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Dopamine Transients in the Ventral Tegmental Area Attenuate Aversive Prediction Error

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Abstract

Dopamine Transients in the Ventral Tegmental Area Attenuate Aversive Prediction Error

Ashraf Mahmud

Prediction-error, the discrepancy between real and expected outcomes, drives associative learning. It is best exemplified in the blocking paradigm. In *blocking*, impairment in learning about the predictive relation between a cue (e.g., a clicker) and an outcome (e.g. footshock) is observed when this learning takes place in the presence of a good predictor (e.g. a light) for the same outcome. Small prediction-error generated by the light leads to impairment in learning about the clicker-footshock relationship. The mere presentation of the two stimuli in compound in the absence of pre-training of one of those stimuli does not yield blocking. That is, in the so-called overshadowing control condition, the clicker is presented in compound with the light that was not previously associated with the footshock. This arrangement leads to robust learning about the clicker due to the presence of a maximum prediction-error. Dopamine (DA) in the ventral tegmental area (VTA) has been implicated in *reward* prediction-error (RPE). Evidence suggests an opposing role of DA in fear and reward. Here we undertook several experiments aimed at elucidating the role of VTA DA neurons in aversive prediction-error (APE). We used a powerful behavioural and theory-driven approach by combining blocking and the corresponding overshadowing control in the context of aversive (fear) learning along with optogenetics. We used the Th-cre+/- rats in order to exercise fine temporal control over VTA DA neurons during aversive learning. Taken together, our results provide evidence that optical stimulation of VTA DA neurons and their terminals in the nucleus accumbens (NAc) at the time of expected shock

augmented the blocking effect by attenuating APE and further impaired learning about the blocked cue. We did not observe such an effect in the overshadowing control nor many neural control groups.

Keywords: Prediction-error, Optogenetics, Dopamine, Learning and Memory, Fear Conditioning, Pavlovian Conditioning, Ventral Tegmental Area, Aversive Prediction-error

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Dopamine Transients in the Ventral Tegmental Area Attenuate Aversive Prediction Error

Introduction

Adaptive functioning critically depends on our ability to predict the future, to know where there is danger and where there is food. In the laboratory, this learning is modelled using Pavlovian (classical) conditioning. In Pavlovian conditioning, an initially neutral stimulus is paired with a biologically significant event or unconditioned stimulus (US). Following such (often repeated) pairing, the neutral stimulus becomes a conditioned stimulus (CS) because it elicits behavioural responding indicative of the expectation of the US. While the development of the conditioned behavioural response to the CS requires CS-US pairings, subsequent brief non-reinforced presentations of the CS are used to test the strength of the associative learning. That is, the conditioned response (CR) is taken as an indication of the strength of the association between the CS and US. For example, in laboratory rats, a light paired with a footshock becomes a fear-eliciting stimulus. This is often evidenced in freezing, a species-specific (i.e., rats) fear response which is also used as a behavioural index of the strength of the association between the light and the shock. Importantly, once the association has been established, the light elicits freezing in the absence of the footshock.

Historically, the temporal co-occurrence of the CS and the US was considered to be critical for associative learning (Guthrie, 1935). More recent research (e.g., Kamin, 1968, Rescorla, 1968, Wagner et al., 1968) has uncovered that associative learning critically depends on the presence of a prediction error, the discrepancy between the real and expected outcomes (Bush & Mosteller, 1953, Rescorla & Wagner, 1972). Learning is greatest when the prediction error is large. That is, the greater the discrepancy between real and expected outcomes the greater the learning and vice versa.

The blocking effect (Kamin, 1968) best illustrates the role of prediction-error in associative learning. The blocking design consists of two groups, Block and an overshadowing Control. During Phase 1 of the experiment, the Block group receives pairings between a CS (e.g., light) and a US (e.g. footshock), whereas the overshadowing Control group receives no such training. During Phase 2 of the experiment, both groups receive pairings between an audiovisual compound (e.g., light and clicker presented simultaneously) and the US. Critically, for the overshadowing Control group the light and clicker are both completely novel, but for the Block group, only the clicker is novel. That is, for the Block group the light is already trained to signal the US. Of greatest interest is the amount learned about the clicker-shock relationship in each group. This is probed during a non-reinforced test of the clicker. This test reveals that the overshadowing Control group expresses a higher level of fear to the clicker compared to the Block group despite equivalent pairings between the clicker and shock during Phase 2. These data are important for two reasons. Firstly, they provide evidence that temporal contiguity is not sufficient to drive learning, rather learning is driven by prediction error (see below). Secondly, these data underscore that learning about cue-outcome relationships does not take place in isolation and is influenced by the presence of other *predictors* of the same outcome.

The Rescorla-Wagner Model, Prediction Error, Blocking and Overshadowing

The Rescorla-Wagner model is a model of Pavlovian conditioning that describes the learning that takes place between a CS and US during a conditioning trial in terms of the changes in associative strength (V) between a CS and a US (Rescorla, & Wagner, 1972). The model states that the unexpected occurrence of the US leads to learning. That is, the presence of a prediction error drives increases in associative strength. As the association between the CS and the US reaches maximum or asymptote, the prediction-error is reduced, and once the prediction-

error reaches zero, no more learning takes place. The blocking effect occurs because prior training with the light means that at the start of compound training in Phase 2, the light already predicts the arrival of the US. That is, the prediction error is small. This smaller prediction error limits the amount of associative strength that can be acquired by the novel clicker. As a result, responding to the clicker alone on the test is low. Thus, in blocking learning about the predictive relationship between the novel cue (e.g., a clicker) and the outcome (e.g. footshock) is reduced in the presence of a good predictor (e.g. a light) for the same outcome.

In contrast to blocking, in an overshadowing Control, neither of the stimuli comprising the compound predict the US. Therefore, at the start of compound conditioning in Phase 2 the prediction error is maximal and therefore learning takes places between each of the cues and the US. As a result, responding to the clicker alone on the test is high. Importantly, this overshadowing Control condition is the best comparison group for blocking for three reasons. Firstly, similarly to blocking it ensures that training takes place in the presence of another stimulus. Secondly, it equates the number of pairings between the clicker and the US. Thirdly, it presents a condition in which the small prediction error generated in blocking is compared to an identical compound training procedure in which the prediction error is large, thereby isolating the role of prediction error in each group but controlling for temporal contiguity and any training-independent inter-stimulus competition.

Neural Mechanisms of Prediction Error.

One of the greatest questions in neuroscience has been to uncover the neural mechanisms that drive learning. As mentioned earlier, behavioural data provide strong evidence that prediction error is critical for learning. One of the most influential discoveries of our time has been the electrophysiological profile of the ventral tegmental area (VTA) and substantia nigra

(SN) dopamine (DA) neurons during associative learning about rewarding outcomes. Specifically, VTA/SN DA neurons increase their firing to unexpected rewards (juice) but not (or not as much) to the same reward when it is predicted by antecedent cues (Schultz, Dayan, & Montague, 1997). This suggests that VTA/SN DA neurons do not code for the absolute value of rewards or they would fire the same irrespective of whether a reward is expected or unexpected. Interestingly, the VTA/SN DA signal travels earlier in time to the best predictor of the reward (Schultz et al., 1997), which is in line with trial-based (Rescorla & Wagner, 1972) and time-based (Temporal Difference Reinforcement Learning (TDRL), Sutton & Barto, 1990) prediction-error theories. Finally, again as predicted by these models, the omission of an expected reward results in a drop or inhibition of the VTA/SN DA neuronal response (Schultz et al., 1997).

To confirm that the VTA/SN DA signal is critical for reward prediction error (RPE), Waelti, Dickinson, and Schultz (2001) recorded the firing of DA neurons during a blocking paradigm. Critically, use of the blocking paradigm helped differentiate the role of RPE from that of mere CS-US pairing. They employed stimulus A which predicted a reward (A+ trials) and a control stimulus B which did not predict any rewarding event (B- trials) in Phase 1. During the training, Firing of DA neurons increased to the predictor of reward (i.e., A) but not the cue that did not predict reward (i.e., B). In Phase 2, two stimuli (X and Y) were presented in compound in equal numbers of trials with A and B (AX+ and BY+) respectively. In AX+ trials, A already predicted the reward, thus, the presentation of the reward in these trials generated little prediction-error. On the other hand, in BY+ control trials, the reward was not predicted by B, thus prediction-error was large. Notably, DA neurons responded differently in these trials at the time of the reward. DA neurons increased their firing rate at the time of reward following the BY compound but not (or very little) following the AX compound. Test trials of X and Y confirmed

that learning about stimulus X was blocked compared to the control stimulus Y, and VTA DA neurons showed higher level of firing to Y which was established as a good predictor of reward compared to X, which was blocked and therefore not as a good predictor for reward (Waelti, Dickinson, & Schultz, 2001). These data highlight that the activity of VTA DA neurons tracks changes in reward prediction error. These important findings have also been replicated in monkeys (Bayer, & Glimcher, 2005; Nakahara, Itoh, Kawagoe, Takikawa, & Hikosaka,, 2004) and rodents (Day, Roitman, Wightman, & Carelli, 2007; Roesch, Calu, & Schoenbaum, 2007; Cohen, Haesler, Vong, Lowell, & Uchida, 2012; Starkweather, Babayan, Uchida, & Gershman, 2017).

These *correlational* data are also supported by recent *causal* investigations into the role of DA in RPE (Sharpe et al., 2017; Watabe-Uchida, Eshel, & Uchida, 2017; Eshel, Tian, Bukwich, & Uchida, 2016; Steinberg et al., 2013). With the recent advancement of optogenetics and Cre-recombinase-driver rat lines (Th-cre+/-), the field is able to target and induce DA transients at specific time points, which allows us the temporal precision needed to study the role of VTA DA neurons in RPE (Witten et al., 2011). Specifically, Steinberg and colleagues (2013) used the Th-cre+/- rats to target DA neurons and examine their role in RPE using the blocking paradigm. In their experiment, Steinberg and colleagues (2013) optically stimulated VTA DA neurons in the Th-cre+/- rat at the time of expected reward delivery in Phase 2 of a blocking paradigm. The hypothesis was that enhancing DA activity at the time of the expected reward would increase the normally small RPE and this would encourage learning about the normally blocked cue and reward. This is what they found: Responding to the blocked cue following stimulation was higher compared to a blocking control. Taken together, these correlational and causal studies suggest that VTA DA neurons encode for reward prediction-error and stimulation

of those neurons in Phase 2 in a blocking paradigm in reward leads to enhanced learning about the blocked cue (i.e., increase in prediction-error).

The role of VTA DA neurons in associative fear learning is not as clear as its role in RPE. Rather, the involvement of DA in appetitive and aversive settings seems to be contradictory. For example, electrophysiological data, including single cell recordings of different populations of DA neurons in VTA/SN show both inhibition and excitation upon the presentation of an aversive US or a CS predicting an aversive event (Matsumoto, & Hikosaka, 2009; Tovote, Fadok, J & Lüthi, 2015; Brischoux, Chakraborty, Brierley, & Ungless, 2009). Moreover, although stimulation of VTA DA neurons and presentation of reward increases DA release in the nucleus accumbens (NAc) and other downstream structures (Cheer et al., 2007; Witten et al., 2011; Brown, McCutcheon, Cone, Ragozzino, & Roitman, 2011; Parker et al., 2016), the opposite seems to be the case in fear (Badrinarayan et al., 2012. Mccutcheon, Ebner, Loriaux, & Roitman, 2012). These downstream structures receive DA inputs from the VTA (Watabe-Uchida, Zhu, Ogawa, Vamanrao, & Uchida, 2012; Haber, Fudge, & McFarland, 2000). Further, *antagonism* of DA receptors in structures downstream of the VTA (i.e. NAc, and amygdala) during Phase 2 of the blocking paradigm enhanced learning about the blocked cue in fear (Iordanova et al, 2006; Iordanova, 2010). Thus, taken together, these data point to a role of the VTA DA in aversive prediction-error (APE).

The Present Thesis.

Here we undertook an examination aimed at elucidating the role of VTA DA neurons in APE. We used a combination of blocking and overshadowing with an aversive (footshock) US along with optogenetics in Th-cre+/- rats in order to exercise fine temporal control over VTA DA neurons during learning. Our aim was to make a direct comparison between the roles of

VTA DA in learning about rewards versus aversive events. Evidence suggests an opposing role of DA in fear and reward (e.g., Matsumoto, & Hikosaka, 2009; Iordanova et al, 2006; Iordanova, 2010), therefore we expected VTA DA stimulation at time of the expected footshock in blocking to have the opposite effect to that reported in reward. That is, we expected to see an augmentation of the blocking effect. Combining this temporal control of VTA DA neurons with the blocking paradigm (and overshadowing) will allow us to test prediction error and temporal contiguity against one another while controlling for stimuli exposure between the blocking group and the control group (Iordanova, 2009). We report that stimulation of VTA DA neurons and their terminals in NAc at the time of expected shock augmented the blocking effect in fear by attenuating APE and further impaired learning about the blocked cue. We did not observe such an effect in overshadowing and many neural controls.

Experiment 1. Dopamine transients augment the blocking effect.

Pharmacologically reducing DA transmission using the DA antagonist flupenthixol in the VTA target sites, the NAc (Iordanova et al., 2006) and the amygdala (Iordanova, 2010) during Phase 2 of a blocking paradigm prevented blocking. That is antagonizing DA receptors in the NAc or amygdala encouraged learning about a normally blocked cue and footshock. Interestingly, an accumbal infusion of amphetamine, which increases DA in the extracellular space, augmented the blocking effect (Iordanova et al., 2006). These data were taken as evidence that DA at VTA terminal sites modulates aversive prediction error (APE). The aim of the present experiment was to determine the involvement of the VTA during APE using the blocking paradigm. Specifically, we optically stimulated VTA DA neurons at time of an expected footshock (Phase 2) in a blocking design and tested to see how much was learned about the normally blocked cue and the footshock US. This design paralleled a very similar design used to

show that optical stimulation of VTA DA neurons in a blocking design using a sucrose reward as a US encouraged learning about the normally blocked cue and the expected reward (Steinberg et al., 2013). Our data show that VTA DA neurons enhanced the blocking effect in fear.

Materials and Methods

Subjects

Twelve male transgenic Th-cre+/- rats (Long-Evans background) that expressed Cre recombinase under the control of the tyrosine hydroxylase promoter (Th-cre+/-) and Thirteen wild-type littermates (Th-cre-/-) were used in Experiment 1. All rats were bred in-house. Rats were distributed into different groups by counterbalancing body weight, age, and litters. Before the surgery, rats were housed in pairs in standard clear shoebox cages in a humidity and temperature-controlled environment under reverse light-dark conditions (12:12 h light-dark cycle; lights off at 8:00 a.m.). Experimental sessions were run 3-4 hours after the onset of the dark cycle. Rats were at least 3 months old before the surgeries. After the surgeries, the rats were individually housed. Rats had *ad libitum* access to water throughout the experiments and approximately 23g food per rat was given to prevent excessive fat gain (when the body weight reached about 450g) and maintain healthy adult body weight during the virus expression wait time (4 weeks in Experiment 1, Experiment 2 and Experiment 3, and 8 weeks in Experiment 4) after the surgeries. All rats were treated in accordance with the approval granted by the Canadian Council on Animal Care and the Concordia University Animal Care Committee.

Surgeries

Surgeries were performed under isoflurane (1–2% at 0.8 litres/minute) anesthesia and aseptic conditions. Penicillin (450,000 IU/rat) and analgesic (Anafen, Ketoprofen, 0.2 ml/rat;

intraperitoneal injection; CDMV, St. Hyacinthe, QC) were administered pre-operatively and saline (5 ml/rat, s.c.) for hydration was administered during the surgery. Standard stereotaxic surgical procedures were used for viral infusion and implantation of the optical fibre (Witten et al., 2011). Briefly, a Cre-dependent virus with channelrhodopsin-2 (ChR2) (AAV5-Ef1α-DIO-ChR2-eYFP; University of North Carolina Viral Vector Core, Chapel Hill, NC) was infused bilaterally in the VTA at the following coordinates relative to bregma and skull surface: AP: -5.4 & - 6.2; ML: ± 0.8 (Experiment 1, Experiment 2, Experiment 4) & ML: + 0.8 (Experiment 3); DV: -8.3 & -7.2, using a custom-made 31 gauge needle. A 1.0 μl of virus was infused at a rate of 0.1μL/minute for 10 minutes at each infusion site using a syringe pump (Harvard Apparatus). The injector was left in place for an additional 10 mins before it was slowly moved up to the dorsal site of injection or out of the brain. In Experiments 1-3, an in-house made optical implant with an optical fibre (a 200 μm core, Thorlabs) epoxied in a ceramic ferrule (Fiber Instrument Sales) was unilaterally implanted in the right VTA at a 10° angle at the following coordinates relative to bregma and skull surface: AP: - 5.8; ML: + 2.2; DV: -8.12. The optical implant/cannula was secured with jeweller's screws, acrylic and dental cement in all animals. Rats were given *ad libitum* access to food and water for two weeks post-surgery and an oral antibiotic (Cephalexin, 15 mg/kg; CDMV, St. Hyacinthe, QC) prophylactically for 5 days.

Apparatus

Behavioural sessions were conducted in standard operant conditioning chambers (25.4 cm W × 31.8 L × 26.7 cm H; Med Associates, St. Albans, VT, USA), enclosed in wooden cabinets each equipped with a ventilation fan. The background noise in the chambers was approximately 55dB. Each chamber consisted of a stainless steel grid floor, modular left and

right walls, and Perspex back wall, front door and ceiling. The grid floor was connected to a shock generator that delivered a continuous scrambled footshock.

Two white cue lights (28V DC, 100 mA stimulus light) were positioned 10 cm below the ceiling on the left and right panels, and a magazine was located on the centre panel, mounted 10 cm above the stainless metal grid floor and a red house light (28V DC, 100 mA stimulus light with red replacement lens cover) was placed above the food magazine, 15 cm below the ceiling on the centre panel. A mechanical clicker located below the white cue light on the right panel of the left wall. The behavioural sessions were videotaped using an infrared light-sensitive video-camera (Med Associates), mounted on the back wall of the wooden compartment and behind the Perspex back wall of the chamber.

In experiments involving optical stimulation, the optical implant mounted on the head of the rat was connected to a patch cord built in-house using a ceramic sleeve (Fiber Instrument Sales) covered with black tape (to prevent the emission of the laser light illuminating the chamber and acting as a CS itself). The patch cord was connected to an optical commutator (Doric Lenses). The commutator was held by a metal arm secured to a metal pole screwed on the ceiling of the conditioning chamber. A Doric manufactured patch cord connected the commutator to a DPSS 473 nm laser (Shanghai Laser & Optics Century Co., Ltd.). A computer running Med PC IV (Med Associates) software controlled the optical stimulation via an optical Arduino made in-house.

Stimuli.

The auditory stimulus used in all experiments was a 30s 10 Hz 75dB mechanical clicker and the visual stimulus was a 30s 20Hz flashing light. The unconditioned stimulus was a 0.5mA

1s footshock. The optical stimulation was a 1s 20Hz 18-22 mW (on average 20 mW: adjusted for the pulse amplitude and waveform using an oscilloscope and a power meter).

Behavioural Procedures

The experiment consisted of 4 phases: habituation, conditioning during Phase 1 and Phase 2, and non-reinforced Tests.

Habituation. On Day 0 rats were habituated to the auditory and visual stimuli to minimize unconditioned responses to novel stimuli. The habituation session lasted one day and consisted of two presentations of each cue (clicker and flashing light) 5 minutes upon placement in the experimental chambers. The cues were presented two times each for 30s with an intertrial interval (ITI) of 2 minutes and the session lasted for a total of 16 minutes.

Phase 1. On each of Days 1-2, rats in the Blocking groups received three pairings between the flashing light and footshock for a total of 6 such pairings across Phase 1. The first light-shock pairing took place 5 minutes upon placement in the conditioning chamber, and successive pairings were separated by an average of 5 minutes ITI (range: 240-360 s). The last light-shock pairing occurred 4 minutes prior to the end of the training session. Following conditioning, rats received context exposure session during which the rats were brought and placed in the operant chambers for 30 minutes approximately 3.5 hours after the training sessions to reduce freezing to the background cues. Rats in the Control group did not receive Phase 1 conditioning and were merely handled outside the laboratory.

Phase 2. Phase 2 lasted one Day (Day 4) and all rats irrespective of group membership received two pairings between the flashing light and clicker presented in compound and footshock. All rats received context exposure session in a manner identical to that described for

Phase 1. *Optical Stimulation.* The Group Block-Shock ($N = 8$) was optically stimulated for 1s during the expected footshock, whereas the Group Block-ITI ($N = 4$) was optically stimulated for 1s during the ITI. The Group Block ($N = 6$) and the Group Control ($N = 7$) did not receive any surgery or optical stimulation.

Tests. Rats were tested for fear of the clicker and flashing light on Days 6 and 7, respectively. The test session consisted of eight 30s non-reinforced presentations of the conditioned cues (light or clicker) 1 minute apart. Each test session consisted of a 5 minutes acclimation period prior to the first presentation of a cue. Rats were removed from the conditioning chambers following the last (eighth) presentation of the cue.

Histology

After the completion of each experiment, rats were euthanized with a lethal dose of sodium pentobarbital (120 mg/rat) and perfused intracardially with 0.9% sodium chloride, followed by 4% paraformaldehyde (Sigma-Aldrich, Missouri, USA). Fixed brains were cut into 60-µm sections with a cryostat (Thermo Scientific) and examined under a fluorescence microscope (NikonTi, Nikon, Japan) to determine the extent of viral spread and confirm the placement of the optical fibre tip and infusion cannula. In all experiments, data of rats with good viral expression and placement of the ferrule(s) and cannula were included.

Scoring and Statistics

All sessions were videotaped and scored offline. Freezing behaviour was scored on a second-by-second basis with a timestamp procedure in which each rat was observed and scored as either freezing or moving. Freezing was defined as the absence of all movements, except for those related to breathing (Blanchard & Blanchard, 1969). A percentage score was calculated for

each rat which consisted of the time spent freezing over the total observation time for a stimulus. The data were analyzed using planned orthogonal contrasts (PSY, 2000, UNSW; SPSS, version 23; GraphPad Prism 7). Significance was set at the 0.05.

Results

*Histology***.** The brains of all rats in the experiment were perfused and sliced coronally through the VTA. All rats showed expression of eYFP indicative of the transfection of Th positive neurons with channelrhodopsin in the VTA. Figure 3 shows a representative expression eYFP in the VTA. Detailed outline for the minimum and maximum extent of viral spread in the cohort along with optical fiber placements is currently in progress.

Behaviour. Statistical analyses and graphical representations of the data from the conditioning sessions of Phase 1 and Phase 2 are presented in the Appendix section of this thesis.

The main data of interest are those obtained during the clicker and light non-reinforced test sessions. Figure 1 shows the percent time spent freezing to the clicker on test for all groups averaged across trials. Pre-training of the light-shock relationship blocked subsequent learning about the clicker-shock relationship. Rats trained to fear the clicker in the absence of light-shock pre-training (i.e., Control group) showed higher levels of freezing to the clicker compared to rats trained to fear the clicker in the presence of the pre-trained light (i.e., Block groups; $F_{1,21}$ = 19.093, CI{1.032:2.906}). Interestingly, optical stimulation of VTA DA neurons at time of the expected shock further attenuated learning about the clicker-shock relationship. Freezing was significantly attenuated in the Group Block-Shock compared to Groups Block and Block-ITI ($F_{1,21}$ = 13.927, CI{0.791:2.782}). Optical stimulation during a random time point during the compound conditioning session did not affect blocking as freezing did not differ between Group Block and Group Block-ITI ($F_{1,21} < 1$, CI{-1.217:1.468}).

Figure 2 shows the percent time spent freezing to the light on test for all groups averaged across trials. Pre-training with the light and shock in Phase 1 resulted in higher levels of fear to the light compared to no pre-training. Groups Block showed a higher level of freezing than Group Control ($F_{1,21} = 6.320$, CI{-2.070:-0.196}). Optical stimulation of VTA DA neurons during the expected shock did not affect learning about the pre-trained light and shock relationship compared to Groups Block and Block-ITI ($F_{1,21} < 1$, CI{-0.979:1.012}). Unexpectedly, groups Block-ITI showed a lower level of fear to the pre-trained light compared to group Block ($F_{1,21} = 6.774$, CI{0.338:3.022}). This was likely due to differences in pre-training (see Appendix for Experiment 1: differences in fear to the light at the end of Phase 1).

Discussion

Experiment 1 provided evidence that stimulation of VTA DA neurons at the time of expected shock augmented the blocking effect. Before considering the likely psychological effect that stimulation of VTA DA neurons has on learning in the blocking paradigm, we sought to replicate and extend this finding using another set of parameters with extensive training in Phase 1 and Phase 2 that still yields blocking. In addition, we wanted to determine if laser artifacts at time of US presentation may have influenced our results and whether VTA DA stimulation during the ITI does indeed affect fear to the pre-trained light on the test. Therefore in Experiment 2 we again stimulated VTA DA neurons at time of the expected shock in blocking (Group Block-Shock) or during the ITI (Group Block-ITI), as well as added a new group (Group Block-Green) which received stimulation with green (532nm) light as opposed to the standard blue (473nm) light for channelrhodopsin. The latter group allowed to test whether laser artifacts affected our results.

Figure 1. Experiment 1: Clicker Test

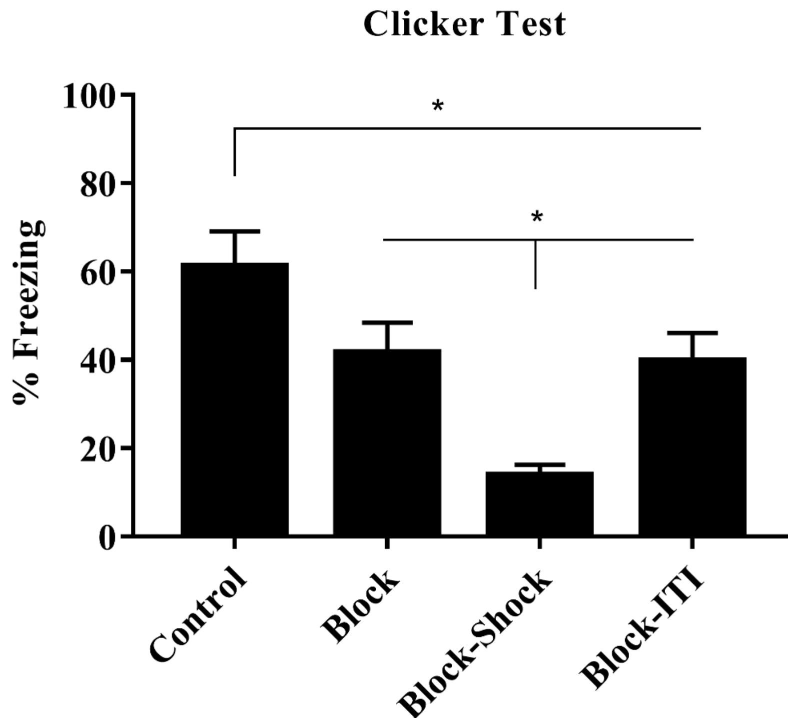


Figure 1. The percent time spent freezing to the clicker on test for all groups averaged across trials. Data are presented as means and error bars represent SEM.

Pre-training of the light-shock relationship blocked subsequent learning about the clicker-shock relationship as the Control group showed higher levels of freezing to the clicker compared to rats in the blocking groups that were pre-trained with the light. Interestingly, optical stimulation of VTA DA neurons at time of the expected shock further attenuated learning about the clicker-shock relationship as freezing was significantly attenuated in the Group Block-Shock compared to Groups Block and Block-ITI. However, optical stimulation during the ITI did not affect blocking as freezing did not differ between Group Block and Group Block-ITI.

Figure 2. Experiment 1: Light Test

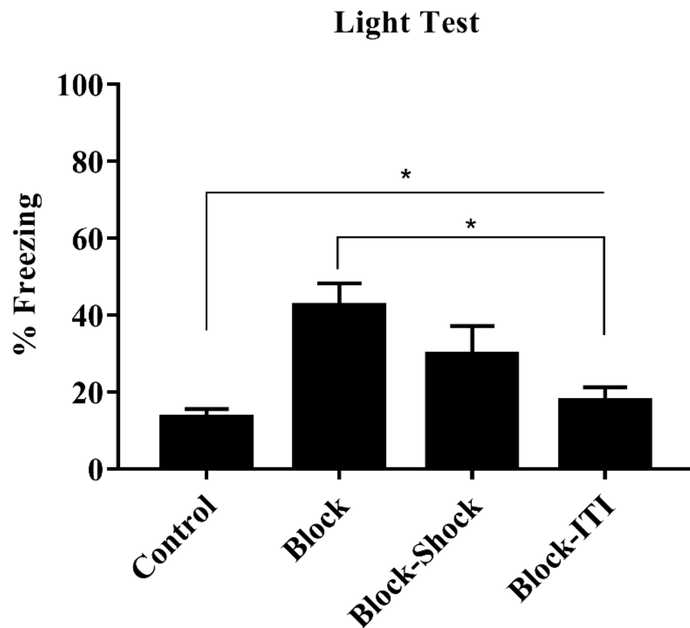


Figure 2. The percent time spent freezing to the light on test for all groups averaged across trials. Data are presented as means and error bars represent SEM.

Pre-training with the light and shock in Phase 1 resulted in higher levels of fear to the light compared to the groups with no pre-training as Groups Block showed a higher level of freezing than Group Control. Optical stimulation of VTA DA neurons during the expected shock did not affect learning about the pre-trained light and shock relationship compared to Groups Block and Block-ITI. Unexpectedly, groups Block-ITI showed a lower level of fear to the pre-trained light compared to group Block, likely due to differences in pre-training.

Histology Figure

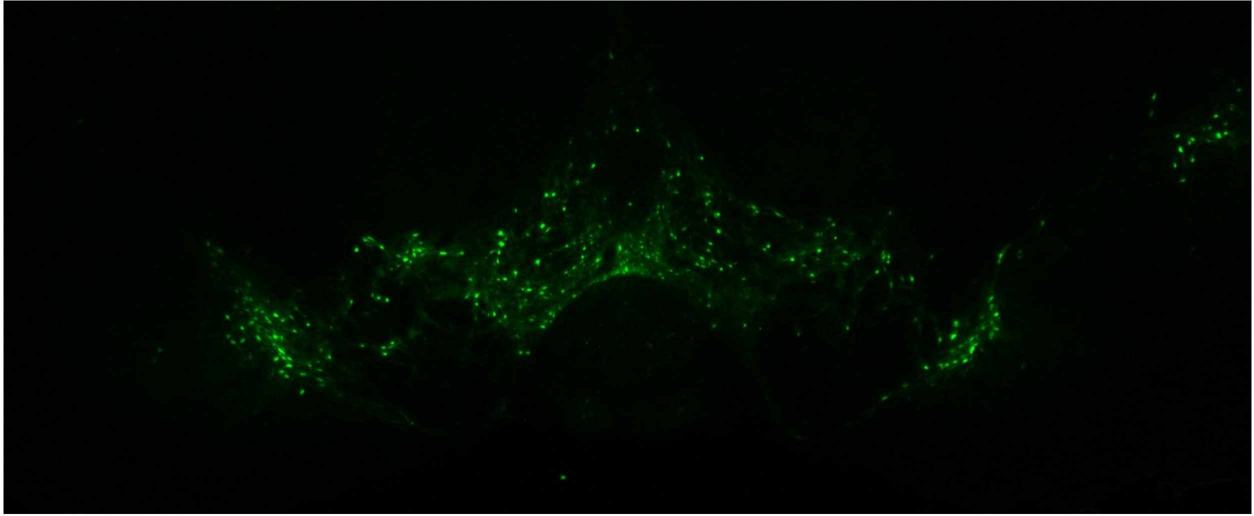


Figure 3. Representation of bilateral virus expression in VTA for the rats included in the analysis in all groups. Detailed outline for the minimum and maximum extent of viral spread in the cohort along with optical fiber placements is currently in progress.

Experiment 2. DA transients augment the blocking effect regardless of experimental parameters.

In this Experiment, we wanted to replicate and confirm the findings reported in Experiment 1 using experimental parameters that allowed for stronger conditioning in Phase 1 and Phase 2. Consequently, in Experiment 2 we extended the training phases by an extra day in both Phase 1 (3 days) and Phase 2 (2 days). In addition, we considered a control group which received stimulation of an inapt wavelength (532 nm; green laser) during the expected shock in Phase 2, thus TH positive neurons should not be excited. This control group allowed us to control for the laser and opsin artifacts during the expected shock delivery.

Materials and Methods

All materials and methods used in this experiment were identical to those described above in Experiment 1 unless stated otherwise.

Subjects

Twelve male transgenic Th-cre+/- rats (Long-Evans background) that expressed Cre recombinase under the control of the tyrosine hydroxylase promoter (Th-cre+/-) were used in Experiment 2. Rats were treated in accordance with the procedures described in Experiment 1 above.

Behavioural Procedures

The experiment consisted of 4 phases: habituation, conditioning during Phase 1 and Phase 2, and non-reinforced Tests.

Habituation. The habituation session was identical to that described for Experiment 1.

Phase 1. Only rats in the blocking groups received Phase 1 training. This phase was identical to that described in Experiment 1 with the exception that it lasted for three days, thus yielding a total of nine conditioning trials. Each condition session was followed by context exposure in the manner described above. Rats in the Control group did not receive Phase 1 conditioning and were merely handled outside the laboratory.

Phase 2. During Phase 2, all rats received compound training in a manner identical to that described in Experiment 1 with the exception that this phase lasted two days and yielding four compound-shock pairings. Each training day was followed by context exposure in the manner described above. *Optical Stimulation.* VTA DA neurons in the Group Block-Shock ($N = 4$) were optically stimulated for 1s during the expected footshock using a blue laser and the Group Block-Green ($N = 4$) was stimulated for 1s during the expected footshock using a green laser, whereas the Group Block-ITI ($N = 4$) was optically stimulated during the ITI for 1s using a blue laser.

Tests. The test sessions were identical to those described for Experiment 1.

Results

Histology. In progress.

Behaviour. Statistical analyses and graphical representations of the data from the conditioning sessions of Phase 1 and Phase 2 are presented in the Appendix section of this thesis.

As in Experiment 1, the main data of interest are those obtained during the clicker and light non-reinforced test sessions. Figure 4 shows the percent time spent freezing to the clicker on test for all groups averaged across trials. Similar to Experiment 1, stimulation of VTA DA neurons at the time of expected shock in a blocking design augmented the blocking effect. Freezing to the clicker was lower in Group Block-Shock compared to the two control Blocking

groups (Block-ITI and Block-Green, $F_{1,9} = 9.084$, CI{-3.231:-0.460}). No differences were obtained between Groups Block-Green and Block-ITI ($F_{1,9} < 1$, CI{-2.156:1.043}), thus laser or opsin artifact did not modulate the blocking effect.

Figure 5 shows the percent time spent freezing to the light on test for all groups averaged across trials. No differences were found between the Groups. Freezing to the light in group Block-Shock did not differ from that of the two control Blocking groups (Groups Block-Green and Block-ITI, $F_{1,9} < 1$, CI{-1.385:1.385}). Groups Block-Green and Block-ITI did not differ from each other ($F_{1,9} = 1.235$, CI{-0.814:2.385}).

Figure 4. Experiment 2: Clicker Test

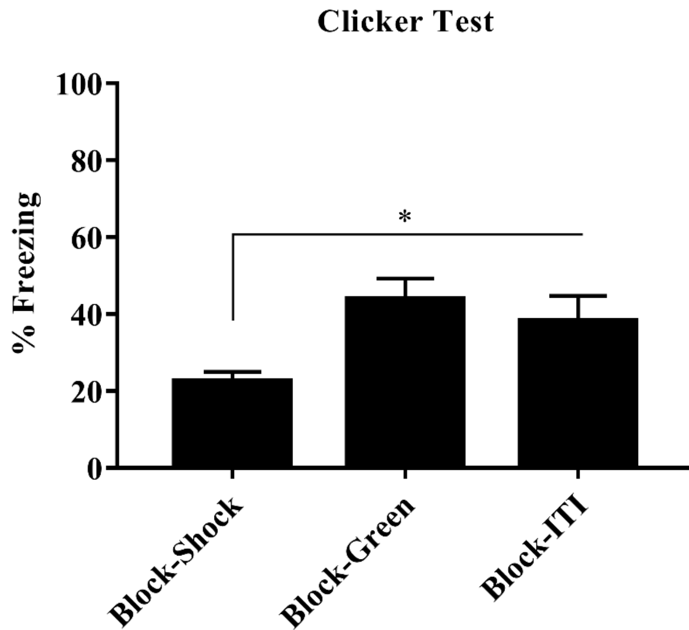


Figure 4. The percent time spent freezing to the clicker on test for all groups averaged across trials. Data are presented as means and error bars represent SEM.

Stimulation of VTA DA neurons at the time of expected shock in a blocking design augmented the blocking effect as freezing to the clicker was lower in Group Block-Shock compared to the two control Blocking groups (Block-ITI and Block-Green). No differences were obtained between Groups Block-Green and Block-ITI, thus laser or opsin artifact did not modulate the blocking effect.

Figure 5. Experiment 2: Light Test

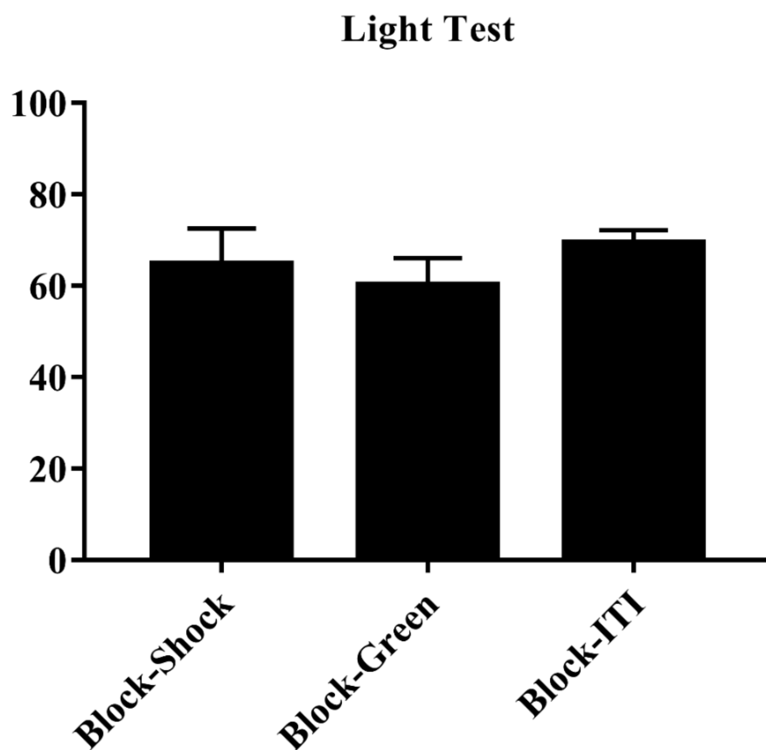


Figure 5. The percent time spent freezing to the light on test for all groups averaged across trials. Data are presented as means and error bars represent SEM.

No differences were found between the Groups as freezing to the light in group Block-Shock did not differ from that of the two control Blocking groups (Groups Block-Green and Block-ITI). Groups Block-Green and Block-ITI did not differ from each other.

Discussion

In Experiment 2, we replicated our results in Experiment 1 using a behavioural paradigm with an extended conditioning in both Phase 1 and Phase 2, which suggests that DA transients augment the blocking effect regardless of experimental parameters. We specifically controlled for the laser and opsin artifacts in Experiment 2 and found that the laser and opsin artifact did not affect the blocking effect. Moreover, data from Experiment 2 provide evidence that fear to the light is not affected by any of the neural manipulations.

Experiments 1 and 2 provided evidence that stimulation of VTA DA neurons at the time of expected shock augmented the blocking effect. Two somewhat similar possibilities for this effect could be that VTA DA stimulation reduced the aversiveness of the footshock US or if VTA DA carries a rewarding signal then it could have counter-conditioned the footshock US. Either of the possibilities would result in a reduced fear response elicited by the conditioned clicker, but not necessarily to the pre-trained light as the additional conditioning in Phase 1 would ensure high levels of fear to that cue. Alternatively, optical stimulation of VTA DA during the expected footshock could have reduced the APE thus further augmenting the blocking effect as seen by the low levels of fear to the clicker compared to the other blocking groups. The aim of Experiment 3 was to test these possibilities.

Experiment 3. DA transients do not modulate overshadowing.

Experiments 1 and 2 showed that stimulation of VTA DA neurons during the expected shock augmented the blocking effect regardless of experimental parameters. Evidence suggests that optical stimulation of DA neurons could have a rewarding effect (Tsai et al., 2009; Rossi et al., 2013), thereby potentially counter-conditioning the footshock US or reducing its

aversiveness. In this experiment, we sought to determine if the optical stimulation of VTA DA neurons used in Experiments 1 and 2 augmented blocking by altering the aversive properties of the footshock US. To do this, we used the exact same footshock US and exact same stimulation parameters in an overshadowing control procedure. Overshadowing is used in blocking studies as a comparison to show how normal learning would proceed if the prediction error was maximal. If VTA DA reduces the aversive properties of the footshock US, then we should see lower levels of freezing to the clicker *and* to the light in the groups stimulated during the unexpected shock. We found no such difference.

Materials and Methods

All materials and methods used in this experiment were identical to those described above in Experiment 1 and Experiment 2 unless stated otherwise.

Subjects

Twenty-three male transgenic Th-cre+/- rats (Long-Evans background) that expressed Cre recombinase under the control of the tyrosine hydroxylase promoter (Th-cre+/-) and three wild-type littermates (Th-cre-/-) were used. Rats were treated in accordance with the procedures described in the General Materials and Methods section above.

Behavioural Procedures

The experiment consisted of 3 phases: habituation, conditioning, and non-reinforced tests. Each of these phases was identical to those described above for Experiments 2 unless stated otherwise.

Habituation. The habituation session was identical to that described for Experiment 1.

Conditioning. During *Conditioning*, all rats received compound training in a manner identical to that described in Experiment 1 with the exception that this phase lasted two days and yielding four compound-shock pairings. Each training day was followed by context exposure.

Optical stimulation. In Phase 2, VTA DA neurons in the Group Control-Shock ($N = 8$) were optically stimulated for 1s during the unexpected footshock using a blue laser and the Group Control-Green ($N = 8$) was stimulated for 1s during the unexpected footshock using a green laser, whereas the Group Control-ITI ($N = 7$) was optically stimulated during the ITI for 1s using a blue laser. The Group Control ($N = 3$) did not receive any surgery or optical stimulation.

Tests. The test sessions were identical to those described for Experiment 1.

Results

Behaviour. Statistical analyses and graphical representations of the data from the conditioning sessions of Phase 2 are presented in the Appendix section of this thesis.

The main data of interest are those obtained during the clicker and light non-reinforced test sessions. Figure 6 shows the percent time spent freezing to the clicker on test for all groups averaged across trials. We found that VTA DA transients do not modulate overshadowing as there was no difference between the Group Control and the other groups that received optical stimulation (i.e., Groups Control-Shock, Control-Green and Control-ITI; $F_{1,22} < 1$, CI{-0.986:1.561}). Stimulation at the time of unexpected shock did not modulate the fear response as freezing did not differ between the Group Control-Shock compared to the Groups Control-ITI and Control-Green ($F_{1,22} < 1$, CI{-.560:1.257}) and freezing did not differ between the Groups Control-ITI and Control-Green ($F_{1,22} = 1.168$, CI{-1.633:0.514}).

Figure 7 shows the percent time spent freezing to the light on test for all groups averaged across trials. Optical stimulation of VTA DA neurons during the unexpected shock did not affect learning about the non-salient stimulus in overshadowing Control as freezing in the Group Control did not differ compared to the Groups that received optical stimulation (i.e., the Groups Control-Shock, Control-ITI and Control-Green; $F_{1,22} < 1$, CI{-0.1.763:0.784}). Stimulation at the time of unexpected shock did not modulate the fear response as freezing did not differ between the Group Control-Shock compared to the Groups Control-ITI and Control-Green ($F_{1,22} < 1$, CI{-1.171:.647}). Moreover, the Groups Control-ITI and Control-Green did not differ from each other ($F_{1,22} < 1$, CI{-1.536:0.610}).

Figure 6. Experiment 3: Clicker Test

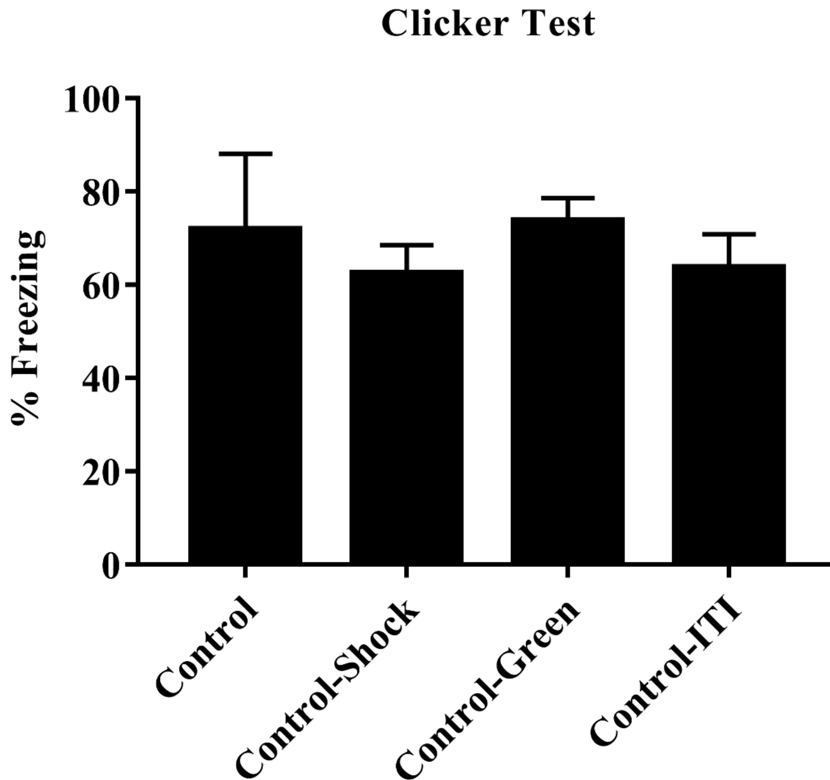


Figure 6. The percent time spent freezing to the clicker on test for all groups averaged across trials. Data are presented as means and error bars represent SEM.

VTA DA transients do not modulate overshadowing as there was no difference between the Group Control and the other groups that received optical stimulation (i.e., Groups Control-Shock, Control-Green and Control-ITI). Stimulation at the time of unexpected shock did not modulate the fear response as freezing did not differ between the Group Control-Shock compared to the Groups Control-ITI and Control-Green, and freezing did not differ between the Groups Control-ITI and Control-Green.

Figure 7. Experiment 3: Light Test

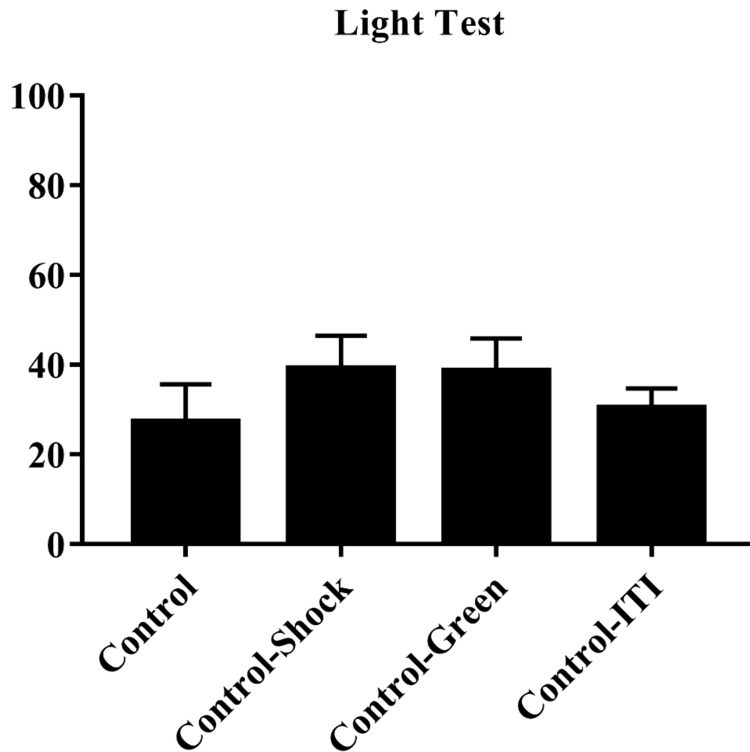


Figure 7. The percent time spent freezing to the light on test for all groups averaged across trials. Data are presented as means and error bars represent SEM.

Optical stimulation of VTA DA neurons during the unexpected shock did not affect learning about the non-salient stimulus in overshadowing Control as freezing in the Group Control did not differ compared to the Groups that received optical stimulation (i.e., the Groups Control-Shock, Control-ITI and Control-Green). Stimulation at the time of unexpected shock did not modulate the fear response as freezing did not differ between the Group Control-Shock compared to the Groups Control-ITI and Control-Green. Moreover, the Groups Control-ITI and Control-Green did not differ from each other.

Histology Figures

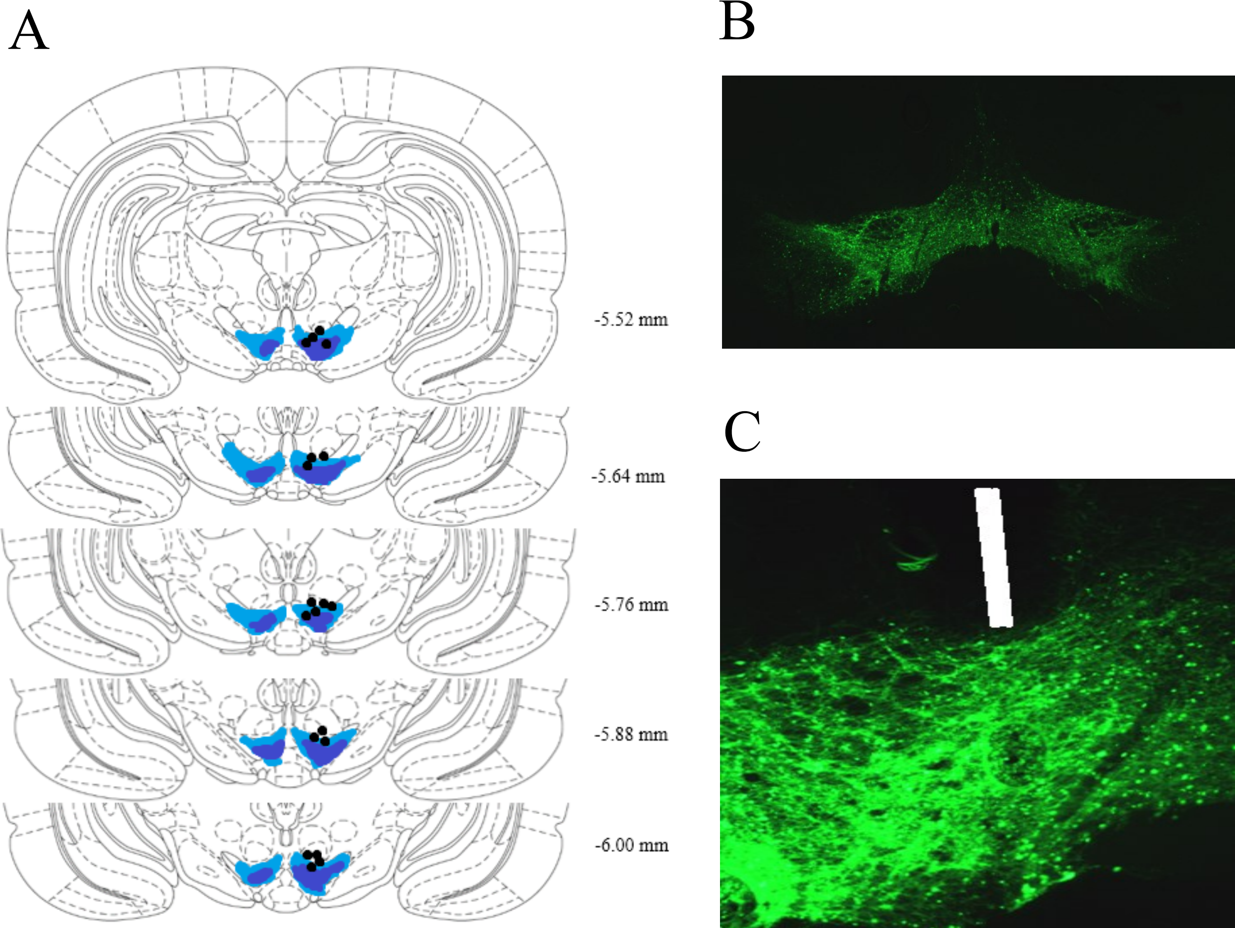


Figure 8. A) Representation of the unilateral fiber placements and bilateral virus expression in VTA for the rats included in the analysis in all groups. Fiber implants (black circles) were in the vicinity of ChR2 (blue) expression in VTA. Where light shading represents the maximal and dark shading represents the minimal spread of viral expression. B) A coronal brain slice showing a representative viral expression throughout the VTA for rats in Experiment 3. C) A coronal brain slice showing a representative viral expression and placement of the ferrule in the VTA for rats in Experiment 3 (60-µm sections)

Discussion

Experiment 3 provided evidence that VTA DA neurons do not modulate predictive learning in an overshadowing Control condition when prediction error is high. These data provide evidence that DA stimulation does not modulate fear conditioning by altering the aversive properties of the footshock US or by counterconditioning this US, or we would have seen similar effects of optical stimulation in the overshadowing control as we do in blocking. The alternative possibility suggested above is that VTA DA stimulation reduced APE. It must be noted that VTA DA may not have the same effect on learning in overshadowing if it acts on APE. When prediction error is at maximum gains in associative strength are large, which precludes detecting differences between the groups. Any reduction in APE as a result of VTA DA stimulation would be difficult to detect when starting with a maximal APE. Therefore, the blocking design is the best way to examine the effect of neural manipulations on PE.

Taken together, the Experiment 1, 2, and 3 provided important evidence that VTA DA neurons modulate APE. As mentioned earlier, neuropharmacological manipulations in the nucleus accumbens, a VTA DA target area, also modulate APE. In the following experiment, we wanted to test the idea that the VTA-NAc pathway is critically involved in APE.

Experiment 4. DA transients in NAc augment the blocking effect.

We further wanted to uncover the neural circuitry that underlies attenuation of APE in fear by the optical stimulation of VTA DA neurons during the expected footshock. VTA neurons heavily project to NAc (Björklund, & Dunnett, 2007; Ikemoto, 1997), and DA neurons in the NAc has been found to modulate reward prediction error signals (Flagel et al. 2011, Hart et al., 2014; Stuber et al., 2008; Wenzel et al., 2014). Moreover, DA transmission in the NAc modulates predictive learning in fear (Iordanova, et al., 2006). The aim of the present experiment

was to examine whether the VTA to NAc projection is involved in APE using the fear blocking design. We infused a viral vector carrying ChR2 into VTA cell bodies and implanted an optical fibre into the VTA terminals in the NAc. By stimulating the VTA DA terminals in the NAc during the expected shock in Phase 2 of blocking we could determine if this specific pathway was involved in augmenting the blocking effect and reducing APE. For a subset of rats, we blocked the firing of DA cell bodies in VTA using TTX, a sodium channel blocker, to rule out the possibility of antidromic conduction.

Materials and Methods

All materials and methods used in this experiment were identical to those described above in Experiment 1 and Experiment 2 unless stated otherwise.

Subjects

Twenty male transgenic Th-cre+/- rats (Long-Evans background) that expressed Cre recombinase under the control of the tyrosine hydroxylase promoter (Th-cre+/-) and twelve wild-type littermates (Th-cre-/-) were used. Rats were treated in accordance with the procedures described in the General Materials and Methods section above.

Surgeries

All surgical procedures were similar to the Experiment 1. Except in Experiment 4, the optical ferrule was implanted into the right nucleus accumbens (AP: +1.7; ML: +1.3; DV: -7) in all rats. For a subset of rats ($N = 6$), a guide cannula (AP: -5.8; ML: +2.2; DV: -7.12; Plastic One) was also implanted in the right VTA to allow for drug infusion.

Drug

Sodium-channel blocker tetrodotoxin (TTX) was dissolved in 0.9% sterile saline and was infused unilaterally (1 µM/0.5 µl/rat) over 2 minutes into the right VTA (a guide cannula: AP: -5.8; ML: + 2.2; DV: -7.12) using an injector that reached 1 mm beyond the tip of the cannulae (AP: - 5.8; ML: + 2.2; DV: -8.12). The injector was connected via tubing to 10-µl Hamilton syringe (Hamilton Co., Reno, NV) mounted on an infusion pump (Harvard Apparatus, South Natick, MA). The injector was left in place for 1 more minute after the infusion. This concentration of TTX used in this experiment has been found to induce behavioural and neurobiological changes in rats previously (Fuchs et al., 2005; Zhang et al., 2017; Martin, & Ghez, 1999).

Behavioural Procedures

The experiment consisted of 4 phases: habituation, conditioning during Phase 1 and Phase 2, and non-reinforced Tests. Each of these phases was identical to those described above for Experiments 2. *Optical stimulation.* In Phase 2, DA terminals in NAc of VTA DA neurons in the Group Block-Shock (*N* = 7) were optically stimulated for 1s during the expected footshock, whereas the Group Block-ITI (*N* = 7) was optically stimulated during the ITI for 1s. DA terminals in NAc of the Group TTX (*N* = 6) were also optically stimulated for 1s during the expected footshock, however, the cell bodies of VTA DA neurons were blocked using TTX. The group Block (*N* = 6) and the Group Control (*N* = 6) did not receive any surgery or optical stimulation.

Results

Histology. See below.

Behaviour. Statistical analyses and graphical representations of the data from the conditioning sessions of Phase 1 and Phase 2 are presented in the Appendix section of this thesis.

The main data of interest are those obtained during the clicker and light non-reinforced test sessions. Figure 9 shows the percent time spent freezing to the clicker on test for all groups averaged across trials. Pre-training of the light-shock relationship blocked subsequent learning about the clicker-shock relationship. Rats trained to fear the clicker in the absence of light-shock pre-training (i.e., Control group) showed higher levels of freezing to the clicker compared to rats trained to fear the clicker in the presence of the pre-trained light (i.e., Block groups; $F_{1,27}$ = 57.856, CI{2.517:4.377}). Interestingly, optical stimulation of VTA DA terminals in NAc at time of the expected shock further attenuated learning about the clicker-shock relationship as freezing was significantly lower in the Groups Block-Shock and Block-TTX compared to the Groups Block and Block-ITI ($F_{1,27}$ = 10.091, CI{-2.057:-0.443}). Optical stimulation during a random time point during the compound conditioning session did not affect blocking as freezing did not differ between the Groups Block and Block-ITI ($F_{1,27} < 1$, CI{-1.607:0.676}). Optical stimulation of DA terminals in NAc did not induce antidromic conduction as freezing did not differ between the Groups Block-Shock and Block-TTX ($F_{1,27} < 1$, CI{-1.008:1.276}).

Figure 10 shows the percent time spent freezing to the light on test for all groups averaged across trials. Pre-training with the light and shock in Phase 1 resulted in higher levels of fear to the light compared to no pre-training as Group Control showed lower levels of freezing than the blocking groups (i.e., Block, Block-Shock, Block-ITI, and Block-TTX; $F_{1,27}$ = 12.797,

CI{-2.551:-0.691}). Optical stimulation of VTA DA terminals in NAc during the expected shock (i.e., the Groups Block-Shock and Block-TTX) did not affect learning about the pre-trained light and shock relationship compared to groups Block and Block-ITI ($F_{1,27} < 1$, CI{-0.792:0.822}). There was no difference in freezing to the light between Groups Block-Shock and Block-TTX ($F_{1,27} < 1$, CI{-1.359:0.924}), however, Group Block-ITI froze higher than Group Block ($F_{1,27} = 5.740$, CI{-2.474:-0.191}).

Discussion

Experiment 4 revealed that stimulation of DA terminals in NAc at time of expected shock augmented the blocking effect similar to the stimulation of VTA DA neurons in Experiment 1 and 2. We blocked the cell bodies of VTA DA neurons while stimulating the terminals in NAc and confirmed that the VTA-NAc pathway is responsible for attenuation of prediction-error in fear. It is unclear why there was more fear to the light in the group that received optical stimulation of the VTA-NAc pathway during the ITI. We will follow this up in a subsequent experiment.

Figure 9. Experiment 4: Clicker Test

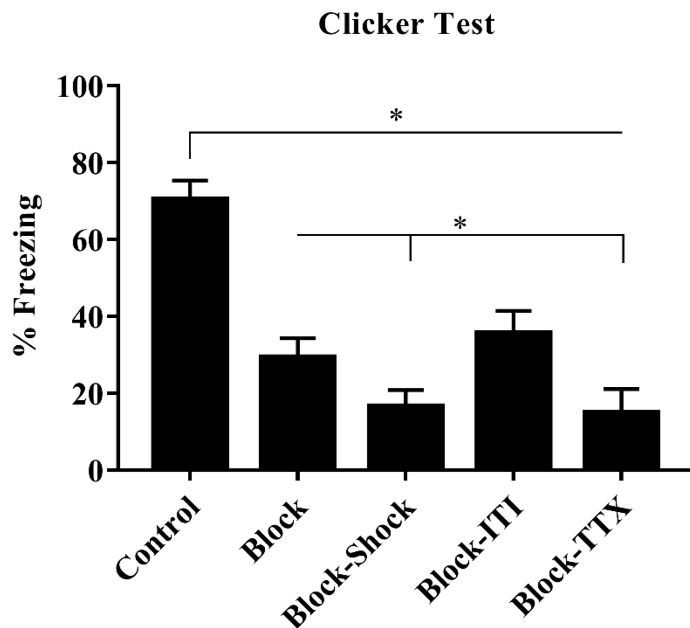


Figure 9. The percent time spent freezing to the clicker on test for all groups averaged across trials. Data are presented as means and error bars represent SEM.

Pre-training of the light-shock relationship blocked subsequent learning about the clicker-shock relationship as freezing to the clicker was higher in the Group Control training compared to rats in the Block groups. Interestingly, optical stimulation of VTA DA terminals in NAc at time of the expected shock further attenuated learning about the clicker-shock relationship as freezing was significantly lower in the Groups Block-Shock and Block-TTX compared to the Groups Block and Block-ITI. Optical stimulation during the ITI did not affect blocking as freezing did not differ between the Groups Block and Block-ITI. Optical stimulation of DA terminals in NAc did not induce antidromic conduction as freezing did not differ between the Groups Block-Shock and Block-TTX.

Figure 10. Experiment 4: Light Test

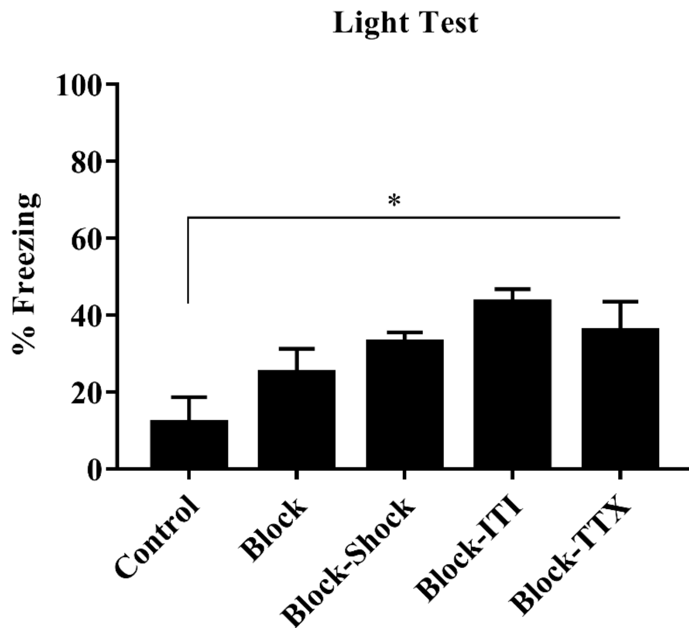
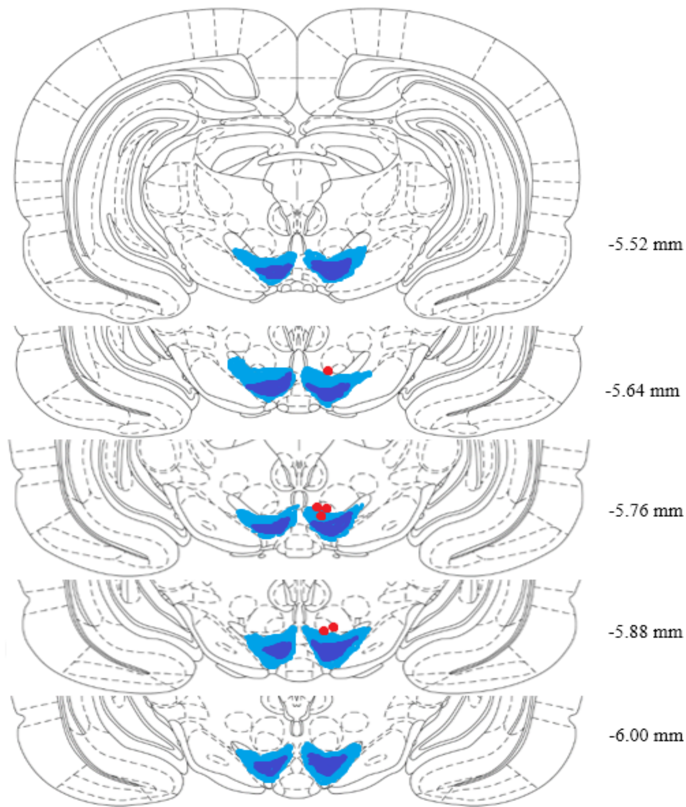


Figure 10. The percent time spent freezing to the light on test for all groups averaged across trials. Data are presented as means and error bars represent SEM.

Pre-training with the light and shock in Phase 1 resulted in higher levels of fear to the light compared to no pre-training as Group Control showed lower levels of freezing than the blocking groups (i.e., Block, Block-Shock, Block-ITI, and Block-TTX). Optical stimulation of VTA DA terminals in NAc during the expected shock (i.e., the Groups Block-Shock and Block-TTX) did not affect learning about the pre-trained light and shock relationship compared to groups Block and Block-ITI. There was no difference in freezing to the light between Groups Block-Shock and Block-TTX, however, Group Block-ITI froze higher than Group Block.

Histology Figures

A



B

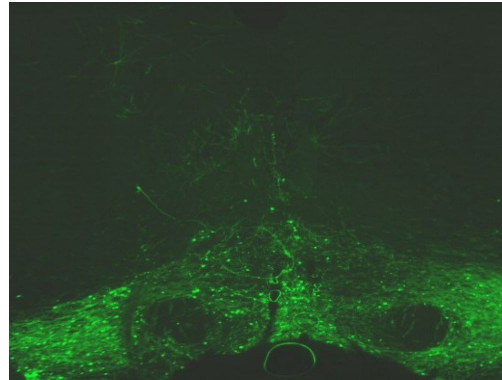


Figure 11. A) Representation of the bilateral virus expression in VTA and unilateral placement of the cannula for the rats included in the analysis in all groups. Light shading represents the maximal and dark shading represents the minimal spread of viral expression. B) A coronal brain slice showing a representative viral expression throughout the VTA for rats in Experiment 4 (60-μm sections).

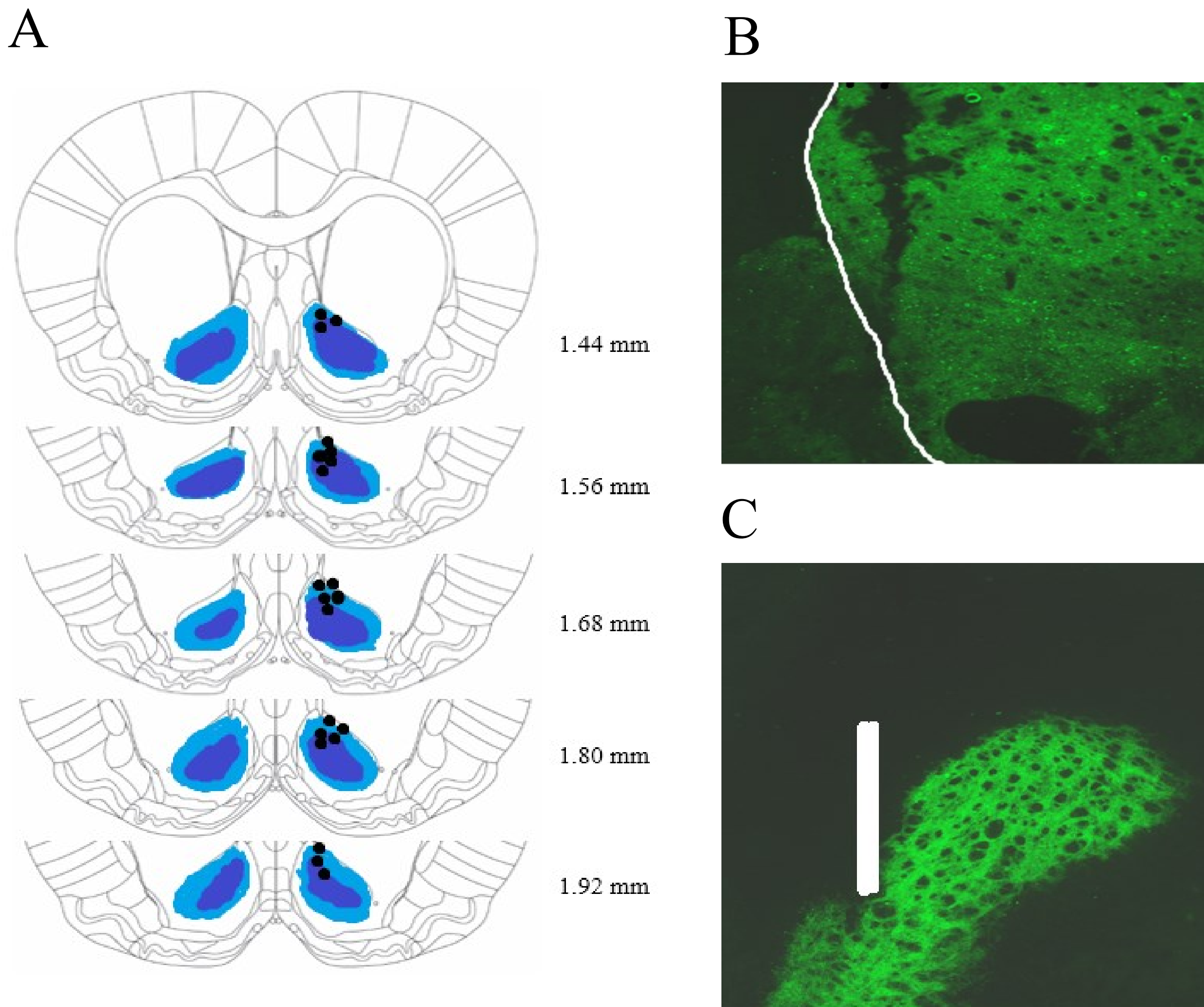


Figure 12. A) Representation of the bilateral virus expression and unilateral ferrule placements in NAc for the rats included in the analysis in all groups. Fiber implants (black circles) were in the vicinity of ChR2 (blue) expression in NAc. Light shading represents the maximal and dark shading represents the minimal spread of viral expression. B) Example of a representative viral expression in the NAc (inside the white border) for the rats in Experiment 4 (60-µm sections) C) Example of a representative viral expression and placement of the ferrule (white bar) in the NAc for the rats in Experiment 4 (60-µm sections).

General Discussion

The overarching goal of this thesis was to elucidate the role of VTA DA neurons in fear learning. We used a powerful behavioural and theory-driven approach by combining blocking and overshadowing designs with optogenetics and elegantly modulated firing in VTA DA neurons with temporal precision during learning. Our approach allowed for a direct comparison of the role of VTA DA in appetitive and aversive learning. Taken together, our results provide evidence that optical stimulation of VTA DA neurons and their terminals in NAc at the time of expected shock augmented the blocking effect by attenuating APE and further impaired learning about the blocked cue.

Our results are in contrast to the role of VTA DA neurons in reward. Stimulation of VTA DA neurons and their terminals in the NAc has been found to encode RPE and various features such as probability, magnitude, timing, and subjective value of a reward (Enomoto et al., 2011; Hart, Rutledge, Glimcher, & Phillips, 2014; Lak, Stauffer, & Schultz, 2014; see Watabe-Uchida et al., 2017 for a review). Specifically, optical stimulation of VTA DA in the Th-cre+/- rat at time of expected reward delivery in Phase 2 of blocking increased the normally small RPE and enhanced learning about the normally blocked cue and reward (Steinberg et al., 2013). Using the similar parameters in fear, that is, optical stimulation of VTA DA neurons and their terminals in the NAc at time of expected reward delivery in Phase 2 of blocking reduced learning about the blocked cue and the footshock US, thus augmented the blocking effect. A prediction that follows from these data is that that optical *inhibition* of VTA DA neurons at the time of expected footshock in Phase 2 of the blocking paradigm would lead to *unblocking*, i.e., a robust learning to the clicker.

One possible explanation for the further impairment we have observed is that optical stimulation of DA neurons at the time of expected footshock during compound training decreased the aversive value or intensity (i.e., aversiveness) of the footshock. Such changes in the US would result in less conditioning to the clicker. Similarly, another possibility is that DA stimulation acts as a rewarding event (Tsai et al., 2009; Rossi et al., 2013) and counter-conditioned the footshock US, again serving to lower the fear acquired by the clicker during learning. Our data, however, does not support this idea as we did not observe the corresponding effects on learning in the standard overshadowing control conditioning (Experiment 3) when the shock was unexpected and prediction error was at maximum. This suggests that DA stimulation is unlikely to change the aversiveness of the footshock or act as a rewarding event to counter-condition the aversive US. Therefore, optical stimulation of VTA DA neurons and their terminals in NAc modulated learning specifically when the effect of prediction error was isolated (in the blocking designs), suggesting a specific effect on aversive prediction-error. Our data also provide evidence that using the standard temporal contiguity paradigm may not be sensitive in revealing effects on associative learning. Further, it is clear form our data that neurobiological mechanisms, learning and behaviour interact in complex, often not linear ways.

Our results are consistent with previous studies in fear (Iordanova et al, 2006; Iordanova, 2010; Li, & McNally, 2015; Budygin et al., 2012; Wenzel et al., 2014; Badrinarayan et al., 2012. Mccutcheon et al., 2012). For example, DA receptor antagonists in structures downstream of the VTA (i.e. NAc, and amygdala) during Phase 2 of the blocking paradigm enhanced learning about the blocked cue (Iordanova et al, 2006; Iordanova, 2010). It should be noted that unlike experiments investigating the role of DA neurons in RPE, only a few studies (Iordanova et al,

2006; Iordanova, 2010) and this thesis used the behavioural designs that allowed investigation of APE and temporal contiguity in fear.

VTA DA neurons may encode for a valence-specific prediction error signal and modulate learning in the opposite way in reward and fear (Matsumoto M, Hikosaka 2009; Matsumoto et al., 2016). For example, it has been found that some DA neurons are activated by the predictor of a reward and some are inhibited by the predictor of an aversive event (Matsumoto, & Hikosaka, 2009). Moreover, the possibility of a valence-specific prediction-error can be supported by the VTA DA neurons due to the heterogeneous nature of the neurons and their projections to different brain areas that are involved in modulation of prediction-error (Lammel et al., 2012; Lammel, Lim, & Malenka, 2014).

Prediction error can influence learning in two ways: *directly* by affecting the processing of the outcome during the ongoing conditioning trial (i.e., if further increments in associative strength can be supported by the same US on that trial) (Rescorla & Wagner, 1972), or *indirectly* by modulating the processing of the predictors of the outcome (Mackintosh, 1975; Pearce & Hall, 1980). An important implication is that the blocking effect is revealed at slightly different time points according to the two theories. According to Rescorla and Wagner (1972), the pre-trained cue blocks learning about the novel cue from the very first trial of conditioning in Phase 2. According of the attentional accounts of learning (Mackintosh, 1975; Pearce & Hall, 1980), the first trial serves to reduce attention to the novel cue and thus block learning on subsequent trials. Put another way, learning about the novel cue proceeds normally on the first compound conditioning trial, but is blocked during subsequent trials. Predictions of these theories (i.e., blocking in the first compound conditioning trials versus subsequent trials) can be tested by precisely stimulating DA neurons or their terminals during the first trial or second trial in Phase 2

in a blocking paradigm. Such investigation will allow us to reconcile other experimental evidence and validate the theories of learning and inform how VTA DA neurons encode for APE across time.

It is still not fully clear how VTA DA neurons calculate aversive prediction-error and which circuit mechanisms are involved. Our results highlight the role of NAc, however, VTA DA neurons also send wide-ranging projections to different brain areas such as medial prefrontal cortex and basolateral amygdala that are involved in fear learning and computation of prediction-error (Morales, & Margolis, 2017; Lammel et al., 2012; Lammel, Lim, & Malenka, 2014). Moreover, VTA GABAergic neurons are also involved in influencing DA neurons in RPE by signalling expected reward (Cohen et al., 2012; Morales, & Margolis, 2017). In addition, subpopulations of VTA GABA and glutamate neurons receive input from and project to the same brain areas as the VTA DA neurons (Morales, & Margolis, 2017) and may interact with specific neuronal networks to modulate behaviours and learning in reward and aversion (Stamatakis et al., 2013; Tan et al., 2012; Wang, Qi, Zhang, Wang, & Morales, 2015). Although we tagged DA neurons using TH-dependent manner, some studies suggest that VTA TH-expressing neurons modulate GABA release (i.e., co-release, suppression) with dopamine (Stamatakis et al., 2013; Morales, & Margolis, 2017). Therefore, there is a possibility of recruiting these subpopulations of neurons in our preparation as these subpopulations of neurons have also been found to project to NAc and often co-release other neurotransmitter along with dopamine (i.e., co-release of dopamine and glutamate within a single axon, or dopamine and GABA from the same vesicle) (Zhang et al., 2015; Root et al., 2014; Berrios et al., 2016; Morales, & Margolis, 2017). Thus, it would be interesting to investigate if results similar to what we have reported are observed using another DA stimulation method (i.e., DAT cre+/- mice).

Although the work presented here signifies an important leap forward in deepening our knowledge of VTA DA neurons and their terminals in NAc in learning about aversive outcomes, future studies are required to disentangle how distinct motivational information and states are encoded by DA neurons. These future attempts will also help refine our understanding of DA function in both fear and reward, and how these functions change in different psychopathologies.

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Appendix

Experiment 1. Dopamine transients augment the blocking effect

Results

Phase 1.

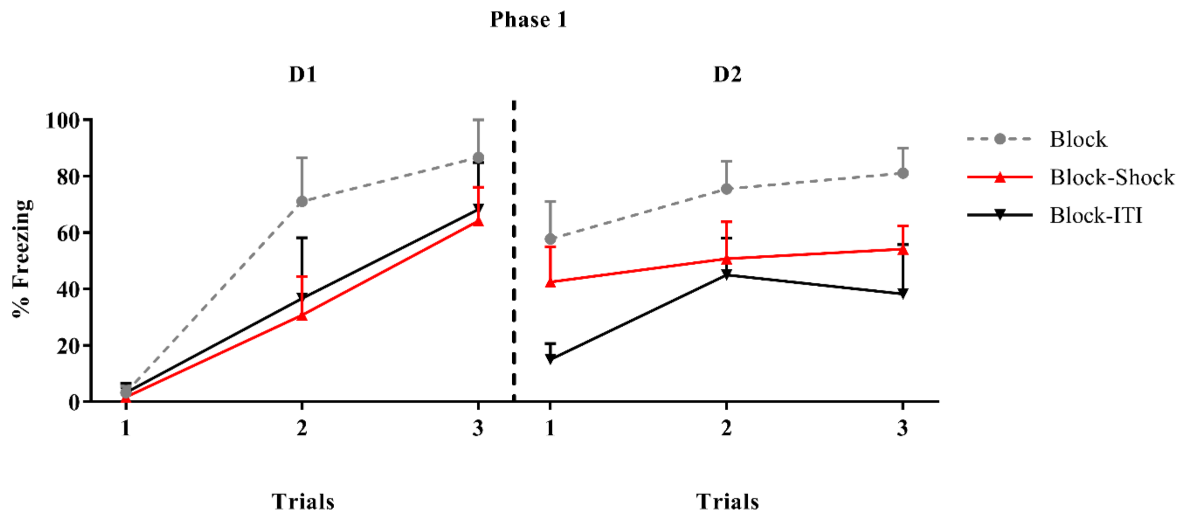

Figure S1. The acquisition of fear across trials on different days of Phase 1. Data are presented as means and error bars represent standard error of the mean (SEM).

Phase 1. Rats conditioned to fear the light showed an increase in fear across trials on Day 1 as indicated by a liner trend across trials ($F_{1,15} = 77.771$, CI{1.776:2.913}). Freezing did not differ between Group Block compared to the Groups Block-Shock and Block-ITI ($F_{1,15} = 2.892$, CI{-0.165:1.469}), nor was there a group X trial interaction ($F_{1,15} = 1.396$, CI{-0.526:1.833}). There was no difference in freezing between the Groups Block-Shock and Block-ITI ($F_{1,15} < 1$, CI{-1.111:0.851}), nor was there a group X trial interaction ($F_{1,15} < 1$, CI{-1.499:1.332}).

Similarly to Day 1, rats conditioned to fear the light showed an increase in fear across trials on Day 2 as indicated by a liner trend across trials ($F_{1,15} = 5.493$, CI{0.060:1.262}).

Freezing was higher in the Group Block compared to the Groups Block-Shock and Block-ITI ($F_{1,15} = 6.650$, CI{0.180:1.894}) but there was no group X trial interaction ($F_{1,15} < 1$, CI{-1.052:1.448}). There was no difference in freezing between Groups Block-Shock and Block-ITI ($F_{1,15} = 1.333$, CI{-0.471:1.586}), nor was there a group X trial interaction ($F_{1,15} < 1$, CI{-1.897:1.104}).

Phase 2.

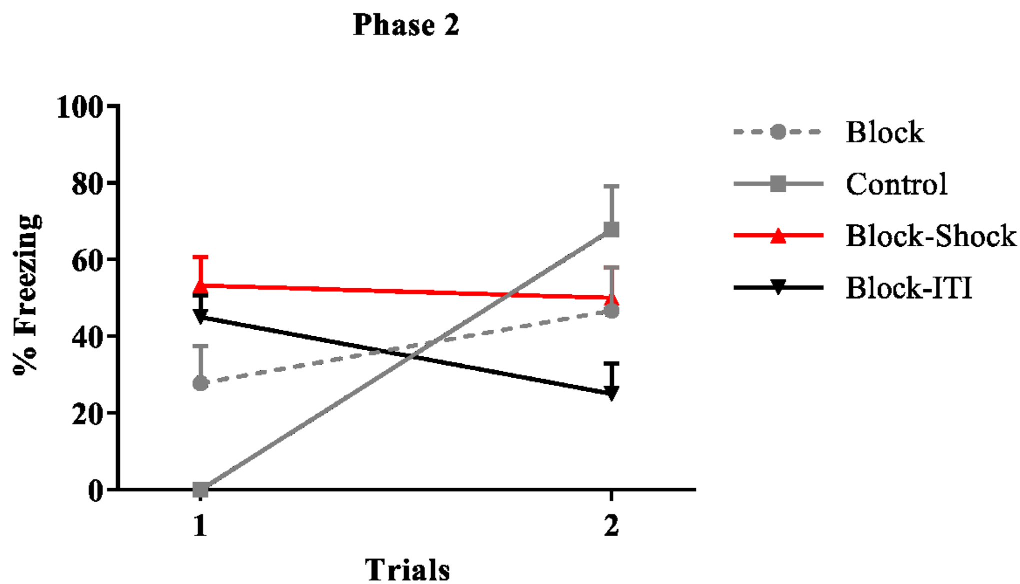

Figure S2. The acquisition of fear across trials on Day 1 of Phase 2. Data are presented as means and error bars represent standard error of the mean (SEM).

Phase 2. Rats conditioned to fear the light and clicker compound showed an increase in fear across trials during Phase 2 as indicated by a liner trend across trials ($F_{1,21} = 5.487$, CI{0.072:1.210}). Freezing did not differ between the Control groups and the blocking groups (i.e., Groups Block, Block-Shock and Block-ITI; $F_{1,21} < 1$, CI{-0.821:0.656}) nor was there a group X trial interaction ($F_{1,21} < 1$, CI{-1.014:1.591}). Freezing did not differ between the Group Block compared to the groups that were optically stimulated (i.e., Groups Block-Shock and

Block-ITI; $F_{1,21}$ = 2.556, CI{-1.342:0.175}), but there was a group X trial interaction ($F_{1,21}$ = 26.215, CI{1.956:4.632}). Freezing did not differ between the Group Block-Shock and Block-ITI ($F_{1,15}$ = 2.709, CI{-0.199:1.711}) nor was there a group X trial interaction ($F_{1,21} < 1$, CI{-0.928:2.441}).

Experiment 2. DA transients augment the blocking effect regardless of experimental parameters.

Results

Phase 1.

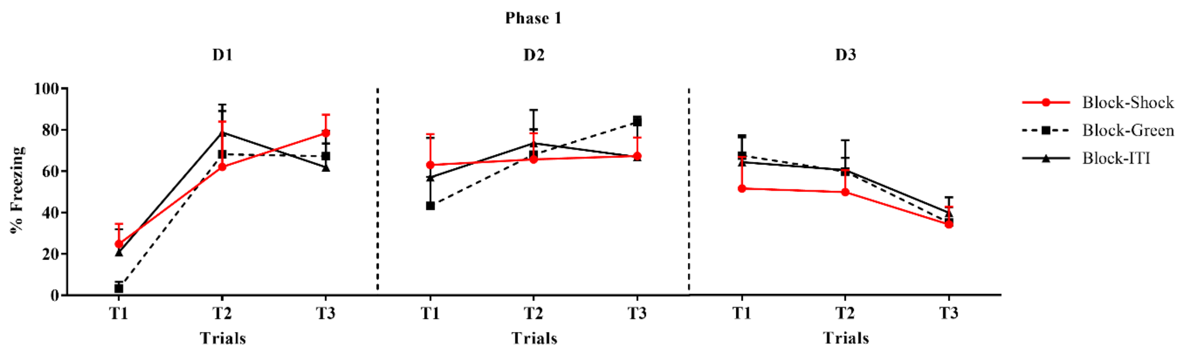

Figure S3. The acquisition of fear across trials on different days of Phase 1. Data are presented as means and error bars represent standard error of the mean (SEM).

Phase 1. Rats conditioned to fear the light showed an increase in fear across trials on Day 1 as indicated by a liner trend across trials ($F_{1,9}$ = 42.736, CI{1.261:2.595}). Freezing did not differ between the Group Block-Shock compared the Groups Block-Green and Block-ITI ($F_{1,9} < 1$, CI{-0.582:0.954}) nor was there a group X trial interaction ($F_{1,9} < 1$, CI{-1.378:1.452}). Freezing did not differ between the Group Block-Green and Block-ITI ($F_{1,9} < 1$, CI{-1.164:0.609}) nor was there a group X trial interaction ($F_{1,9}$ = 1.335, CI{-0.799:2.468}).

Rats conditioned to fear the light showed no difference in fear across trials on Day 2 as indicated by no liner trend across trials ($F_{1,9} = 2.809$, CI{-0.229:1.539}). Freezing did not differ between the Group Block-Shock compared to the Groups Block-Green and Block-ITI ($F_{1,9} < 1$, CI{-0.978:0.977}) nor was there a group X trial interaction ($F_{1,9} < 1$, CI{-2.626:1.125}). Freezing did not differ between the Group Block-Green and Block-ITI ($F_{1,9} < 1$, CI{-1.159:1.098}) nor was there a group X trial interaction ($F_{1,9} = 1.332$, CI{-1.061:3.271}).

Rats conditioned to fear the light showed a decrease in fear across trials on Day 3 as indicated by a liner trend across trials ($F_{1,9} = 20.578$, CI{-1.730:-0.579}). Freezing did not differ between the Group Block-Shock compared to the Groups Block-Green and Block-ITI ($F_{1,9} < 1$, CI{-1.619:0.754}) nor was there a group X trial interaction ($F_{1,9} < 1$, CI{--0.708:1.734}). Freezing did not differ between the Group Block-Green and Block-ITI ($F_{1,9} < 1$, CI{-1.407:1.334}) nor was there a group X trial interaction ($F_{1,9} < 1$, CI{-1.779:1.040}).

Phase 2.

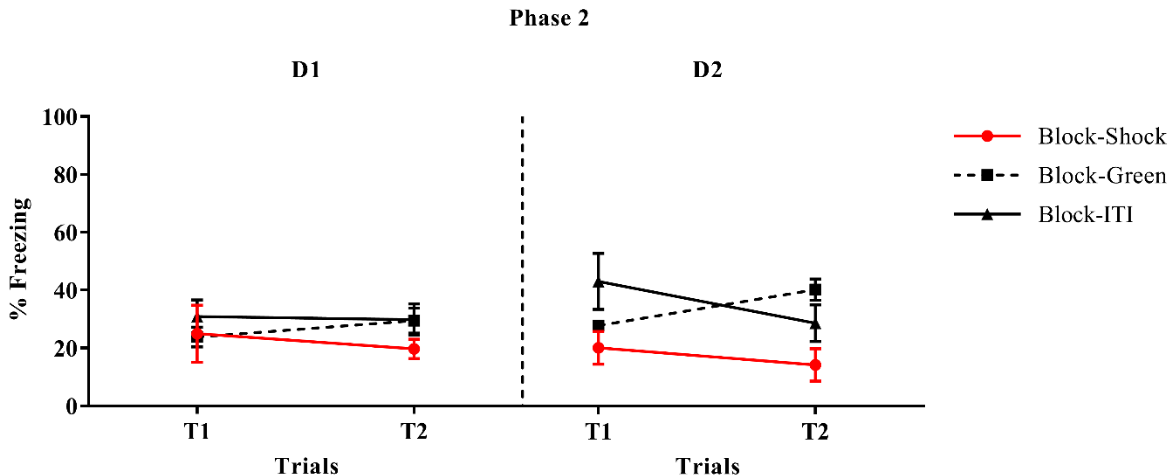

Figure S4. The acquisition of fear across trials on different days of Phase 2. Data are presented as means and error bars represent standard error of the mean (SEM).

Phase 2. Rats conditioned to fear the light and clicker compound showed no difference in fear across trials on Day 1 as indicated by no liner trend across trials ($F_{1,9} < 1$, CI{-1.079:1.041}). Freezing did not differ between the Group Block-Shock compared to the groups that were optically stimulated (i.e., Groups Block-Green and Block-ITI; $F_{1,9} = 2.176$, CI{-1.337:0.282}) nor was there a group X trial interaction ($F_{1,9} < 1$, CI{-2.896:1.601}). Freezing did not differ between the Group Block-Green and Block-ITI ($F_{1,9} < 1$, CI{-1.254:0.615}) nor was there a group X trial interaction ($F_{1,9} < 1$, CI{-2.014:3.178}).

Rats conditioned to fear the light and clicker compound showed no difference in fear across trials on Day 2 as indicated by no liner trend across trials ($F_{1,9} < 1$, CI{-1.141:0.698}). Freezing was lower in the Group Block-Shock compared to the other groups that were optically stimulated (i.e., Groups Block-Green and Block-ITI; $F_{1,9} = 11.694$, CI{-2.470:-0.503}) nor was there a group X trial interaction ($F_{1,9} < 1$, CI{-2.362:1.540}). Freezing did not differ between the Group Block-Green and Block-ITI ($F_{1,9} < 1$, CI{-1.287:0.984}) nor was there a group X trial interaction ($F_{1,9} = 5.070$, CI{-0.011:4.495}).

Experiment 3. DA transients do not modulate overshadowing.

Results

Conditioning.

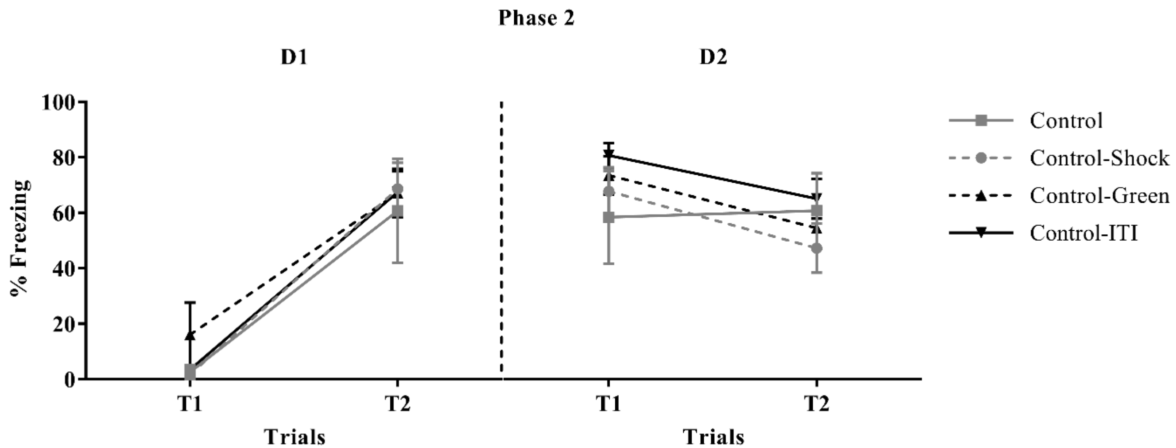

Figure S5. The acquisition of fear across trials on different days of Phase 2. Data are presented as means and error bars represent standard error of the mean (SEM).

Phase 2. Rats conditioned to fear the light and clicker compound showed an increase in fear across trials on Day 1 as indicated by a liner trend across trials ($F_{1,22} = 108.905$, CI{2.180:3.261}). Freezing did not differ between the Group Compound compared to the groups that were optically stimulated (i.e., Groups Control-Shock, Control-Green, and Block-ITI; $F_{1,22} < 1$, CI{-1.270:0.744}) nor was there a group X trial interaction ($F_{1,22} < 1$, CI{-1.672:1.444}). Freezing did not differ between the Group Control-Shock compared to the Groups Control-Green, and Block-ITI ($F_{1,22} < 1$, CI{-0.871:0.567}) nor was there a group X trial interaction ($F_{1,22} < 1$, CI{-0.687:1.537}). Freezing did not differ between the Groups Block-ITI and Control-Green ($F_{1,22} < 1$, CI{-0.571:1.127}) nor was there a group X trial interaction ($F_{1,22} < 1$, CI{-1.898:0.729}).

Rats conditioned to fear the light and clicker compound showed a decrease in fear across trials on Day 2 as indicated by a liner trend across trials ($F_{1,22}$ = 9.586, CI{-1.029:-0.204}). Freezing did not differ between the Group Compound compared to the Groups Control-Shock, Control-Green, and Block-ITI ($F_{1,22}$ < 1, CI{-1.369:0.883}) nor was there a group X trial interaction ($F_{1,22}$ = 2.821, CI{-0.226:2.154}). Freezing did not differ between the Group Control-Shock compared to the Groups Control-Green, and Block-ITI ($F_{1,22}$ = 1.738, CI{-1.314:0.293}) nor was there a group X trial interaction ($F_{1,22}$ < 1, CI{-1.002:0.696}). Freezing did not differ between the Groups Block-ITI and Control-Green ($F_{1,22}$ < 1, CI{-1.362:0.536}) nor was there a group X trial interaction ($F_{1,22}$ < 1, CI{-1.1690:0.846}).

Experiment 4. DA transients in NAc augment the blocking effect.

Results

Phase 1.

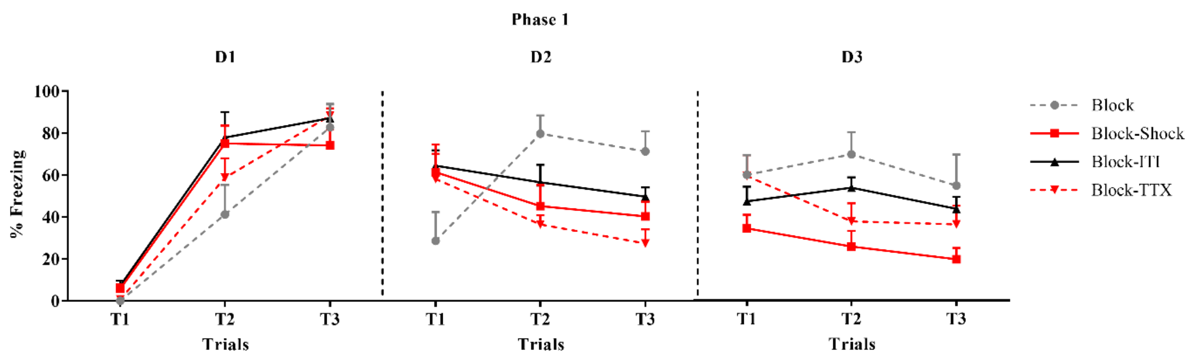

Figure S6. The acquisition of fear across trials on different days of Phase 1. Data are presented as means and error bars represent standard error of the mean (SEM).

Phase 1. Rats conditioned to fear the light showed an increase in fear across trials on Day 1 as indicated by a liner trend across trials ($F_{1,22}$ = 399.738, CI{3.539:4.358}). Freezing did not

differ between the Group Block compared to the Groups Block-Shock, Block-ITI, and Block-TTX ($F_{1,22}$ = 3.000, CI{-1.252:0.112}) nor was there a group X trial interaction ($F_{1,22}$ < 1, CI{-0.766:1.174}). Freezing did not differ between the Group Block-ITI compared to the Groups Block-Shock and Block-TTX ($F_{1,22}$ = 1.089, CI{-0.341:1.033}) nor was there a group X trial interaction ($F_{1,22}$ < 1, CI{-0.882:1.073}). Freezing did not differ between the Groups Block-Shock and Block-TTX ($F_{1,22}$ < 1, CI{-0.935:0.694}) nor was there a group X trial interaction ($F_{1,22}$ = 2.905, CI{-0.206:2.111}).

Rats conditioned to fear the light showed no difference in fear across trials on Day 2 as indicated by no liner trend across trials ($F_{1,22}$ = 1.155, CI{-0.739:0.235}). Freezing did not differ between the Group Block compared to the Groups Block-Shock, Block-ITI, and Block-TTX ($F_{1,22}$ = 1.820, CI{-0.251:1.186}), but there was a group X trial interaction ($F_{1,22}$ = 24.408, CI{1.593:3.899}). Freezing did not differ between the Group Block-ITI compared to the Groups Block-Shock and Block-TTX ($F_{1,22}$ = 2.157, CI{-0.211:1.237}) nor was there a group X trial interaction ($F_{1,22}$ <1, CI{-0.689:1.634}). Freezing did not differ between the Groups Block-Shock and Block-TTX ($F_{1,22}$ <1, CI{-1.210:1.237}) nor was there a group X trial interaction ($F_{1,22}$ <1, CI{-1.783:0.970}).

Rats conditioned to fear the light showed a decrease in fear across trials on Day 3 as indicated by a liner trend across trials ($F_{1,22}$ = 5.154, CI{-1.042:-0.047}). Freezing was higher in the Group Block compared to the Groups Block-Shock, Block-ITI, and Block-TTX ($F_{1,22}$ = 7.358, CI{0.239:1.794}), but there was no group X trial interaction ($F_{1,22}$ < 1, CI{-0.774:1.582}). Freezing did not differ between the Group Block-ITI compared to the Groups Block-Shock and Block-TTX ($F_{1,22}$ = 2.494, CI{-0.187:1.379}) nor was there a group X trial interaction ($F_{1,22}$ = 1.575, CI{-0.469:1.906}). Freezing did not differ between the Groups Block-Shock and Block-

TTX ($F_{1,22} = 3.499$, CI{-0.091:1.766}) nor was there a group X trial interaction ($F_{1,22} < 1$, CI{-1.797:1.018}).

Phase 2.

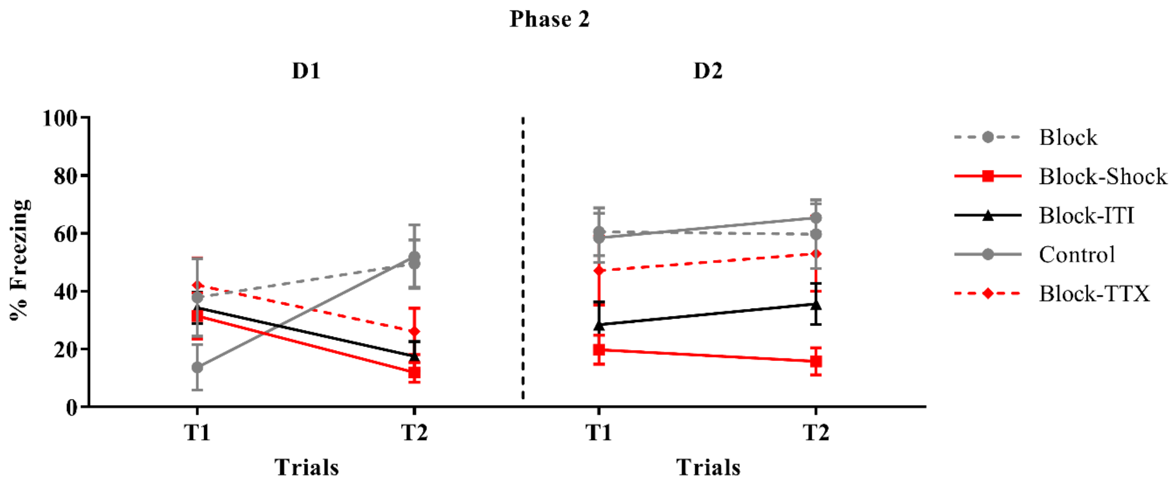

Figure S7. The acquisition of fear across trials on different days of Phase 2. Data are presented as means and error bars represent standard error of the mean (SEM).

Phase 2. Rats conditioned to fear the light and clicker compound showed no difference in fear across trials on Day 1 as indicated by no liner trend across trials ($F_{1,27} < 1$, CI{-0.455:0.410}). Freezing did not differ between the Group Control compared to the blocking groups (i.e., Groups Block, Block-Shock, Block-ITI, and Block-TTX; $F_{1,27} < 1$, CI{-0.677:0.818}), but there was a group X trial interaction ($F_{1,27} = 18.946$, CI{1.240:3.452}). Freezing was higher in the Group Block compared to the groups that were optically stimulated (i.e., Groups Block-Shock, Block-ITI, and Block-TTX; $F_{1,27} = 4.548$, CI{0.032:1.567}), and there was a group X trial interaction ($F_{1,27} = 6.438$, CI{0.269:2.543}). Freezing did not differ between the Group Block-ITI compared to the Groups Block-Shock and Block-TTX ($F_{1,27} < 1$, CI{-0.872:0.677}) nor was there a group X trial interaction ($F_{1,27} < 1$, CI{-1.097:1.194}).

Freezing did not differ between the Groups Block-Shock and Block-TTX ($F_{1,27} = 1.820$, CI{-1.521:0.314}) nor was there a group X trial interaction ($F_{1,27} < 1$, CI{-1.530:1.186}).

Rats conditioned to fear the light and clicker compound showed no difference in fear across trials on Day 2 as indicated by no liner trend across trials ($F_{1,27} < 1$, CI{-0.180:0.461}). Freezing was higher in the Group Control compared to the blocking groups (i.e., Groups Block, Block-Shock, Block-ITI, and Block-TTX; $F_{1,27} = 6.253$, CI{0.183:1.852}), but there was no group X trial interaction ($F_{1,27} < 1$, CI{-0.593:1.046}). Freezing was higher in the Group Block compared to the groups that were optically stimulated (i.e., Groups Block-Shock, Block-ITI, and Block-TTX; $F_{1,27} = 8.867$, CI{0.387:2.103}), but there was no group X trial interaction ($F_{1,27} < 1$, CI{-1.018:0.667}). Freezing did not differ between the Group Block-ITI compared to the Groups Block-Shock and Block-TTX ($F_{1,27} < 1$, CI{-0.951:0.778}) nor was there a group X trial interaction ($F_{1,27} < 1$, CI{-0.560:1.137}). Freezing was lower in the Group Block-Shock compared to the Group Block-TTX ($F_{1,27} = 8.976$, CI{-2.521:0.472}), but there was no group X trial interaction ($F_{1,27} < 1$, CI{-1.460:0.552}).

Raw Data

Experiment 1. Dopamine transients augment the blocking effect.

Phase 1 & Phase 2

		Phase 1						**Phase 2**	
		D1			**D2**			**D1**	
Group	**Rat #**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**
Block	**1**	13.33	100.00	100.00	93.33	93.33	60.00	33.33	20.00
	2	6.67	93.33	100.00	86.67	73.33	100.00	0.00	40.00
	3	0.00	100.00	100.00	20.00	66.67	100.00	53.33	40.00
	4	0.00	33.33	100.00	80.00	100.00	100.00	0.00	73.33
	CH5	0.00	86.67	100.00	40.00	86.67	53.33	53.33	86.67
	9	0.00	13.33	20.00	26.67	33.33	73.33	26.67	20.00
Control	**5**							0.00	53.33
	6							0.00	20.00
	7							0.00	20.00
	8							0.00	73.33
	10							0.00	93.33
	11							0.00	93.33
	WT2							0.00	73.33
Block-Shock	**CH1**	13.33	13.33	66.67	6.67	6.67	40.00	33.33	20.00

	CH2	0.00	80.00	100.00	93.33	53.33	53.33	53.33	40.00
	CH3	0.00	53.33	93.33	53.33	100.00	66.67	33.33	86.67
	CH4	0.00	93.33	100.00	93.33	86.67	80.00	86.67	73.33
	CH1	0.00	0.00	60.00	13.33	40.00	46.67	80.00	53.33
	CH2	0.00	6.67	20.00	33.33	6.67	13.33	46.67	60.00
	CH3	0.00	0.00	60.00	40.00	86.67	86.67	60.00	40.00
	CH4	0.00	0.00	13.33	6.67	26.67	46.67	33.33	26.67
Block-ITI	**CH5**	13.33	93.33	93.33	13.33	66.67	40.00	40.00	46.67
	CH6	0.00	0.00	73.33	26.67	33.33	86.67	46.67	13.33
	CH7	0.00	6.67	86.67	20.00	66.67	6.67	60.00	26.67
	CH8	0.00	46.67	20.00	0.00	13.33	20.00	33.33	13.33

Clicker Test & Light Test

Group	Rat #	Clicker Test	Light Test
Block	**1**	32.50	61.67
	2	56.67	47.50
	3	30.83	30.00
	4	65.83	47.50
	CH5	41.67	46.67
	9	22.50	20.83
Control	**5**	77.50	25.00
	6	63.33	15.00
	7	29.17	8.33
	8	72.50	10.83
	10	89.17	16.67
	11	45.00	9.17
	WT2	52.50	8.33
Block-Shock	**CH1**	6.67	20.00
	CH2	20.83	50.00
	CH3	8.33	70.83
	CH4	9.17	18.33
	CH1	18.33	35.83
	CH2	15.83	15.00

	CH3	24.17	17.50
	CH4	8.33	10.83
Block-ITI	**CH5**	44.17	23.33
	CH6	36.67	21.67
	CH7	54.17	18.33
	CH8	24.17	7.50

Experiment 2. DA transients augment the blocking effect regardless of experimental parameters.

Phase 1

		Phase 1								
		D1			**D2**			**D3**		
Group	**Rat #**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**
Block-Shock	**1**	18.97	93.73	65.63	25.33	76.40	58.27	37.37	40.10	33.87
	2	37.53	92.07	93.53	90.37	96.20	58.93	30.50	31.73	31.80
	4	0.00	62.77	60.53	53.40	40.37	58.43	41.67	47.67	14.57
	5	43.03	0.00	94.20	83.53	50.20	94.20	96.93	80.13	56.47
Block-Green	**3-1**	0.00	88.30	57.43	43.63	58.17	87.83	70.80	48.37	39.07
	3-2	13.13	83.03	71.73	65.53	71.63	88.83	87.60	64.47	54.20
	6-1	0.00	95.53	99.00	60.40	100.00	76.93	67.47	49.77	27.03
	6-2	0.00	6.00	41.03	3.47	42.67	81.73	44.20	76.57	20.07
Block-ITI	**1**	0.00	95.37	73.73	40.37	29.37	47.27	40.57	39.33	41.77
	2	31.13	39.90	79.83	76.33	70.53	28.10	46.13	33.43	21.77
	4	47.43	81.00	28.37	12.83	94.83	92.47	74.33	74.60	58.47
	5	4.93	98.83	66.00	99.03	99.80	100.00	96.43	94.63	37.50

Phase 2

		Phase 2			
		D1		D2	
Group	Rat #	T1	T2	T1	T2
Block-Shock	**1**	13.40	22.17	28.37	0.00
	2	53.60	9.93	3.40	10.50
	4	10.77	21.50	26.30	23.40
	5	22.00	25.23	22.33	22.83
Block-Green	**3-1**	33.00	22.83	24.60	46.03
	3-2	17.87	35.70	32.57	30.60
	6-1	19.30	21.03	25.87	38.30
	6-2	24.90	38.17	28.17	45.83
Block-ITI	**1**	48.03	28.87	31.43	24.70
	2	25.90	27.80	24.20	22.63
	4	23.27	17.97	48.77	47.30
	5	26.23	44.40	67.63	19.80

Clicker Test & Light Test

Group	Rat #	Clicker Test	Light Test
Block-Shock	**1**	21.67	61.67
	2	15.83	87.50
	4	27.50	53.33
	5	25.00	56.67
Block-Green	**3-1**	60.00	57.50
	3-2	40.00	61.67
	6-1	37.50	75.00
	6-2	38.33	46.67
Block-ITI	**1**	50.00	63.33
	2	33.33	66.67
	4	47.50	75.83
	5	22.50	71.67

Experiment 3. DA transients do not modulate overshadowing.

Phase 2

		Phase 2			
		D1		**D2**	
Group	**Rat #**	**T1**	**T2**	**T1**	**T2**
Compound	**S01**	0.00	94.27	75.83	57.33
	S02	0.00	29.33	25.03	39.17
	S03	7.60	58.40	74.37	85.57
Control-Shock	**S01**	8.03	92.10	75.43	45.87
	S02	0.00	96.33	89.87	50.37
	S03	0.00	61.80	79.90	51.87
	S04	0.00	77.87	79.13	67.07
	16	0.00	96.03	96.83	93.90
	9	0.00	23.73	29.87	21.57
	5	0.00	45.23	54.40	18.53
	4	5.67	55.87	36.50	28.20
Control-Green	**S01**	95.83	96.70	95.50	89.17
	S02	0.00	46.60	54.50	51.90
	S03	4.57	29.77	70.73	62.87
	S04	10.93	86.87	86.67	53.43

	1	0.00	79.10	80.90	76.57
	2	17.03	55.40	81.90	31.27
	11	0.00	51.20	36.27	42.30
	15	0.00	91.33	81.20	28.40
Block-ITI	**S01**	6.57	94.40	94.20	78.10
	S03	7.53	58.70	76.50	46.93
	S04	0.00	69.83	81.27	92.13
	12	0.00	91.93	97.97	45.07
	8	0.00	66.33	76.63	81.00
	13	0.00	43.00	73.93	48.33
	17	10.13	47.87	63.93	63.70

Clicker Test & Light Test

Group	Rat #	Clicker Test	Light Test
Comound	**S01**	90.58	41.25
	S02	39.39	12.17
	S03	85.55	28.17
Control-Shock	**S01**	67.10	37.68
	S02	43.47	1.44
	S03	58.11	46.18
	S04	63.24	41.54
	16	78.43	48.81
	9	92.81	74.39
	5	40.99	34.02
	4	55.54	29.35
Control-Green	**S01**	54.07	34.38
	S02	85.81	44.60
	S03	39.26	8.87
	S04	43.63	28.98
	1	85.19	31.87
	2	62.84	23.00
	11	75.15	40.23

	15	62.98	14.88
Block-ITI	**S01**	66.59	41.41
	S03	58.33	16.93
	S04	63.19	45.51
	12	97.33	64.28
	8	74.33	18.83
	13	87.73	39.97
	17	79.90	67.21

Experiment 4. DA transients in NAc augment the blocking effect.

Phase 1

		Phase 1								
			D1			D2			D3	
Group	**Rat #**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**
Block	**20**	0.00	68.70	95.83	4.37	96.80	78.07	77.63	90.17	79.57
	21	0.00	91.83	99.03	82.10	88.57	91.87	93.80	86.13	87.00
	26	0.00	11.73	98.07	0.00	98.53	88.10	52.27	90.50	73.43
	27	0.00	8.13	74.67	4.33	87.70	86.97	28.77	78.27	71.03
	12	0.00	16.93	29.83	23.63	45.03	41.57	49.53	34.47	0.00
	13	0.00	49.73	98.70	57.40	61.60	41.43	58.27	38.97	18.23
Block-Shock	**6**	9.57	99.40	58.77	4.40	11.63	11.27	6.70	12.13	4.73
	7	10.77	98.60	99.57	92.50	71.23	52.50	51.70	18.63	12.90
	9	0.00	91.33	57.77	56.43	36.07	29.83	18.77	18.03	23.83
	1	14.00	43.80	48.63	49.47	28.40	29.57	36.13	29.20	35.50
	4	6.30	65.87	72.97	95.57	53.60	45.33	30.37	25.20	17.93
	2	0.00	75.20	95.20	94.97	87.50	68.77	43.73	67.87	40.27
	7	0.00	51.03	86.10	36.30	28.23	44.70	53.83	9.33	2.80
Block-ITI	**5**	0.00	14.43	51.57	27.63	90.77	40.27	72.27	53.33	58.63
	4	11.63	99.53	84.77	71.93	46.33	55.30	54.40	70.23	53.60

	10	8.73	97.87	100.00	60.60	45.97	60.00	52.80	42.73	44.70
	3	14.40	99.10	99.10	82.10	34.67	67.50	40.97	44.00	28.17
	8	3.13	92.43	99.67	72.63	57.33	49.67	59.50	44.57	25.40
	10	13.00	54.07	80.93	83.43	37.60	36.73	37.50	74.43	63.17
	6	0.00	87.53	93.87	53.07	83.17	38.73	13.93	47.87	32.77
Block-TTX	**11**	0.00	46.57	97.40	27.07	25.30	33.17	51.13	52.43	41.77
	12	0.00	50.17	70.53	66.37	48.93	29.00	40.63	9.87	8.43
	13	0.00	24.50	88.63	21.40	21.33	6.63	66.23	59.03	31.90
	14	0.00	67.70	94.23	80.67	39.97	49.63	79.87	58.00	74.53
	16	4.40	77.90	98.73	56.97	39.93	9.50	27.03	21.53	35.20
	17	0.00	85.37	80.07	96.07	43.47	36.43	91.70	26.10	26.30

Phase 2

		Phase 2			
		D1		D2	
Group	Rat #	T1	T2	T1	T2
Block	**20**	57.60	53.37	49.07	19.77
	21	71.23	83.63	87.77	91.60
	26	0.00	35.67	41.73	54.93
	27	6.80	29.53	74.80	71.13
	12	19.20	36.67	71.43	87.30
	13	72.57	58.17	38.23	33.60
Block-Shock	**6**	0.00	0.00	4.00	0.00
	7	29.70	5.07	11.20	17.43
	9	71.27	15.70	3.47	0.00
	1	23.50	12.47	31.20	23.17
	4	34.13	19.23	31.67	15.83
	2	29.23	25.97	22.10	34.37
	7	32.17	5.07	34.60	19.63
Block-ITI	**5**	33.33	13.80	5.07	13.43
	4	53.57	0.00	41.90	32.03
	10	20.27	7.97	0.00	23.57

	3	25.10	23.67	17.00	30.13
	8	27.93	21.63	46.83	27.33
	10	23.77	13.77	37.57	57.97
	6	55.80	41.87	50.83	64.77
Block-TTX	**11**	40.83	38.00	72.33	33.80
	12	76.73	16.70	11.33	34.50
	13	19.63	28.30	33.80	48.70
	14	60.33	58.13	80.23	84.10
	16	38.13	6.70	64.67	98.87
	17	16.97	9.07	20.30	17.63
Control	**22**	0.00	37.37	31.17	67.37
	23	5.97	73.13	75.13	53.43
	24	0.00	62.97	68.17	53.60
	25	15.20	18.33	32.13	65.43
	18	9.90	87.53	72.70	85.87
	19	50.93	32.23	71.37	66.60

Clicker Test & Light Test

Group	Rat #	Clicker Test	Light Test
Block	**20**	14.02	13.96
	21	20.37	8.73
	26	38.85	33.90
	27	35.12	20.38
	12	45.52	51.00
	13	22.45	22.12
Block-Shock	**6**	33.58	28.85
	7	11.73	39.44
	9	5.45	29.80
	1	14.91	25.88
	4	23.76	31.41
	2	24.59	45.64
	7	2.48	29.13
Block-ITI	**5**	20.25	43.99
	4	51.51	55.09
	10	35.86	54.90
	3	39.46	29.45
	8	10.80	37.80
	10	38.57	41.66
	6	52.99	40.28

Block-TTX	**11**	11.06	42.86
	12	5.72	15.09
	13	4.21	42.89
	14	43.33	66.30
	16	4.04	27.57
	17	20.71	20.48
Control	**22**	51.46	0.84
	23	74.99	6.03
	24	76.49	4.10
	25	85.47	43.78
	18	60.32	1.82
	19	73.52	15.53