonical view on striatal function is that D1-MSNs encode positive valence/reward whereas D2-MSNs encode negative/aversive responses[13–16]. In the dorsomedial striatum, D1-MSN optogenetic stimulation induces persistent reinforcement, while D2-MSN stimulation induces transient punishment[16]. In the same direction, stimulation of NAc D1-MSNs enhances cocaine-mediated conditioning, whereas optical stimulation of D2-MSNs suppresses it[15]. Yet, recent studies suggest that these two subpopulations may exert a concurrent action in reward-related

behaviors[17–21]. For example, in dorsolateral striatum, both MSN subpopulations are involved in positive reinforcement, but support different action strategies[21]. We have shown that activation of either type of NAc MSNs during cue exposure strongly enhances motivational drive towards natural rewards[18,20], suggesting that D2-MSNs do not exclusively modulate negative stimuli.

Our hypothesis to explain this conundrum was that both D1- and D2-MSNs can convey positive and negative stimuli through different patterns of activation and consequent changes in downstream target regions, such as the VP, to which both subpopulations project, or the VTA, only innervated by D1-MSNs. Our results show that both MSN subpopulations can drive reward and aversion and differentially modulate cocaine conditioning. These divergent behavioural outputs are associated with MSN pattern of stimulation and consequent downstream electrophysiological effects.

Materials and Methods

Methods are described in more detail in supplementary information.

Animals

Male and female D1-cre (line EY262, Gensat.org) and D2-cre (line ER44, Gensat.org) were used. All animals were maintained under standard laboratory conditions: artificial 12h light/dark cycle, temperature of 21±1ºC and 60% relative humidity; mice were given standard diet and water *ad libitum*. All behavioral experiments were performed during the light period of the cycle.

Health monitoring was performed according to FELASA guidelines. All procedures were conducted in accordance with European Union regulations (Directive 2010/63/EU). Animal facilities and the people directly involved in animal experiments were certified by the Portuguese regulatory entity DGAV. All protocols were approved by the Ethics Committee of the Life and Health Sciences Research Institute and by DGAV (#19074).

Constructs, virus injection and cannula implantation for optogenetic manipulation

Cre-inducible AAV5/EF1a-DIO-hChR2(H134R)-eYFP, AAV5/EF1a-DIO-eNpHR-eYFP and AAV5/EF1a-DIO-eYFP were obtained directly from the UNC Gene Therapy Center (University of North Carolina, NC, USA). AAV5 vectors titers were 3.7-6x10^{12} viral molecules/ml as determined

by dot blot.

Stereotaxic surgery was performed as described in supplementary information. For optical stimulation in the NAc, 500nl of virus was unilaterally injected into the NAc of D1- and D2-cre mice (coordinates from bregma[22]: +1.3mm anteroposterior (AP), +0.9mm mediolateral (ML), -4.0mm dorsoventral (DV)), and an optic fiber was implanted using the same coordinates (with the exception of DV: -3.9mm). For optical stimulation+drug delivery in terminals, the guide cannula was implanted in the VTA (-3.2mm AP, +0.5mm ML, and 4.5mm DV) or the VP (0.1mm AP, +1.6mm ML, and -3.9mm DV).

Optical manipulation was performed using a 473nm (ChR2) or 589nm (NpHR) DPSS lasers, which were controlled using a pulse generator (Master-8; AMPI, New Ulm, MN, USA). Stimulation parameters: brief: 1s, 12.5ms pulses at 40Hz; prolonged: 1s, 12.5ms pulses at 40Hz; inhibition: 10s constant light.

Place Preference Tests

The conditioned place preference (CPP) and Real-time place preference (RTPP) protocol were previously described[15,23,24]; and are described in detail in supplementary material. Briefly, in the CPP with optical stimulation, animals were exposed to one pre-test session (15min), two conditioning sessions (30min; 1 day with and 1 day without stimulation), and one post-test session (15min). In the CPP with cocaine (5mg/kg) + optical stimulation, animals were exposed to one pre-test session (20min), two days of conditioning – morning session with cocaine + optical stimulation and afternoon session with saline + no optical stimulation (30min each session), and one post-test session (20min).

In the RTPP, a single 15min session was performed, in which one of the chambers was associated with optical stimulation.

***In vivo* single cell electrophysiology**

In brief, a recording electrode coupled with a fiber optic patch cable was placed in the NAc (+1.3mm AP, +0.9mm ML, and 3.5mm to 4.2mm DV); VP (-0.12mm AP, +1.6mm ML, and -3.5mm to 4mm DV) and in the VTA (-3.2mm AP, +0.5mm ML, and 4mm to 4.8mm DV). Spikes of single neurons were recorded and further analyzed. All details in supplementary information.

Drugs

Drugs or vehicle were delivered 20 minutes before animals performed the CPP test, through a fluid system chronically implanted in the VP or VTA. Naltrindole 0.1µg (Nal, δ-opioid receptor (DOR) antagonist, Sigma) and norbinaltorphimine 1µg (nor-BNI, κ-opioid receptor (KOR) antagonist, Sigma).

Immunofluorescence

Paraformaldehyde-fixed sections were incubated with specific antibodies against eYFP (1:200, Ab1218, Abcam), D1R (1:200, NB110-60017, Novus), or D2R (1:400, sc-5303, Santa Cruz Biotechnology). Positive cells within the brain regions of interest were quantified by confocal microscopy. Details in Sup. Material.

Statistical analysis

Prior to any statistical comparison between groups, normality tests (Shapiro-Wilks (S-W)) were performed for all data analysed. Results are presented as mean±SEM. Statistical details can be found throughout the results description; these include the value of the statistical tests used and exact p-value. The n for each experiment is indicated in figures' legends. More details in supplementary information.

Results

Brief optical stimulation of NAc D1- or D2-MSNs induces preference

To date, there is still controversy regarding which NAc MSN subpopulation encodes reward and aversion, so we used optogenetics to specifically manipulate NAc D1- or D2-MSNs activity. D1- or D2-cre mice were injected in the NAc with AAV5 containing a cre-dependent channelrhodopsin (ChR2, optical stimulation), hallorodopsin (NpHR, optical inhibition) or eYFP (control group) (Figure 1a). This approach successfully transfected ~60% of NAc $D1^+$ cells ($eYFP^+/D1R^+$ cells) and ~50% of $D2^+$ cells ($eYFP^+/D2R^+$ cells) (Figure 1b; Supplementary Figure 1a).

We next evaluated the reinforcing properties of NAc D1- or D2-MSN modulation, using an unbiased CPP paradigm, a non-contingent conditioning paradigm (experimenter-induced), in which one of the chambers was associated with optical stimulation (or inhibition) (Figure 1c).

D1-MSN brief optical stimulation (1s stimulus – 40 pulses of 12.5ms at 40Hz, every minute) induced preference for the stimulus-associated (ON) chamber (Figure 1d, U=0.0, p=0.0003; Figure 1e, t_6=6.2, p=0.0008), in agreement with the proposed pro-reward role of this subpopulation[15,16]. Surprisingly, brief optogenetic stimulation of D2-MSNs also induced preference for the stimulus-associated chamber (Figure 1d; t_{15}=9.0, p<0.000), with all animals showing preference for the ON side (Figure 1e; t_8=12.4, p<0.000). D1-eYFP and D2-eYFP control mice showed no preference (Supplementary Figure 1b).

Optogenetic inhibition of D1-MSNs (10s of constant light at 5mW, every minute) triggered aversion to the ON chamber (Supplementary Figure 2; t_8=3.6, p=0.0065). A similar result was observed with D2-MSN optical inhibition, D2-eNpHR mice showed aversion to the stimulus chamber (t_8=3.3, p=0.0296).

Prolonged optical stimulation of NAc D1- or D2-MSNs induces aversion

Given that our results differ from the study by Lobo *et al*, which showed that D1- or D2-MSN optogenetic activation (3min at 10Hz) did not induce place preference (a finding that we replicated– Supplementary Figure 3); and that D2-MSN activation even abolished cocaine conditioning effects[15], we hypothesized that distinct patterns of stimulation led to different behavioral outcomes, explaining the discrepancies between studies.

Hence, we performed the CPP with prolonged stimulation of NAc MSNs – 60s, 12.5ms pulses at 40Hz, stimulus given every other minute. Prolonged stimulation of D1-MSNs induced aversion, since D1-ChR2 mice decreased their preference for the ON chamber (Figure 1f, U=8.0, p=0.0205; Figure 1g, t_6=2.5, p=0.0485). Prolonged stimulation of D2-MSNs also induced aversion to the stimulus-associated chamber (Figure 1f, t_{15}=3.6, p=0.0024; Figure 1g, t_8=8.3, p<0.000).

We also tested other optical stimulation protocols; 10Hz stimulation of either subpopulation did not induce preference, as expected[15,25]; whereas 20Hz stimulation induced a similar result as 40 Hz stimulation (Supplementary Figure 4).

Because our results were surprising, we added a second behavioral readout to evaluate the impact of D1- and D2-MSN stimulation in reward/aversion. So, we used the real-time place preference (RTPP) paradigm (Figure 1h), which also measures the reinforcing properties of a

stimulus, but it is dependent on subject's choice (contingent). Optical stimulation (12.5ms light pulses at 40Hz) occurred whenever the animal was in the ON box.

D1-MSN stimulation induced aversion to the ON side (Figure 1i,k-l; t_6=4.3, p=0.0051). Similarly, D2-MSN optical stimulation also induced aversion to the ON side (Figure 1j,k-l; t_8=7.5, p<0.001). As all animals were subjected on average to 60s of stimulation per entry in the ON chamber (Supplementary Figure 1c-d), these results corroborate the prolonged stimulation data of the CPP test.

Neither brief nor prolonged optical stimulation induced significant differences in locomotion (Supplementary Figure 5).

Differential effect of D1- and D2-MSN optical stimulation on cocaine CPP

Our data contrasted with the results of optical activation of D1- or D1-MSN in the context of cocaine conditioning[15]. Thus, we performed the classic CPP using cocaine (5mg/kg) as the conditioned stimulus together with brief or prolonged stimulation of NAc D1- or D2-MSNs (Figure 2a).

As anticipated, ChR2 and eYFP groups were conditioned by cocaine (Figure 2b; saline *vs* cocaine: D1-eYFP, t_5=7.2, p=0.0008; D1-ChR2, t_8=7.7, p<0.0001; D2-eYFP, t_4=9.0, p=0.0008; D2-ChR2, t_8=13.9, p<0.0001). Interestingly, brief optical stimulation of D1- or D2-MSNs significantly heightened cocaine preference as observed in the difference of time spent in cocaine chamber – saline chamber (Figure 2c; D1-eYFP *vs.* D1-ChR2, U=2.0, p=0.0016; D2-eYFP *vs.* D2-ChR2, U=3.0, p=0.007). Conversely, prolonged D1-MSN stimulation had no effect in enhancing cocaine conditioning (Figure 2e; t_{13}=0.2, p=0.8278). Prolonged D2-MSN stimulation decreased cocaine-conditioning effects (Figure 2e; t_{13}=2.4, p=0.0332), as previously reported with 3 min 10Hz stimulation of this subpopulation[15].

Cocaine induced the same locomotion effects in all groups (Supplementary Figure 6).

NAc electrophysiological correlates

Our behavioral data indicated that D1- and D2-MSNs drive both reward and aversion, depending on their stimulation period. To understand the functional impact of different periods of

stimulation, we performed *in vivo* single unit electrophysiological recordings in the NAc (Figure 3a). Different NAc neuronal populations - putative MSNs (pMSN), cholinergic interneurons (pCIN) and fast-spiking GABAergic interneurons (pFS), were identified based on characteristic waveforms and basal firing rate[26,27] (Figure 3a-b).

Brief stimulation of both D1- and D2-MSNs increased activity of the majority of MSNs (77% and 71%, respectively). Prolonged stimulation elicited the opposite response, with most of MSNs decreasing activity during stimulation (71% for D1- and 52% for D2-MSN prolonged stimulation) (Figure 3c-d).

D1-MSN brief optical stimulation increased average firing rate of pMSNs (Figure 3e; $F_{2,126}$=18.6, p<0.0001, *post hoc* before *vs* during p<0.0001); firing rate returned to basal levels after stimulation period (Supplementary Figure 7 shows the recordings of 30min protocol). This effect was also shown by the temporal variation of the activity of the cells (Figure 3f). MSNs were divided into those that increase activity (>20% from of baseline) during stimulation - pMSNs A, and other type of response - pMSNs B (Figure 3f).

Despite the fact that there is a higher percentage of MSNs decreasing firing rate in the prolonged D1-MSN stimulation in comparison with brief stimulation, we observe the same net effect, i.e., there is a net increase in average firing rate of MSNs (Figure 3g; $F_{3,189}$=10.6, p<0.0001, *post hoc* before *vs* during (1s) p<0.000, *post hoc* before *vs* during (59s) p=0.007). These neurons return to baseline activity after stimulation period (Figure 3h; pMSNs A: KS=1.0, p<0.001; pMSN B: KS=0.9, p<0.001; Supplementary Figure 8).

Regarding D2-MSN optical stimulation, a significant increase in MSN firing rate was also observed with 1s stimulation (Figure 3i; $F_{2,116}$=13.9, p<0.000, *post hoc* before *vs* during p<0.000; Figure 3j; pMNS A, p<0.000; pMSN B, p=0.05; Supplementary Figure 7). Prolonged stimulation of D2-MSNs resulted in increased average firing rate (Figure 3k; $F_{3,174}$=8.8, p=0.001, *post hoc* before *vs* during (1s) p=0.0056, *post hoc* before *vs* during (59s) p=0.0013), also evident in the temporal variation of the activity of these neurons (Figure 3l; pMSN A, KS=1.0, p<0.001; pMSN B, KS=0.9, p<0.001; Supplementary Figure 8).

No major differences in the average firing rate of pCINs and pFSs were found, though these results need to be interpreted carefully because of the low number of neurons recorded (Supplementary Figure 9).

Electrophysiological effects in the VP and VTA in response to D1-MSN stimulation

Our results suggested that brief or prolonged optogenetic stimulation of either subpopulation did not elicit a remarkably distinct electrophysiological response in the NAc, so downstream regions could be mediating the divergent behavioral effects. Thus, we evaluated the neuronal activity of the VTA, directly innervated by D1-MSNs, and the VP[28–30] innervated by both MSN subpopulations[30]. VP GABAergic neurons also provide a tonic inhibitory input to the VTA[30–33] (Figure 4a).

Brief stimulation of D1-MSNs significantly decreased the average firing rate of 85% of identified VP putative GABAergic (pGABAergic) neurons (Figure 4c-d; $F_{2,78}$=17.4, p<0.001, *post hoc* before *vs.* during p=0.0003; Supplementary Figures 7, 10). Accordingly, temporal variation of VP activity shows a decrease in activity during optical stimulation (Figure 4d; baseline vs. stimulus, p<0.000).

In the VTA, cells were separated in putative dopaminergic (pDAergic) and pGABAergic neurons (Figure 4b), according to their firing rate and waveform duration[34–36]. Brief stimulation of D1-MSNs increased average firing rate of 89% of pDAergic neurons; in contrast, 83% of pGABAergic neurons decreased activity (Figure 4e-f; pDAergic: $F_{2,66}$=8.8, p=0.0023, *post hoc* before *vs.* during p=0.0025; pGABAergic: $F_{2,22}$=12.9, p=0.0019, *post hoc* before *vs.* during p=0.0098; Supplementary Figures 7, 10). Temporal variation revealed an opposite response of VTA pDAergic and pGABAergic neurons (Figure 4f; pDAergic and pGABAergic: baseline *vs.* stimulus, p<0.000; Supplementary Figure 7).

Conversely, prolonged D1-MSN stimulation decreased firing rate of VP neurons during the first 2s of stimulation (Figure 4l-m; $F_{3,159}$=13.9, p<0.000, *post hoc* before *vs.* during (1s) p=0.0002); those neurons return to baseline activity after that (Figure 4m; KS=0.6, p<0.000; Supplementary Figure 8).

In the VTA, prolonged D1-MSN activation led to an increase in average firing rate of pDAergic neurons during the first second of stimulation (Figure 4n-o; $F_{3,99}$=3.9, p=0.0203, *post hoc* before *vs.* during (1s) p=0.0233; Supplementary Figure 8), and no major changes thereafter. When analyzing the response throughout time, pDAergic neurons present a marked increase in activity in the first second of stimulation that then decrease to levels comparable to baseline (with the exception of few time points) as stimulation continues (Figure 4o; KS=0.6, p<0.000). Conversely, VTA pGABAergic neurons activity was decreased in the first second of stimulation (Figure 4n-o; $F_{3,33}$=10.5, p=0.0037, *post hoc* before *vs.* during (1s) p=0.0055), and then normalized, except for some time points (Figure 4o; KS=0.4, p=0.002).

Altogether, this data indicates that brief D1-MSN stimulation decreases VP activity and enhances VTA dopaminergic activity. Prolonged stimulation induces similar effects but only during the first seconds of stimulation, and then the activity of these brain regions *quasi* normalizes.

Electrophysiological effects in the VP and VTA in response to D2-MSN stimulation

NAc D2-MSNs do not project to the VTA directly, but are able to control midbrain activity indirectly through the VP[30] (Figure 4g).

Accordingly, brief stimulation of D2-MSNs decreased activity of 87% of VP neurons (Figure 4h-i; $F_{2,122}$=9.7, p=0.0014, *post hoc* before *vs.* during p=0.0109). Temporal variation of VP neurons shows this effect time-locked to optical stimulation (Figure 4i; baseline *vs.* stimulus, p<0.000; Supplementary Figure 7). This occurred prior to the increase in activity of 90% of VTA pDAergic neurons (Figure 4j-k: $F_{2,80}$=11.9, p=0.0010, *post hoc* before *vs.* during p=0.0025; Figure 4k: baseline *vs.* stimulus, p<0.000). Latency to fire of pDAergic neurons was 139.5ms, indicative of polysynaptic modulation (Supplementary Figure 10). The majority of pGABergic neurons presented a decrease in activity during stimulation, that was significantly different from baseline (Figure 4j-k; $F_{2,57}$=7.5, p=0.0228, *post hoc* before *vs.* during p=0.0273).

Contrariwise, prolonged optogenetic stimulation of D2-MSNs decreased average firing rate of VP neurons during the first 2s of stimulation, and increased VP activity after that period (Figure 4p-q; $F_{3,183}$=16.9, p<0.000, *post hoc* before *vs.* during (1s) p=0.0163, *post hoc* before *vs.* during (59s)

p=0.0081; Supplementary Figure 8). When analyzing the response throughout time, this increase in activity after the initial 2s inhibition was marked (Figure 4q; KS=0.4, p<0.000).

Regarding VTA, prolonged activation of D2-MSNs caused an increase in VTA pDAergic firing rate in the first second of stimulation and a decrease thereafter (Figure 4r; $F_{3,120}$=19.5, p<0.000, *post hoc* before *vs.* during (1s) p=0.0037, *post hoc* before *vs.* during (59s) p=0.0003; Supplementary Figure 8). This effect was particularly evident in the temporal analysis of VTA activity (Figure 4s; KS=0.4, p=0.003). In contrast, prolonged D2-MSN stimulation did not cause major differences in pGABAergic net neuronal activity (Figure 4r-s).

Our results indicate that D2-MSN brief stimulation leads to increase in VTA dopaminergic activity, caused by indirect inhibition of VP GABAergic neurons. Inversely, prolonged stimulation of D2-MSNs causes a significant increase in VP activity, which likely contributes for the decrease in VTA dopaminergic signaling (after the first seconds of stimulation).

D1- and D2-MSN-induced place aversion is mediated by activation of distinct opioid receptors

We next explored the mechanism underlying the aversive effect induced by prolonged stimulation of MSNs. D1-MSNs express and co-release dynorphin (KOR ligand), whereas D2-MSNs express and co-release enkephalin (DOR ligand)[30,37–39]. These opioids can have complex pre- and post-synaptic effects, and have been associated with reward and aversion[40,41].

We injected a cre-inducible ChR2 virus in the NAc of D1- or D2-cre mice, and implanted in the VTA or VP, respectively, a hybrid cannula that allows drug delivery and optical stimulation (Figure 5a, g). We next performed the CPP test with optical stimulation of either MSN subpopulation in animals previously injected in the VTA or VP with KOR or DOR antagonists (Figure 5b, h).

D1-MSN-VTA or D2-MSN-VP terminal stimulation recapitulated the effects of D1- or D2-MSNs soma stimulation, respectively (Figure 5), supporting the involvement of VTA and VP downstream targets in the observed behavioral phenotype.

VTA injection of nor-BNI (KOR antagonist) had no impact in animals submitted to D1-MSN brief stimulation (Figure 5c-d), but occluded the behavioral effects of prolonged stimulation since D1-ChR2 stimulated animals no longer manifest aversion to the ON side (Figure 5e-f; D1-ChR2

veh vs. D1-ChR2 nor-BNI, t_{10}=7.9, p=0.000). As a control experiment, we also injected nor-BNI injection in the VP of D2-MSN prolonged stimulation group, and observed no effect (Supplementary Figure 11).

Injection of naltrindole (DOR antagonist) in the VP did not affect D2-MSN brief stimulation-induced effects in behavior (Figure 5i-j). Conversely, it abolished the aversive effect caused by prolonged D2-MSN optical stimulation (D2-ChR2 veh vs. D2-ChR2 NaI, t_{10}=6.2, p=0.0001). As anticipated, naltrindole injection in the VTA had no effect whatsoever in prolonged D1-MSN stimulation (Supplementary Figure 11).

Discussion

Here we show that optogenetic activation of NAc D1- or D2-MSNs (soma or terminals) induces reward or aversion, depending on the characteristics of MSN stimulation protocol. Brief stimulation induced positive reinforcement and enhanced cocaine conditioning; whilst prolonged stimulation of either subpopulation induced aversion. Importantly, we show that these distinct stimulation protocols elicit divergent electrophysiological responses in downstream targets, the VTA and VP.

The VTA-NAc pathway is crucial to integrate neural information from the cortex/thalamus and facilitate selection of actions that achieve reward and avoid aversive outcomes[11,42,43], thus it is not surprising that dysfunction of this pathway underlies some neuropsychiatric disorders. For example, depression and addiction are characterized by a marked dysfunction of NAc both in animal models and humans[44–49], which highlights the importance of studying this region in more detail.

Classical views on striatal function propose a dichotomous role for D1- and D2-MSNs (both in dorsal striatum and NAc) in encoding rewarding and aversive signals[50,51]. In the dorsal striatum, optogenetic activation of D1-MSNs induces preference, whereas D2-MSN stimulation aversion[16]. Similarly, in the NAc, optogenetic activation of D1-MSNs enhanced cocaine conditioning whereas D2-MSNs activation abolished cocaine effects[15], which lead to the assumption that these neurons also convey opposing valence information. Yet, recent evidence suggested that this view is too simplistic. For example, in dorsolateral striatum, both MSN subpopulations are involved in positive

reinforcement, but support different action strategies[21]. In line with this, we have shown that brief optogenetic activation of D1- or D2-MSNs enhanced motivational drive towards natural rewards[18,20]. Others have shown that in the ventrolateral striatum, both MSN subpopulations are active during specific stages of a motivation task[19].

Our hypothesis was that both MSNs can drive reward and aversion through differential modulation of downstream target regions, namely the VP and VTA. D1-MSNs preferentially innervate VTA GABAergic neurons[52,53], which in turn disinhibit local dopaminergic neurons[54,55], although some reports indicate that they can synapse onto both GABAergic and dopaminergic VTA neurons via selective activation of different GABA receptors, or depending on the subregion of the NAc[56,57]. Our electrophysiological data appears to support a direct inhibition of VTA GABAergic neurons and a later activation (higher latencies– Supplementary Figure 9) of dopaminergic neurons induced by D1-MSN brief optogenetic stimulation. These results are line with the work of Kupchik *et al*. which has also shown a preferential innervation of VTA GABA neurons by D1-MSNs from the core region[30] (in this study we mostly target core (and part of dorsal medial shell) region). But it is also important to refer that some dopaminergic neurons also present inhibitory responses, suggesting a monosynaptic input by D1-MSNs. A recent study has elegantly shown that different NAc shell subregions can preferentially innervate either VTA GABA or DA neurons[58], emphasizing the need to perform a detailed and systematic anatomical and electrophysiological characterization of NAc-VTA inputs.

In addition, D1-MSNs stimulation also inhibited 87% of recorded VP neurons, more than the previoulsy 50% reported using patch in slices[30], which is probably explained by technical differences between the two electrophysiological apaproaches. Nevertheless, the observed inhibiton of the VP to VTA tonic inhibitory input can also partially contribute for the observed increase in VTA dopaminergic activity.

Regarding D2-MSNs, brief stimulation inhibited VP activity, disinhibiting VTA dopaminergic activity, in accordance with VP innervation to VTA dopaminergic neurons[31,33,59]. In line with this, a recent pharma-optogenetic study from our team showed that the increase in motivation due to D2-MSNs stimulation is dependent on VTA dopaminergic tone and consequent D1R and D2R activation in the NAc[20]. Though the VTA is likely involved, one cannot exclude the contribution of

other VP output regions such as the subthalamic nucleus (STN), which has been proposed to play a critical role in ascertaining reward value and magnitude[60], and that can and exert an opposite control on cocaine and natural rewards[61].

Regardless of additional mechanisms, one final outcome of both D1- or D2-MSNs brief stimulation was a substantial increase in VTA dopaminergic activity, known to trigger robust behavioral conditioning[23,62], supporting the place preference of D1- and D2-MSN brief stimulation. In this context, in future studies it will be crucial to evaluate real time dopamine levels in the NAc (and PFC) of stimulated animals.

Contrary, aversion was observed when MSN stimulation was longer (60s), and in the case of D2-MSNs, it also decreased cocaine conditioning effects. Prolonged D1-MSN activation increased average firing rate of dopaminergic neurons but only during the first second of stimulation. Conversely, VTA GABAergic neurons activity decreased in the first second of stimulation, and then normalized, which was surprising since we predicted a sustained decrease in activity[38]. This suggested that adaptive synaptic mechanisms were occurring, and our hypothesis was that in these conditions, D1-MSNs were co-releasing dynorphin, an endogenous KOR ligand. that has been associated with aversion and negative reinforcement[63,64]. For example, intra-VTA injection of KOR agonists induces robust conditioned place aversion[63]. Supporting our hypothesis, blocking KOR in the VTA abolished the aversive effect of D1-MSN prolonged stimulation, suggesting that the aversive effect was indeed mediated by dynorphin. Moreover, we observed an initial increase in VTA dopaminergic firing rate, and then the activity quasi normalizes, except for some timepoints, which present subtle activity changes. One hypothesis is that this reflects the net signal of a complex interaction between KOR activation (which decreases activity of dopaminergic neurons *in vitro* and *in vivo*[65,66]), and the reduction in VP inhibitory tone to VTA.

D2-MSN prolonged activation did not cause major electrophysiological differences in VTA GABAergic neurons. In contrast, we observed an increase in dopaminergic firing rate in the first second of stimulation that inverted to a significant decrease in activity afterwards. These electrophysiological findings support the aversive effect observed in the CPP, since it has been shown that reducing VTA dopaminergic activity triggers aversion[67]. In addition, D2-MSN prolonged

stimulation reduced cocaine rewarding effects, proposing that the recruitment of this subpopulation may serve as a protective mechanism in drug-exposed individuals[68].

Our *in vivo* electrophysiological data resembles a very elegant *ex vivo* study showing that HFS (100 pulses at 100Hz 20s) induced LTD at D2-MSNs-to-VP synapses[38]. This effect was mediated by co-release of enkephalin (which only occurs at HFS), that acted presynaptically at DOR in D2-MSNs terminals, decreasing GABA exocytosis[38]. Though our stimulation protocol was different, one hypothesized that it could also induce the co-release of enkephalin, decreasing inhibitory transmission at VP synapses, supporting the observed increase in VP activity after the initial seconds of stimulation. In agreement, we show that intra-VP injection of DOR antagonist abolished the aversive effects of D2-MSN prolonged stimulation (but not of brief).

Optogenetics has been extensively used to dissect neuronal circuits, but it also poses great challenges. It is important to use stimulation protocols that generate a neuronal response closest to the physiological one, which is remarkably difficult, as these are not always characterized. *In vivo* recordings show that NAc neurons are relatively quiet (<5Hz), and change firing rate in response to discrete reinforcing stimuli (predictive stimuli or reward itself) in <1s (or during very few seconds)[69,70]. Considering this, one could argue that prolonged stimulation could lead to artificial effects not (usually) observed in natural conditions. Yet, in the context of cocaine self-administration, prolonged excitation/inhibition of a fraction of NAc neurons can also occur[71,72]. So, we believe that it is crucial to perform additional studies evaluating the activity pattern of MSNs in freely moving animals during different rewarding/aversive tasks in order to better understand how the two subpopulations work to generate behavior.

This study also highlights the importance of testing different stimulation/inhibition parameters and evaluating their impact not only in the manipulated region, but also in downstream targets and in behaviour. Although the increases in firing rate of NAc neurons of brief and prolonged stimulation are similar to those found in freely moving animals in response to natural rewards[69,70,73], the evoked effects in the VTA and VP were clearly different between the two stimuli.

In this work, we contributed to explain contradictory results in the field, by showing that optogenetic stimulation of NAc D1- or D2-MSNs can drive both reward and aversion, depending on their stimulation pattern. In addition, we show that cocaine conditioning is also differentially

affected by manipulation of these NAc subpopulations. This work highlights that the striatal functional organization is more complex than classically proposed, and that additional studies are needed to disentangle the contribution of each subpopulation in valenced behaviors, which is of utmost importance to better comprehend neuropsychiatric disorders such as depression and addiction, that present marked striatal dysfunction[44–46,48,49].

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary information is available at MP's

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Figure Legends

Figure 1. Bidirectional effect of NAc D1- and D2-MSNs in reinforcement. (**a**) Strategy used for optogenetic manipulation. A cre-dependent ChR2 or eYFP was injected unilaterally in the NAc of D1-cre or D2-cre transgenic mice. (**b**) Representative immunofluorescence of eYFP and D1R (left panel) or D2R (right panel). (**c**) Schematic representation of optogenetic stimulation parameters in the CPP test (1s or 60s of 12.5ms pulses at 40 Hz) . (**d**) Brief (1s) optical stimulation of NAc D1- or D2-MSNs induces place preference ($n_{D1\text{-}eYFP}$=8, $n_{D1\text{-}ChR2}$=7; $n_{D2\text{-}eYFP}$=8, $n_{D2\text{-}ChR2}$=9). (**e**) Increased preference for the stimulus-associated chamber (ON) in all D1-ChR2 and D2-ChR2 animals. (**f**) Prolonged (60s) optical stimulation of NAc D1- or D2-MSNs induces aversion ($n_{D1\text{-}eYFP}$=8, $n_{D1\text{-}ChR2}$=7; $n_{D2\text{-}eYFP}$=8, $n_{D2\text{-}ChR2}$=9). (**g**) Aversion to the ON chamber in D1-ChR2 and D2-ChR2 animals. (**h**) Schematic representation of the real-time preference apparatus. Whenever animals are on the "ON chamber", they receive optogenetic stimulation (12.5 ms pulses at 40Hz) that ceases only when animals cross to the OFF side. Representative track of (**i**) D1-ChR2 and D1-eYFP animals, and (**j**) D2-ChR2 and D2-eYFP animals. (**k-l**) Both D1-ChR2 and D2-ChR2 mice spent less time in the stimulus-associated box ($n_{D1\text{-}eYFP}$=7, $n_{D1\text{-}ChR2}$=8; $n_{D2\text{-}eYFP}$=8, $n_{D2\text{-}ChR2}$=9). *p<0.05, **p<0.01, ***p<0.001. Data is represented as mean ± SEM.

Figure 2. Brief and prolonged D1- and D2-MSN stimulation differentially modulate cocaine conditioning. (**a**) Representation of the CPP cocaine 5mg/kg+optical stimulation (1s or 60s) paradigm. (**b**) Cocaine induces preference in all groups as expected; and brief optical activation of D1- or D2-MSNs further enhances cocaine reinforcing effects (**c**). In comparison to YFP groups, D1-ChR2 or D2-ChR2 spend more time in the cocaine+ brief stimulus chamber ($n_{D1\text{-}eYFP}$=6, $n_{D1\text{-}ChR2}$=9; $n_{D2\text{-}eYFP}$=5, $n_{D2\text{-}ChR2}$=9). (**d**) Cocaine induces preference in all groups. Prolonged optical activation of D1-MSNs does not change the reinforcing properties of cocaine, whereas D2-MSN prolonged stimulation decreases cocaine reward (**e**) ($n_{D1\text{-}eYFP}$=6, $n_{D1\text{-}ChR2}$=9; $n_{D2\text{-}eYFP}$=6, $n_{D2\text{-}ChR2}$=9). **p<0.01, ***p<0.001. Data is represented as mean ± SEM.

Figure 3. Electrophysiological response of NAc neurons to brief or prolonged stimulation.

(**a**) Schematic representation of NAc electrophysiological recordings. (**b**) NAc neurons were separated into 3 categories according to firing rate and waveform characteristics: putative medium spiny neurons (pMSNs), cholinergic interneurons (pCINs) and fast spiking GABAergic interneurons (pFS). (**c**) In D1-cre stimulated mice, 90.1% of recorded cells were classified as pMSNs (64/71 cells), 5.6% as pCINs and 4.3% as pFS interneurons. During D1-MSNs brief (**c'**) or prolonged (**c''**) stimulation, 77% and 44% of the pMSNs increased their activity respectively. (**d**) In D2-cre stimulated mice, 85.5% of recorded cells were pMSNs (59/69 cells), 8.7% pCINs and 5.8% pFS interneurons. D2-MSNs brief (**d'**) or prolonged (**d''**) stimulation increased the activity in 71% and 52% of the pMSNs respectively. (**e**) D1-MSNs brief stimulation increased average firing rate of MSNs. (**f**) Temporal variation of the normalized activity of pMSNs that increase (pMSN A) and decrease (pMSN B) activity during the stimulation period (blue). (**g**) D1-MSNs prolonged stimulation increased average firing rate of MSNs. (**h**) Temporal variation of the normalized activity of pMSNs showing the distinct pattern of response during stimulation (blue). (**i**) D2-MSNs brief stimulation increased the average firing rate of MSNs. (**j**) Temporal variation of the normalized activity of pMSNs showing the response during stimulation. (**k**) Prolonged D2-MSNs stimulation increased average firing rate of MSNs throughout the stimulation period. (**l**) Temporal variation of the normalized activity of pMSNs showing the response during stimulation. *$p<0.05$. Data is represented as mean ± SEM.

Figure 4. Distinct electrophysiological responses of VP and VTA during brief or prolonged MSNs stimulation. (**a**) NAc D1-MSN circuitry. (**b**) Representative waveform of a VP pGABAergic neuron, and VTA pGABAergic and pDAergic neurons. Pie charts represent the percentage of each type of response to stimulus; bar graphs represent net firing rate before, during and after optogenetic stimulation; blue stripe in scatterplots represents optogenetic stimulus. (**c**) Brief D1-MSNs optical stimulation decreases the net firing rate of VP pGABAergic neurons. (**d**) Temporal variation of VP activity; note the decrease in activity during optical stimulation (blue). (**e**) The same stimulation increases the net activity of VTA pDAergic neurons; conversely, pGABAergic neurons decrease activity. (**f**) Temporal variation of VTA neurons activity. (**g**) NAc D2-MSNs circuitry. Brief D2-MSNs optical stimulation induces a similar response in VP and VTA regions as D1-MSNs brief

stimulation (**h-k**). (**l**) Prolonged D1-MSNs optical stimulation decreases the average VP firing rate in the first second of stimulation; then net activity normalizes to baseline during the rest of the stimulation. (**m**) Temporal variation of VP activity. (**n**) Prolonged D1-MSNs stimulation induced an increase in the average firing rate of VTA pDAergic neurons in the first second of stimulation, contrary to pGABAergic neurons, that decreased activity. (**o**) Temporal variation of the activity of VTA neurons. Note the opposing patterns of activity in pDAergic and pGABAergic neurons. (**p**) Prolonged optical stimulation of D2-MSNs decreases the activity of VP neurons in the first second of stimulation, and increases it during the rest of the stimulation. (**q**) Temporal variation of VP activity; note the decrease in activity during the first seconds of optical stimulation, and the increase thereafter. (**r**) This same stimulation increased the average firing rate of VTA pDAergic neurons in the first second of stimulation, and decreased their activity after. No changes were found in the activity of VTA pGABAergic neurons. (**s**) Temporal variation of the activity of VTA neurons. "*" Before *vs*. during; "#" during *vs*. after; *p<0.05, **or$^{\#\#}$p<0.01, ***p<0.001. Data is represented as mean ± SEM.

Figure 5. Aversion induced by prolonged MSN stimulation is mediated by opioids. (**a**) A Cre-dependent ChR2 was injected in the NAc of D1-cre mice and a guide cannula was placed in the VTA to allow injection of drugs and optical stimulation of D1-MSN terminals. (**b**) D1-cre mice were injected with nor-BNI (KOR antagonist, 1µg/0.5µl) in the VTA 20 min before the CPP conditioning session with brief or prolonged optical stimulation. In the VTA, KOR is mainly expressed in dopaminergic neurons. (**c-d**) Nor-BNI administration had no effect on D1-MSN brief stimulation-induced CPP ($n_{D1\text{-}eYFPveh}$=5, $n_{D1\text{-}eYFPnor\text{-}BNI}$=5, $n_{D1\text{-}ChR2veh}$=6, $n_{D1\text{-}ChR2veh}$=6). (**e-f**) Nor-BNI occluded D1-MSN prolonged optical induced aversion ($n_{D1\text{-}eYFPveh}$=5, $n_{D1\text{-}eYFPnor\text{-}BNI}$=5, $n_{D1\text{-}ChR2veh}$=6, $n_{D1\text{-}ChR2nor\text{-}BNI}$=6). D1-ChR2 nor-BNI mice did not show preference for any chamber. (**g**) A Cre-dependent ChR2 was injected in the NAc of D2-cre mice and a guide cannula was placed in the VP to allow injection of drugs and optical stimulation of D2-MSN terminals. (**h**) D2-cre mice were injected with Nal (DOR- antagonist 0.1µg/0.5µl) in the VP 20 min before the CPP conditioning session with brief or prolonged optical stimulation. In the VP, DOR is expressed in D2-MSN terminals arising from the NAc. (**i-j**) Nal administration had no effect on D2-MSN brief stimulation-induced CPP ($n_{D2\text{-}}$

$_{eYFPveh}$=5, $n_{D2\text{-}eYFPNal}$=5, $n_{D2\text{-}ChR2veh}$=6, $n_{D1\text{-}ChR2Nal}$=6). (**k-l**) Nal occluded the effect of D2-MSN prolonged optical stimulation, blocking the aversive effect ($n_{D2\text{-}eYFPveh}$=5, $n_{D2\text{-}eYFPNal}$=5, $n_{D2\text{-}ChR2veh}$=6, $n_{D2\text{-}ChR2Nal}$=6). ***p<0.001. Data is represented as mean ± SEM.