

Activating the infralimbic prefrontal cortex promotes the inhibition of appetitive Pavlovian
conditioning following extinction

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Abstract

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Objectives: There is conflicting evidence about the role of the infralimbic prefrontal cortex (IL-PFC) in the extinction of appetitive Pavlovian conditioning. Here, we tested the hypothesis that IL-PFC activity mediates the inhibition of conditioned behaviour following extinction, and predicted that augmenting IL-PFC activity would reduce sucrose-induced reinstatement and spontaneous recovery of Pavlovian conditioned sucrose-seeking.

Methods: Male, Long-Evans rats (Charles River) received bilateral cannulae or unilateral microinfusions of a viral vector coding for Channelrhodopsin-2 (ChR2) and enhanced yellow fluorescent protein (eYFP), or eYFP only, and optical fiber implantation targeting the IL-PFC. During Pavlovian conditioning, presentations of an auditory conditioned stimulus (CS; 10 s, 14 trials/session, VT-140 s) were paired with a 10% *w/v* sucrose solution (0.2 ml; 2.8 ml/session). The same procedure was repeated during extinction and test sessions, except that sucrose was omitted during CS trials. The effect of optically stimulating (473 nm, 5 ms, 20 Hz, 30 mW) IL-PFC neurons during CS trials in spontaneous recovery tests was assessed at 24 h after a single extinction session, and also 26 days later. In two separate groups of rats, the effect of enhancing IL-PFC activity using pre-session microinfusions of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA; 0 or 0.3 nmol/ μ l/side) or optical stimulation during CS trials on sucrose-induced reinstatement of sucrose-seeking behaviour was assessed. The ability of optical stimulation to induce Fos expression was also measured in separate animals.

Results: Optical stimulation of IL-PFC neurons reduced spontaneous recovery after a delay of 26 days, but not 24 h. Reinstatement was reduced by both intra-IL-PFC AMPA and by optical stimulation. Optical stimulation increased Fos expression in ChR2 expressing neurons compared to eYFP controls.

Conclusions: Our results show that the IL-PFC plays a key role in maintaining the inhibition of appetitive, Pavlovian conditioned behaviour following extinction.

Keywords: Extinction, Pavlovian conditioning, infralimbic prefrontal cortex, AMPA, optogenetics, glutamate, learning and memory, disinhibition

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Overview

Extinction refers to the process by which a learned response that is associated with a salient outcome gradually decreases following repeated omission of the outcome. This capacity makes normal behaviour flexible and adaptable, allowing organisms to respond appropriately to changes in their environment. Deficits in extinction can also contribute to pathological states in humans. For example, individuals with substance use disorders experience drug craving when exposed to drug-predictive cues even after symptoms of withdrawal have ceased, and these reactions may facilitate relapse (Conklin & Tiffany, 2002; Field & Duka, 2002; Garavan & Hester, 2007). Complete extinction of conditioned responses to drug-predictive cues would therefore aid in promoting abstinence. Studying processes that are involved in extinction and in the maintenance of response inhibition following extinction thus has considerable clinical relevance. One brain structure that is proposed to be central in extinction is the infralimbic prefrontal cortex (IL-PFC). In addition to its role in the acquisition and consolidation of extinction (Chen et al., 2016; LaLumiere, Niehoff, & Kalivas, 2010; Milad, Vidal-Gonzalez, & Quirk, 2004; Milad & Quirk, 2002; Sierra-Mercado, Padilla-Coreano, & Quirk, 2011), activity in the IL-PFC is believed to be necessary for the long-term inhibition of behaviour following extinction. Studies have shown, for example, that pharmacological inactivation of the IL-PFC following extinction produces a return of conditioned behaviour (Peters, LaLumiere, & Kalivas, 2008), and that rats with IL-PFC lesions show an augmented return of behaviour in tests that promote this effect (Rhodes & Killcross, 2007, 2004). However, there are also findings that fail to support the hypothesis that IL-PFC activity promotes the long-term inhibition of behaviour

(Bossert et al., 2011; Eddy, Todd, Bouton, & Green, 2016; Mendoza, Sanio, & Chaudhri, 2015; Willcocks & McNally, 2013), highlighting a need for more research to clarify this question. These discrepancies may be accounted for by differences in methodology or by drawbacks inherent in the techniques used. In addition, techniques that allow for precise temporal control over the activity of IL-PFC neurons would be advantageous for studying conditioned behaviour triggered by discrete conditioned stimuli. Based on these considerations, the present studies assessed the effect of activating the IL-PFC on the inhibition of an appetitive Pavlovian conditioned response following extinction.

To replicate and extend findings from the literature (LaLumiere et al., 2010; Peters et al., 2008), we microinfused α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) into the IL-PFC to examine the impact of pharmacologically augmenting IL-PFC activity on sucrose-induced reinstatement of responding to a sucrose-predictive cue. Using the same appetitive conditioning task we also used *in vivo* optogenetics to activate IL-PFC neurons that expressed Channelrhodopsin-2 (ChR2) during conditioned stimulus trials in reinstatement and spontaneous recovery tests. In all experiments, we predicted that increasing IL-PFC activity at test would inhibit the return of behaviour. We also assessed the ability of optical stimulation to induce Fos expression in IL-PFC neurons expressing ChR2.

What is extinction?

Extinction refers to the process by which a learned response that is associated with a salient outcome gradually decreases following repeated omission of the outcome. Behaviours acquired through either instrumental or Pavlovian conditioning procedures can undergo extinction. In instrumental conditioning, subjects learn to perform an operant response to obtain

a reinforcer, an outcome that is contingent on the performance of an operant response. If the reinforcer is omitted when the subjects perform the response, the conditioned behaviour gradually decreases (Delamater, 2004; Myers & Davis, 2007, 2002). During Pavlovian conditioning, a conditioned stimulus (CS) comes to predict an unconditioned stimulus (US) with which it has been repeatedly paired. This learning results in the appearance of a conditioned response elicited by the CS. If the CS is then repeatedly presented without the US during extinction trials, subjects will inhibit their responding to the CS.

Interestingly, the decrease in conditioned behaviour during extinction does not necessarily signify that the original learned association between the response and reinforcer, or between the CS and US has been erased. Procedures can be used to elicit a return of the conditioned response following extinction, thereby demonstrating that the original association is still present. At least three manipulations have been found to robustly evoke a conditioned response in the absence of the reinforcer or US following extinction: reinstatement, spontaneous recovery, and renewal (Bouton, 2002; Conklin & Tiffany, 2002). In the reinstatement procedure, following extinction, a test session is conducted in which subjects are exposed to manipulations that can trigger a return of a conditioned response (Shaham, Shalev, Lu, De Wit, & Stewart, 2003; Shalev, Grimm, & Shaham, 2002), such as exposure to a cue (Alleweireldt, Weber, Kirschner, Bullock, & Neisewander, 2002), stress (Lê et al., 1998), or the reinforcer or US (De Vries et al., 1998; Lê et al., 1998; Rhodes & Killcross, 2004; Shaham et al., 1997). Typically, an increase of the performance of the conditioned response in the absence of the reinforcer or US is observed. In spontaneous recovery, the return of the conditioned response results from the passage of time (Bouton, 2002; Conklin & Tiffany, 2002). Specifically, if subjects are given the opportunity to produce a conditioned response after time has elapsed following a period of

extinction, then the conditioned response will emerge once again. Finally, in context-induced renewal, two different contexts can work as “occasion setters” to differentiate between Pavlovian conditioning and extinction phases. When subjects are exposed to the Pavlovian conditioning context following extinction in a different context, then the conditioned response is elicited again, even in the absence of the reinforcer or US (Bossert et al., 2004; Bouton & Bolles, 1979; Bouton et al., 2011; Chaudhri et al., 2008; Crombag & Shaham, 2002).

Experiments in the present thesis used an appetitive Pavlovian conditioning paradigm in which a CS was initially paired with sucrose, and subsequently presented in the absence of sucrose to induce extinction. The conditioned response of interest was entry into a fluid port, in which sucrose was delivered, during the CS. We then conducted reinstatement and spontaneous recovery tests to trigger a return of behaviour. Namely, our objective was to determine if augmenting IL-PFC activity during these tests would promote the inhibition of responding.

A role for the IL-PFC in the acquisition and consolidation of extinction

The IL-PFC is hypothesized to be central in the acquisition and consolidation of extinction. Historically, this hypothesis has been tested using aversive Pavlovian conditioning procedures in which a CS is paired with an aversive stimulus like foot shock, and freezing during CS trials is observed. Studies using this procedure have shown that temporarily inactivating the IL-PFC via microinfusions of muscimol and baclofen (M/B) before the first extinction session resulted in impaired extinction of conditioned freezing during CS trials, relative to a saline control, and in lower recall of extinction 24 h later (Sierra-Mercado et al., 2011). These results suggest that IL-PFC activity is important in both the acquisition and consolidation of extinction. Inhibition of N-methyl-D-aspartate glutamate receptors (Burgos-Robles, Vidal-Gonzalez,

Santini, & Quirk, 2007) or the metabotropic glutamate receptor 5 (Fontanez-Nuin, Santini, Quirk, & Porter, 2011) in the IL-PFC prior to the first extinction session also prevented extinction recall 24 h later, with no effect on behaviour during the extinction session itself, supporting a role for glutamate in the IL-PFC in promoting the consolidation of extinction. Electrical stimulation of the IL-PFC during CS trials in extinction also decreased responding to a shock-predictive CS during a first extinction session, and resulted in greater recall of extinction 24 hrs later (Milad & Quirk, 2002; Milad et al., 2004). These data suggest that the IL-PFC is involved in both the acquisition and consolidation of extinction of a conditioned aversive response.

Studies by LaLumiere et al. (2010) using appetitive conditioning assays paralleled these findings from aversive conditioning studies. Intra-IL-PFC microinfusions of M/B after each of five extinction sessions of cocaine lever-pressing resulted in impaired extinction retention during seven follow-up extinction sessions. Additionally, intra-IL-PFC microinfusions of 4-[2-(Phenylsulphonylamino)ethylthio]-2,6-difluorophenoxyacetamide (PEPA), an AMPA receptor potentiator administered after 15 min extinction sessions enhanced extinction and resulted in increased retention during follow-up 2 h extinction sessions. Likewise, intra-IL-PFC PEPA administration before extinction sessions enhanced the extinction of heroin lever-pressing, compared to saline (Chen et al., 2016). Altogether, these results suggest that the role of the IL-PFC in extinction acquisition and consolidation is similar in appetitive and aversive conditioning.

Contradictory evidence has, however, been put forward. Indeed, inactivating the IL-PFC before the first extinction session of conditioned lever-pressing for cocaine had no effect on extinction acquisition or on extinction recall in a second extinction session 24 h later, suggesting

that IL-PFC activity does not affect either acquisition or consolidation of extinction of instrumental cocaine-seeking (Peters et al., 2008). Pre-session IL-PFC inactivation on the first day of extinction enhanced within-session extinction of responding to a sucrose-predictive Pavlovian conditioned CS, and had no effect on recall of extinction during the following four extinction sessions (Mendoza et al., 2015). These data are in contrast with other findings that also used an appetitive conditioning approach, although they used instrumental conditioning paradigms (Chen et al., 2016; LaLumiere et al., 2010). Thus, while there is strong converging evidence for a role of the IL-PFC in extinction acquisition and consolidation using aversive conditioning procedures, there are more discrepancies in results from studies that used appetitive conditioning procedures. These discrepancies could be related to the nature of the reinforcer or US. It has been suggested that the extinction of aversive and appetitive conditioning recruits different IL-PFC targets, namely the basolateral amygdala and nucleus accumbens shell, respectively (Peters, Kalivas, & Quirk, 2009). However, we have proposed that these discrepancies could also be related to the differences in the nature of the learning (Mendoza et al., 2015). Aversive conditioning studies have relied exclusively on Pavlovian conditioning, whereas appetitive conditioning studies have used both instrumental and Pavlovian conditioning. Thus, there is still need for clarification concerning the role of the IL-PFC in extinction of appetitive conditioning.

The IL-PFC is involved in the suppression of conditioned responding following extinction

There is a growing literature on the role of the IL-PFC in the maintenance of inhibition of appetitive conditioned behaviour following a period of extinction. Lesions of the IL-PFC using ibotenic acid enhanced spontaneous recovery, reinstatement and renewal of Pavlovian

conditioned food-seeking (Rhodes & Killcross, 2007, 2004), while having no effect on responding during prior Pavlovian conditioning and extinction phases compared to sham lesions. Moreover, inactivating specific neuronal ensembles from the IL-PFC that were activated by the extinction of lever-pressing resulted in an increase of operant food-seeking following extinction (Warren et al., 2016). These data suggest that the IL-PFC is involved in inhibiting responding after extinction has already occurred, and that this region plays a role in the retention of extinction. This interpretation was supported by findings showing that inactivating the IL-PFC with M/B before an extinction session, following several sessions of extinction, could in itself reinstate extinguished lever-pressing for cocaine (Peters et al., 2008). The idea that the IL-PFC is an important structure in mediating behaviour after extinction was further strengthened by the finding that IL-PFC activation with AMPA reduced cocaine-induced reinstatement of lever-pressing for cocaine. Collectively, these data suggest that the IL-PFC is necessary to maintain the inhibition of conditioned behaviour after the behaviour is fully extinguished in both Pavlovian and instrumental appetitive paradigms.

The aforementioned possibility has, however, been mitigated by contradictory findings. For example, Willcocks & McNally (2013) failed to find an effect of IL-PFC inactivation before an extinction session on instrumental beer-seeking when trying to replicate findings by Peters et al. (2008). Furthermore, the same researchers found no effect of IL-PFC inactivation on context-induced renewal of operant responding for beer. Another recent study found that even though IL-PFC inactivation increased food-seeking in an extinction context (consistent with Peters et al.), the same procedure reduced food-seeking during renewal in the instrumental conditioning context (Eddy et al., 2016). Interestingly, inactivation of a specific IL-PFC neuronal ensemble activated during exposure to a heroin self-administration associated context reduced renewal of

heroin-seeking, indicating that different portions of the IL-PFC might store different types of associations (Bossert et al., 2011). While the overall evidence seems to agree that the IL-PFC plays an important role in the maintenance of extinction of conditioned behaviour, there is still a need to clarify this involvement. Consequently, in the present experiments we examined the effect of augmenting IL-PFC activity in the continued inhibition of conditioning Pavlovian sucrose-seeking following extinction.

New methods to test old ideas

The contradictory results obtained among the studies cited previously could in part be attributed to methodological limitations. When using intracranial microinfusions the precision of the infusion can be compromised by diffusion to nearby structures that might also support the behaviour of interest. For example, the prelimbic prefrontal cortex is situated directly dorsal to the IL-PFC and also plays a role in modulating conditioned behaviour (Killcross & Coutureau, 2003; Kalivas et al., 2008; Sierra-Mercado et al., 2011). Lesion techniques cause loss of a brain region for a significant part of the experiment, and neuronal adaptations can emerge to compensate for the loss and account for the results. Lesions and pharmacological manipulations also offer no temporal specificity, making it difficult to interpret changes in behaviour over time when studying conditioned responding to a discrete CS.

Techniques that allow researchers to control the timing of neuronal activation or inactivation are advantageous when studying the role of the IL-PFC in the extinction of conditioned responding elicited by a discrete CS. Procedures such as electrical stimulation and optogenetics allow the activation of neurons from a particular brain region during temporally

specific events. However, the optogenetic approach offers an additional advantage in that the activation can be limited to neuronal cell bodies, avoiding the stimulation of fibers of passage.

In the present studies we used *in vivo* optogenetic stimulation to replicate results obtained with AMPA microinfusions to temporally limit the activation of IL-PFC neurons to discrete CS trials during test sessions.

Present research objectives and rationale

Based on the literature described above, we posit that additional research is needed to clarify the role of the IL-PFC in the extinction of appetitive conditioned behaviour. This endeavour would benefit from advancements in neuroscience techniques that allow for a more temporally precise control of neuronal activity.

The present studies tested the hypothesis that IL-PFC activity is involved in maintaining the inhibition of conditioned behaviour after extinction has occurred. Specifically, we assessed the effect of IL-PFC activation on the inhibition of an appetitive Pavlovian conditioned response following extinction. For this purpose, we first trained rats to associate the delivery of sucrose with an auditory CS. Conditioned responding to the CS was then extinguished. Before a reinstatement test session, we infused AMPA, an AMPA glutamatergic receptor agonist, bilaterally into the IL-PFC to test the prediction that this manipulation would diminish sucrose-induced reinstatement of sucrose-seeking. Next, to address the limitations inherent in pharmacological techniques, we used *in vivo* optogenetics to temporally limit the activation of IL-PFC neurons to CS trials at test. Rats received IL-PFC microinfusions of a viral vector containing a sequence coding for ChR2, a cation channel that increases neuronal activity when activated by blue light (473 nm), or a control viral vector. Using this method, we investigated the

effect of unilateral IL-PFC activation on the inhibition of conditioned responding to a discrete CS during the spontaneous recovery and sucrose-induced reinstatement of Pavlovian conditioned sucrose-seeking. Finally, we confirmed the functional properties of light stimulation in IL-PFC neurons expressing ChR2 by measuring cell activation using Fos immunohistochemistry.

Methods

Subjects

Male, Long-Evans rats (Charles River, N= 64) weighing 220-240 g on arrival were housed on a 12 h light/dark cycle, with lights on at 7AM. They were housed in pairs for three days before being single-housed in individual home cages (Ancare, cat. No. R20PC) with wire bar cage lids (Ancare, cat. No. R20SS2B) containing beta chip bedding (aspen, Harlan, cat. No. 7090A) and a nylabone (Bio-Serv, cat. No. K3580). Rats had unrestricted access to food (Agribrands, Charles River, cat. No. 5075) and water throughout experimental procedures. They were handled and weighed daily for one week before the start of procedures. All procedures were approved by the Concordia University Animal Research Ethics Committee, in adherence to the guidelines of the Canadian Council on Animal Care.

Apparatus

General. Behavioural procedures used equipment from Med Associates (St. Albans, VT, USA) and were conducted in conditioning chambers (ENV-009A) that were housed in ventilated, melamine cubicles (53.6 cm x 68.2 cm x 62.8 cm; fans: ENV-025FAC-R). Each chamber comprised two side walls made of stainless steel and a door, back wall and ceiling made of Plexiglas. The floor was made of metal bar rods (ENV-009A-GF) underneath which a waste tray was slid and cleaned with 3% v/v hydrogen peroxide between cohorts of rats. A white noise generator with speaker (76-85 dB, ENV-225SM) was located in the upper left corner of the left wall. A dual cup liquid receptacle (ENV-200R3AM) was located in the center-right portion of the right wall. Polyethylene tubing (Tygon, Fisher Scientific, cat. No. 141691A) connected one of the cups to a fluid syringe mounted on a pump (PHM-100, 3.33 RPM) situated outside of the melamine cubicles. On either side of the fluid receptacle were two infrared detectors (ENV-254-CB) used to measure port entries. In the upper center-left portion of the left wall, a houselight

(75 mW, 100 mA, ENV-215M) provided general lighting during behavioural sessions.

Activation of the houselights, white noise, fluid pumps and laser pulses was controlled by MedPC IV program that was run on a PC.

In-vivo optogenetics. To enable optical stimulation, rats in Experiments 2-4 received a permanently implanted 300 μm optical fiber (Thorlabs, cat. No. BFH37-300) targeting the IL. The optical fiber implant was constructed in house. Its external portion was encased in an alloy ferrule with a 340 μm bore (Fiber Instrument Sales, cat. No. F10061F340), and held together using heat cured epoxy (Precision Fiber Products, cat. No. PFP-353ND-16OZ). It was then polished using silicon carbide and diamond lapping sheets of decreasing coarseness (Thorlabs, cat. No. LFG5P, LF6D, LF3D, LF1D). Light transmission of the optical implant was tested by measuring power output when connected to a laser using a power meter (Thorlabs, cat. No. PM100D) and calculating the percentage of power transmitted. During behavioural sessions, this ferrule was connected to a patch cord (200 μm) by a ceramic split sleeve (Fiber Instrument Sales, cat. No. F18300SSC25). The patch cord was also constructed in-house as per the protocol described by Trujillo-Pisanty et al. (2015). It was connected through a hole cut into the Plexiglas ceiling of the conditioning chamber to a unilateral optical rotary joint (Doric Lenses, cat. No. FRJ-FC-FC) that was fixed to a weighted arm (Med Associates, cat. No. PHM-110-SAI). The weighted arm was attached behind the back wall of the conditioning chamber and used to facilitate free movement of the rats when attached to the patch cord. A 125 μm optical fiber connected the rotary joint to a 150 mW, 473 nm blue laser (Shanghai Laser & Optics Century Co., Model: BL473T3-150) located outside the melamine cubicles.

Surgery

One week after single-housing, standard stereotactic procedures were used to implant a cannula bilaterally (Experiment 1) or to perform unilateral virus microinfusion followed by optical fiber implantation (Experiments 2-4) into the IL-PFC. Rats were anesthetized using 5% isoflurane in oxygen (CDMV, cat No. 108737), their heads shaved, and placed into the stereotactic frame (Stoelting) using blunt ear bars. Anesthesia was then maintained using 2-3% isoflurane with an oxygen flow rate of 800 ml/min. Atropine (0.4 mg/ml, s.c. 0.01 ml/kg, CDMV, cat. No. 102509) was administered to prevent respiratory congestion, tear gel (CDMV, cat. No. 100741) was applied on the eyes and proviodine (10%, CDMV, cat. No. 4568) was used to disinfect the site of the incision, which was made using a scalpel blade (Blade #10, Fisher Scientific, cat. No. 089165A). Lambda and bregma were used as references to estimate a flat skull. Holes for cannulae, implants, injectors and screws were drilled using a micromotor drill attached to one of the stereotactic arms.

Coordinates used to target the IL-PFC were: (from bregma) AP +2.9, ML \pm 0.6, DV -5.1 (from surface of skull). In Experiment 1, double-barreled cannulae (26 ga, Plastics One, cat. No. C235G-1.2-SPC) were implanted 2 mm above the target (DV -3.1) and injectors (33 ga) projecting 2 mm from the tip of the cannulae were used during microinfusions. Dust caps and obturators were used to prevent cannula obstruction. In Experiments 2 to 4, rats received either infusion of a viral vector containing a sequence coding for ChR2 and eYFP (AAV2-CamKIIa-hChR2(H134R)-EYFP, UNC Vector core, $n = 23$) or of a control virus containing the sequence for eYFP only (AAV2-CamKIIa-EYFP, UNC Vector core, $n = 23$). A volume of 0.5 μ l was infused at a rate of 0.075-0.1 μ l/min and the injector was left in place for 15 min to allow for diffusion. The hemisphere chosen for the infusion of the viral vector and the optical fiber

implantation was alternated from rat to rat in Experiment 2. In Experiment 3 and 4, all virus infusions and optical fiber implantations were done in the right hemisphere.

For virus infusion surgeries, a needle (27 ga ¼, Fisher Scientific, cat. No. 1482113B) stripped from its plastic end and blunted using a Dremmel was inserted into polyethylene tubing (PE20, VWR, cat. No. CA-63018-645) connected to a 10 µl Hamilton syringe (Hamilton, cat. No. 1701N 10ul SYR) that was placed on a microinfusion pump (Pump 11 Elite, Harvard Apparatus, cat. No. 704501). The needle was attached to an injector holder (Stoelting, cat. No. 51631) that was held by the stereotactic arm.

Dental acrylic (A-M Systems, cat. No. 525000 (poweder), 526000 (solvent); for optical fiber, Metabond, Patterson Dental, cat. No. 5533484) was applied to the exposed skull to form a head cap and hold in place the optical fiber implant, and 4-5 screws were used to solidify the structure. Buprenorphine (Temgesic, 0.3 mg/ml, 0.01 ml/kg, s.c.) was administered afterward as a painkiller and soft food mixed with sugar was placed in the home cage to help recovery. Behavioural procedures commenced 1-3 weeks after the last rat had received surgery. Recovery time varied between experiments to allow at least five weeks for virus expression before testing with optical stimulation.

Drugs and solutions

(RS)- α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid hydrobromide (AMPA.HBr) (Tocris, cat. No. 1074) was dissolved in saline to obtain a dose of 0.3 nmol/0.3 µl (weight refers to the drug in salt form). AMPA is a full agonist at AMPA glutamatergic receptors (Zhang, Robert, Vogensen, & Howe, 2006) and has been used in previous research to promote IL-PFC activity at doses similar to the one chosen for this study (Bell & Kalivas, 1996; Peters et

al., 2008; Pierce et al., 1996). The solution was mixed one week before use, stored at -20° C in aliquots of 0.5 ml that were thawed before each test. A 10% w/v sucrose solution was prepared once a week by mixing sucrose in tap water, and then stored in the fridge between uses.

Optical stimulation

In Experiments 2 to 4, pulses of blue light (473 nm, 30 mW) were delivered at a frequency of 20 Hz (pulse duration of 5 ms) in trains of 10.2 s, starting 0.2 s before the start of each CS trial and co-terminating with the CS. These parameters were shown to produce strong spike fidelity in neurons expressing ChR2 when measured by *in vitro* electrophysiology (Lacroix, Sparks, Sanio, Chapman, & Chaudhri, 2015).

Microinfusions

In Experiment 1, AMPA (0 or 0.3 nmol/0.3 µl/side) was infused bilaterally into the IL-PFC before the reinstatement test. During microinfusions, 10 µl Hamilton syringes (Fisher Scientific, 1701 RNR, cat. No. 14-815-279) were placed on a Microinfusion Pump 11 Elite (Harvard Apparatus, cat. No. 704501). Rats were restrained by the experimenter and injectors connected to the syringes by tubing (PE50, VWR, cat. No. CA-63019-047A) were inserted into the IL-PFC. The drug was administered over 1 min at a rate of 0.3 µl/min. Following the end of the injection, the injectors were left in place for 2 min to allow for diffusion.

Perfusion

At the end of Experiments 2-4, rats were anesthetized with pentobarbital sodium (Euthanyl, 240 mg/ml, i.p. 0.1 ml/kg) and then transcardially perfused using 0.9% phosphate

buffered saline (PBS) followed by 4% paraformaldehyde (4% PFA) kept on ice. The descending aorta was clamped to limit the circulatory system to the upper body. A needle attached to tubing connected to the perfusion pump was then inserted into the left ventricle. The right atrium was cut to allow evacuation of the fluids. Perfusion details relevant to each experiment are provided below.

Histology

All brains were sliced using a cryostat at -20° C. Sections were mounted onto glass slides (Superfrost, Fisher, cat. No. 1255020) directly in the cryostat (Experiments 1-3) or after immunostaining (Experiment 4).

The placement of injectors and optical fiber implants was analysed in sections that had been processed for Nissl stain using a light microscope (Leica, Leitz Laborlux S). Placement of the ventral tip of injector and optical implant tracks were assessed using a brain atlas (Paxinos & Watson, 1998) and are depicted in schematics of coronal sections of the rat brain in Figure 1.

Virus expression was assessed using a fluorescence light microscope (Leica, DMRA2) equipped with an external electronic power module (Leica CTR MIC), an isolated mercury and xenon discharge lamp power supply (Leistungselektronik Jena GmbH, EBQ 100) and a CoolSNAP HQ CCD camera. The spread of virus expression was determined by visual inspection of sections including the injector track as well as those directly anterior and posterior.

Fos immunohistochemistry

IL-PFC sections from Experiment 4 were subjected to an immunohistochemical staining (IHC) procedure for Fos which consisted of six phases: quenching, preblocking, primary,

secondary, tertiary and quaternary. Before each phase, the sections were rinsed in tris buffered solution (TBS) for four periods of 10 min. For all incubation periods, the well-plates or carousels were left on a rotator at a slow speed.

During the quenching phase, sections were incubated for 30 min at room temperature in a 0.3% hydrogen peroxide solution. For the pre-blocking phase, in order to reduce non-specific binding of antibodies, sections were transferred to a solution containing normal goat serum (NGS, Vector Labs, cat. No. S-1000) in 0.3% triton (Triton X-100, Sigma Aldrich, cat. No. X100) TBS and incubated for 2 h at room temperature. During the primary phase, sections were incubated for 48 h at 4°C in a solution containing NGS and c-Fos antibody (Santa Cruz Biotech, cat. No. SC-52; final concentration 1:5000) in 0.15% triton TBS. Then, for the secondary phase, sections were transferred to a 0.3% triton TBS solution containing NGS and a secondary antibody (biotinylated goat anti-rabbit IgG antibody, Vector Labs, cat. No. BA-1000) for 1 h at 4°C. For the tertiary phase, avidin and biotinylated horseradish peroxidase solutions (ABC Kit, Vector Labs, cat. No. PK-6100) were mixed in 0.3% triton TBS and sections were left to incubate in this solution for 2 h at 4°C. Finally, during the quaternary phase sections were incubated in a TBS solution containing 3, 3'-diaminobenzidine (DAB), hydrogen peroxide and nickel chloride (DAB Kit, Vector Labs, cat. No. SK-4100) for 8 min.

Sucrose exposure on the home cage

In Experiments 1-3, sucrose was given in the home cage in order to habituate the rats to the taste of sucrose. Rats had 48 h (Experiment 2) or 24 h (Experiment 1 and 3) access to a graduated 100 ml cylinder containing sucrose (10% w/v) and water. Sucrose cylinders and water bottles were blocked by rubber stoppers containing metal sipper tubes with ball bearings. The

sucrose cylinders, water bottles and rats were weighed at the beginning of the session, and the sucrose cylinders and water bottles were weighed again upon their removal 24 h later to measure sucrose consumption. Rats in Experiments 1 and 3 consumed on average 74.24 g (SEM = 2.47 g) of 10% sucrose solution.

Experiment 1: Effect of IL-PFC infusions of AMPA on sucrose-induced reinstatement of sucrose-seeking

The day after sucrose exposure on the home cage, rats were wheeled on a cart to the behaviour room and remained there in their home cages for a 20 min habituation session. The same procedure was repeated the next day, but rats were then weighed and handled in the behaviour room. The next day, rats were weighed in the behaviour room and then placed in a designated conditioning chamber for a 20 min habituation session to the conditioning chambers during which the houselights and fans were turned on and port entries were recorded.

Rats were then trained to associate the delivery of sucrose with presentations of an auditory conditioned stimulus (CS, white noise) during ten 37 min Pavlovian training sessions that were conducted Sunday to Friday. The houselights were turned on after a 2 min delay period following the start of the program. There were 14 CS trials (140-VT schedule) and 4 s after the onset of each 10 s CS, the fluid pumps were activated for 6 s and sucrose (0.2 ml; 2.8 ml/session) was delivered. After each session, the ports were checked to ensure that the sucrose solution had been consumed. The number of port entries made by the rats was recorded in each session.

Responding to the CS was extinguished over at least 9 extinction sessions, or until responding during CS trials had reached a criterion of 7 or less normalized port entries during a session (see below for a description of normalized CS port entries). During extinction, CS trials

occurred in the absence of sucrose delivery. The pumps were turned off and no sucrose syringes were attached. These sessions were otherwise identical to the Pavlovian training sessions described above.

To test the effect of IL-PFC activation using AMPA on reinstatement of sucrose-seeking, saline (n= 9) or AMPA (0.3 nmol/0.3 μ l; n= 9) was administered prior to a reinstatement test session. During the delay period preceding the start of the test session, a 0.4 ml drop of sucrose was delivered into the fluid port. No further sucrose delivery occurred afterward. A between-subjects design was used and rats were counterbalanced across treatments such that there were no differences in responding during Pavlovian conditioning and extinction between treatment groups.

Following the experiment, rats were anesthetized with isoflurane (5%) and 0.3 μ l/hemisphere of fast green was bilaterally microinfused (0.1 μ l/min) through the cannula to mark the ventral tip of the injectors. Rats were then decapitated and the brains were kept in formalin for 24 h and then in a 25% w/v sucrose solution for 4-5 days before being sliced in coronal sections at a 60 μ m thickness and processed using a Nissl stain procedure.

Experiment 2: Effect of IL-PFC optical stimulation during CS trials on spontaneous recovery of Pavlovian conditioned sucrose-seeking

After habituation to the room, one habituation session to the conditioning chambers without dummy patch cords, followed by three habituation sessions with dummy patch cords were given. On the last habituation session, 0.5 ml of 10% sucrose was delivered into the fluid ports to incite the rats to check the ports for sucrose in preparation for Pavlovian training. Dummy patch cords were similar to those used to transmit light during tests, except that they did

not contain an optical fiber in order to limit the damage done to the optical fiber implants caused by repeated contact with another optical fiber.

Nine Pavlovian conditioning sessions, as described in Experiment 1, followed by a single extinction session were given. The effect of optical stimulation of IL-PFC neurons on spontaneous recovery of Pavlovian conditioned sucrose-seeking was tested 24 h later, and then 26 days later in rats expressing Chr2 (n=7) or eYFP only (n=7). The procedure during spontaneous recovery test sessions was the same as for extinction, except that rats were connected to patch cords containing an optical fiber and optical stimulation was delivered during each CS trial.

Rats were perfused at the end of the experiment. Brains were post-fixed for 24 h in 4% PFA and then switched to a 25% sucrose solution for 4-5 days. Coronal sections of 60 μm thickness were collected alternately for fluorescence viewing and Nissl staining. Sections used for fluorescence were mounted in Vectashield with DAPI (Vector labs, cat. No. H-1200).

Experiment 3: Effect of IL-PFC optical stimulation during CS trials on sucrose-induced reinstatement of Pavlovian conditioned sucrose-seeking

After habituation to the behaviour room, two habituation sessions to the conditioning chambers alone and two habituation sessions to the conditioning chambers with dummy patch cords were given, as described in Experiment 2. Magazine training was conducted on the last day of habituation to incite the rats to make port entries. During this session, 0.2 ml of sucrose was delivered at regular intervals over 20 min in the absence of any cue presentation.

Nine Pavlovian conditioning sessions, as described in Experiment 1, and 6 extinction sessions were conducted. To test the effect of optical stimulation on reinstatement, rats

expressing ChR2 (n=12) or eYFP only (n=12) were connected to patch cords containing an optical fiber and optical stimulation was delivered during each CS trial. During the first reinstatement test, 0.2 ml of sucrose was delivered in the fluid ports during the delay period preceding the illumination of the houselights. No sucrose was delivered following this drop. Following the first test, rats were retrained over three Pavlovian conditioning and three extinction sessions. A second reinstatement test was then conducted in which 0.2 ml of sucrose was delivered during the delay period as well as during the first CS trial. This was done to evoke a stronger reinstatement effect in rats. Optical stimulation occurred during each CS trial of both test sessions.

Rats were perfused after the last test. Brains were processed in the same way as described for Experiment 2.

Experiment 4: Effect of optical stimulation on Fos expression in the IL-PFC

Rats were given a total of two habituation sessions without patch cords and four 90 min habituation sessions with dummy patch cords. These last four sessions were longer in duration to match the time necessary at test for Fos expression (Kovacs, 1998; Morgan & Curran, 1991). To test the effect of optogenetic activation of IL-PFC neurons on Fos expression, optical stimulation was delivered to rats expressing ChR2 (n=4) or eYFP only (n=4) during a 37 min test session following the same schedule as previously described, except that no CS was presented. This was done to replicate the amount of optical stimulation received at test in Experiments 2-3. Rats were left an additional 50 min in the chambers with the houselights on. After a total delay of 90 min, rats were taken out of the chambers and perfused. Each perfusion was completed in 15-20-min

and brains were post-fixed in a 20% sucrose 4% PFA solution for 2 h and then switched to sucrose for 4-5 days.

When slicing brains, thickness was set at 50 μm and IL-PFC coronal sections were collected alternately for Fos IHC, fluorescence viewing and Nissl staining. Sections saved for Fos IHC were preserved in Watson's cryoprotectant at -20°C until the immunostaining procedure.

Image analysis. Following IHC, brain sections from Experiment 4 were mounted on microscope slides and cover-slipped. Slides were imaged using a light microscope (Leica, DMR) mounted with a camera (Scion) and pictures of the IL-PFC were taken at a 10x magnification. Fos-positive cells were counted using ImageJ. Because sections reserved for IHC included only a third of the IL-PFC sections, it was only possible to obtain one section that included the optical fiber track for each rat for Fos positive cell counts. The anterior-posterior coordinates for the sections were comprised between 3.20 and 2.70. For each region, an area ($370.40\ \mu\text{m} \times 370.40\ \mu\text{m}$) was selected just below the tip of the optical fiber track and total cell counts were recorded for analyses. Counts were repeated by a second researcher who was blind to the experimental groups, and results between both researchers were comparable.

Data integrity

In Experiment 1, three rats in the saline group were excluded from analyses because at least one of the cannulae was outside of the IL-PFC ($n=2$) or because of an adverse reaction following microinfusion at test ($n=1$).

In Experiment 2, one rat in the ChR2 group was excluded because the placement of the optical fiber was outside of the IL-PFC. Another rat in the eYFP group was excluded because its headcap came off before the end of the experiment.

In Experiment 3, two rats were excluded because their headcaps came off during the experiment (ChR2, n=1; eYFP, n=1). Moreover, because of the absence of virus expression, four additional rats in the ChR2 group were dropped from analyses.

In Experiment 4, one rat in the ChR2 group was excluded from analyses because of tissue damage that occurred during the immunohistochemistry procedure and prevented accurate cell counts.

Statistical analyses

For Experiments 1-3, port entries made during behavioural sessions were recorded. Conditioned behaviour was analysed using a normalized measure of port entries elicited during the CS taking into account a baseline of responding. This was done by subtracting port entries made during a 10 s period immediately preceding each CS trial (preCS) from the number of port entries made during each CS trial. Port entries made outside of CS trials were also recorded and analysed as a measure of non-specific responding. Additionally, for each trial, the number of port entries per CS trial, the latency to the first CS-elicited port entry, and the total duration of CS-elicited port entries were analysed. These measures were averaged across blocks of two CS trials to investigate differences in the pattern of responding within test sessions between groups.

Behaviour was analysed with repeated measures ANOVA using behavioural sessions as within-subjects factor and virus or treatment conditions as between-subjects factor. Statistically significant interactions were further analysed using independent samples and paired-samples *t*-

tests. The significance level was $\alpha = .05$ and the Greenhouse-Geisser correction was used whenever Mauchly's test of sphericity reached significance. Analyses were conducted using IBM SPSS Statistics version 23.

Results

AMPA microinfusion in the IL-PFC reduces reinstatement of sucrose-seeking

CS-elicited port entries increased across Pavlovian conditioning sessions (Fig. 2A) [Session, $F(11, 143) = 14.472, p < .001$] with no differences between saline and AMPA groups [Group, $F(1, 13) = .062, p = .807$; Session x Group, $F(11, 143) = 1.082, p = .379$]. Next, all rats experienced at least 9 extinction sessions, across which CS-elicited port entries diminished equivalently in both groups (data not shown) [Session, $F(8, 104) = 27.157, p < .001$; Group, $F(1, 13) = .025, p = .877$; Session x Group, $F(1, 104) = 1.481, p = .221$].

To trigger reinstatement, 0.4 ml of 10% sucrose into the fluid port immediately after the initiation of the Med-PC program, before an extinction session (Fig. 2B). This manipulation increased CS-elicited port entries compared to the preceding extinction session [Phase, $F(11, 143) = 14.472, p < .001$]. Collapsed across phase, CS-elicited port entries did not differ between groups [Group, $F(1, 13) = 2.176, p = .164$]. However, compared to saline, AMPA microinfusion into the IL-PFC reduced reinstatement [Phase x Group, $F(1, 13) = 9.187, p = .010$]. Subsequent independent samples *t*-tests indicated that although the number of CS-elicited port entries was the same in both groups during the last extinction session [$t(13) = .879, p = .395$], intra-IL-PFC AMPA before test reduced CS-elicited port entries compared to saline [$t(13) = -2.534, p = .025$]. Moreover, paired-samples *t*-tests showed that while there was a trend for the AMPA group to make more CS-elicited port entries at test than during the last extinction session [$t(8) = 2.200, p = .059$], this effect reached statistical significance in the saline group [$t(5) = 4.872, p = .005$].

The number of port entries made outside of CS intervals (Total port entries minus CS port entries; Fig. 2C) increased at test compared to the last extinction session [Phase, $F(1, 13) =$

42.458, $p < .001$], with no impact of intra-IL AMPA on this measure [Group, $F(1, 13) = .005$, $p = .945$; Phase x Group, $F(1, 13) = .046$, $p = .834$].

The effects of intra-IL AMPA on behavioural measures obtained for each CS trial, averaged across blocks of 2 trials, are depicted in Figure 2D-F. Overall, AMPA microinfusions resulted in fewer CS-elicited port entries (Fig. 2D) compared to saline [Group, $F(1, 13) = 5.400$, $p = .037$]. The number of CS-elicited port entries did not change across the test session [Block, $F(6, 78) = 2.284$, $p = .097$] in either group [Block x Group, $F(6, 78) = .996$, $p = .403$]. Latency to make the first CS-elicited port entry (Fig. 2E) did not change during the test [Block, $F(6, 78) = .689$, $p = .659$] and was the same in both groups [Group, $F(1, 13) = .595$, $p = .454$; Block x Group, $F(6, 78) = .268$, $p = .950$]. Similarly, duration of CS port entries (Fig. 2F) did not change across the test [Block, $F(6, 78) = 1.410$, $p = .254$] and was the same in both groups [Group, $F(1, 13) = 1.152$, $p = .303$; Block x Group, $F(6, 78) = 2.176$, $p = .105$].

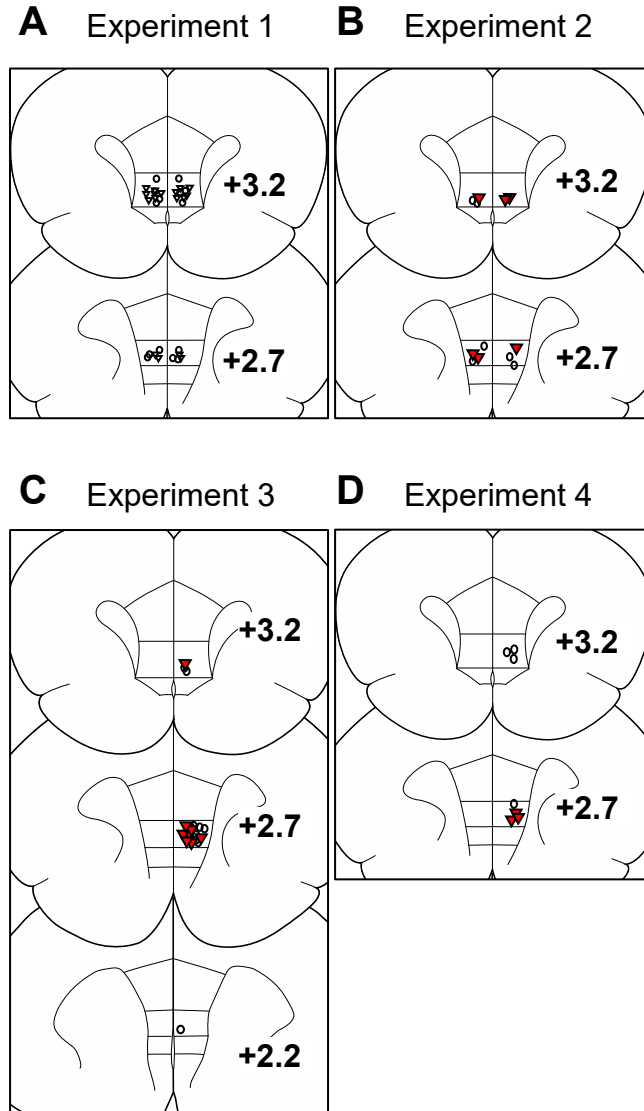
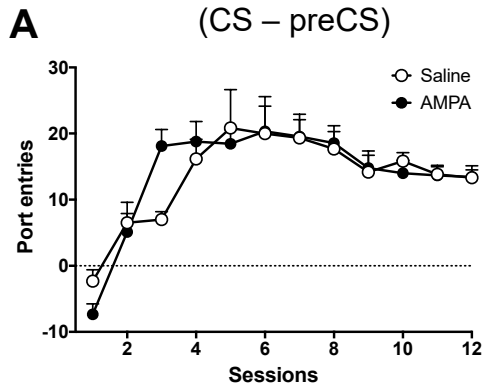
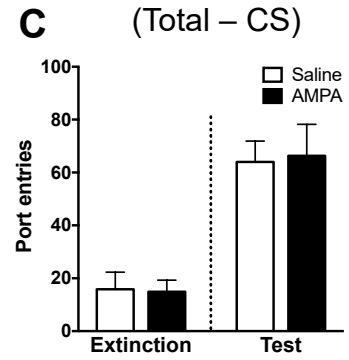
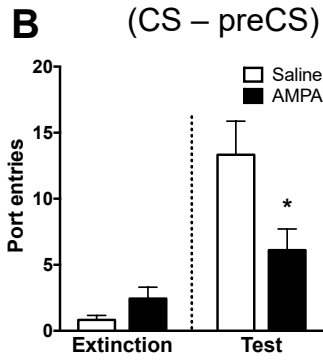


Figure 1. Schematics representing placements of tips of injector tracks or optical fiber implants within coronal sections of the IL-PFC. **A** Placement of tips of injector tracks in Experiment 1 in rats administered AMPA (white triangles) or saline (white circles) at test. **B-C-D** Placement of tips of optical fiber implant tracks in Experiments 2 to 4 in rats in the ChR2 (red triangles) or eYFP (white circles) groups. Numbers indicate AP coordinates from bregma.

Pavlovian conditioning



Extinction and Reinstatement



Reinstatement

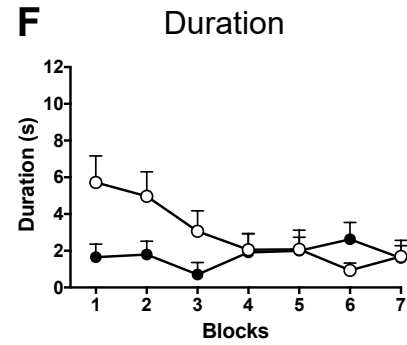
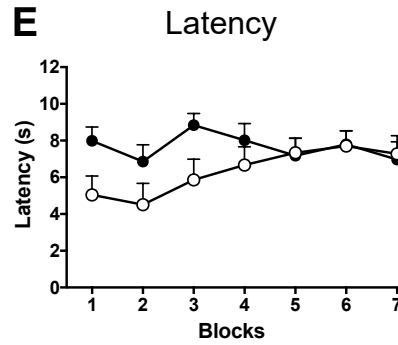
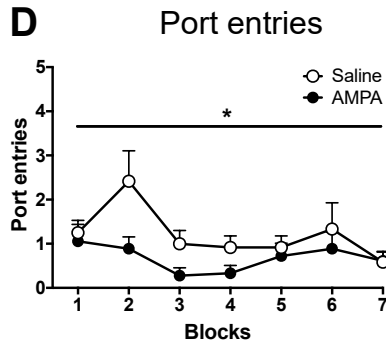


Figure 2. Bilateral microinfusion of AMPA into the IL-PFC reduced sucrose-induced reinstatement of Pavlovian sucrose-seeking. **A** Average (\pm SEM) number of normalized CS port entries across Pavlovian training sessions for saline (white circles) and AMPA (black circles) groups. **B** Average number of normalized CS port entries (CS – preCS) during the last extinction session and the reinstatement test. **C** Average number of port entries made outside of CS trials (Total – CS) during the last extinction session and the reinstatement test. **D** Number of CS-elicited port entries, **E** latency to the first CS-elicited port entry, and **F** duration of CS-elicited port entries averaged across blocks of two CS trials in the saline and AMPA groups at test. * $p < .05$, comparison between the saline and AMPA conditions.

Optogenetic activation of the IL-PFC during CS trials reduces spontaneous recovery

During Pavlovian training (Fig. 3A), CS-elicited port entries increased as a function of session [Session, $F(8, 80) = 16.654, p < .001$] to the same degree in both eYFP control and ChR2 groups [Group, $F(1, 10) = .838, p = .381$; Session x Group, $F(8, 80) = .437, p = .711$]. During a subsequent extinction session (Fig. 3A), the average number of CS-elicited port entries was equivalent in both groups [$t(10) = .907, p = .386$].

Optical stimulation of IL-PFC neurons during CS trials in an extinction session conducted 24 h later (Fig. 3A; Test 1) had no impact on CS-elicited port entries in the ChR2 group, compared to the eYFP group [$t(10) = 1.220, p = .251$]. However, optical stimulation during CS trials in an extinction session that occurred 26 days after the first extinction session (Fig. 3A; Test 2) resulted in fewer CS-elicited port entries in the ChR2 group compared to eYFP controls [$t(10) = 2.592, p = .027$]. The number of port entries made outside of CS trial intervals (Fig. 3B) was equivalent between groups in the first test [$t(10) = -.692, p = .505$] and second [$t(10) = -.731, p = .482$].

Additional analyses were conducted to examine the impact of optical stimulation on the number of port entries per CS, and latency and duration measures averaged across blocks of 2 CS trials. A Test x Group ANOVA conducted on CS-elicited port entries (Fig. 3C) in the last block of test 1 and the first block of test 2 verified that spontaneous recovery occurred only in the eYFP control group. CS-elicited port entries were higher during block 1 of test 2 compared to block 7 of test 1 [Test, $F(1, 10) = 6.785, p = .026$], with the ChR2 group making fewer port entries than the eYFP group [Group, $F(1, 10) = 11.352, p = .007$]. However, a significant Test x Group interaction [$F(1, 10) = 8.138, p = .017$] suggested a greater increase in responding from test 1 to test 2 in the eYFP control group, relative to the ChR2 group. Paired samples *t*-tests

indicated that there was no difference in the number of CS-elicited port entries between block 7 of test 1 and block 1 of test 2 in the ChR2 group [$t(5) = 1.000, p = .363$], but that the eYFP group made more port entries during block 1 of test 2 compared to block 7 of test 1 [$t(10) = -2.750, p = .040$].

Within test 2, optical stimulation of the IL-PFC altered the pattern of CS-elicited port entries across blocks of trials (Fig. 3C). The ChR2 group made fewer CS-elicited port entries than the eYFP group [Group, $F(1, 10) = 6.347, p = .030$]. While responding did not vary overall across blocks [Block, $F(6, 60) = 2.759, p = .073$], there was a difference in the pattern of responding across blocks between both groups [Block x Group, $F(6, 60) = 4.022, p = .024$]. Independent samples t -tests indicated that the ChR2 group made fewer CS-elicited port-entries than the eYFP group during the first block [$t(10) = -3.136, p = .026$], but not the last block [$t(10) = -.559, p = .588$].

Overall latency to the first CS-elicited port entry (Fig. 3D) collapsed across block 7 of test 1 and block 1 of test 2 was longer in the ChR2 group compared to the eYFP group [Group, $F(1, 10) = 12.861, p = .005$], although there was no overall difference in latency across tests [Test, $F(1, 10) = 3.924, p = .076$]. There was a significant interaction [Test x Group, $F(1, 10) = 6.076, p = .033$] suggesting that latency remained the same across tests in the ChR2 group but decreased at the beginning of test 2 in the eYFP group. This effect was verified with independent samples t -tests indicating no difference between groups in latency to the first CS-elicited port entry at the end of test 1 [$t(10) = .048, p = .963$], but significantly longer latencies following optical stimulation of the IL-PFC in ChR2 rats compared to eYFP controls at the start of test 2 [$t(10) = -3.160, p = .025$]. Additionally, paired-samples t -tests indicated no difference in latency

across tests in the ChR2 group, [$t(5) = -1.000, p = .363$], but there was a trend for latency to decrease in the eYFP group [$t(5) = 2.291, p = .071$].

Within test 2, there was no impact of optical IL-PFC stimulation on latency (Fig. 3D). This measure remained stable across the test [Block, $F(6, 60) = 1.631, p = .217$], with no differences between groups [Group, $F(1, 10) = 4.534, p = .059$; Block x Group, $F(6, 60) = 2.215, p = .129$].

A comparison of the duration of CS-elicited port entries (Fig. 3E) during block 1 of test 2 and block 7 of test 1 revealed that overall duration was shorter in the ChR2 than in the eYFP control group [Group, $F(1, 10) = 20.899, p = .001$]. There was no difference in duration across test [Test, $F(1, 10) = 1.324, p = .277$] and no Test x Group interaction [$F(1, 10) = 1.863, p = .202$].

Within test 2, the duration of CS-elicited port entries was lower in the ChR2 group when compared to the eYFP group [Group, $F(1, 10) = 5.925, p = .035$]. Overall duration was stable across blocks [Block, $F(6, 60) = .900, p = .389$] and there was no difference in the pattern of behaviour across blocks in both groups [Block x Group, $F(6, 60) = 1.144, p = .323$].

Pavlovian conditioning and Test

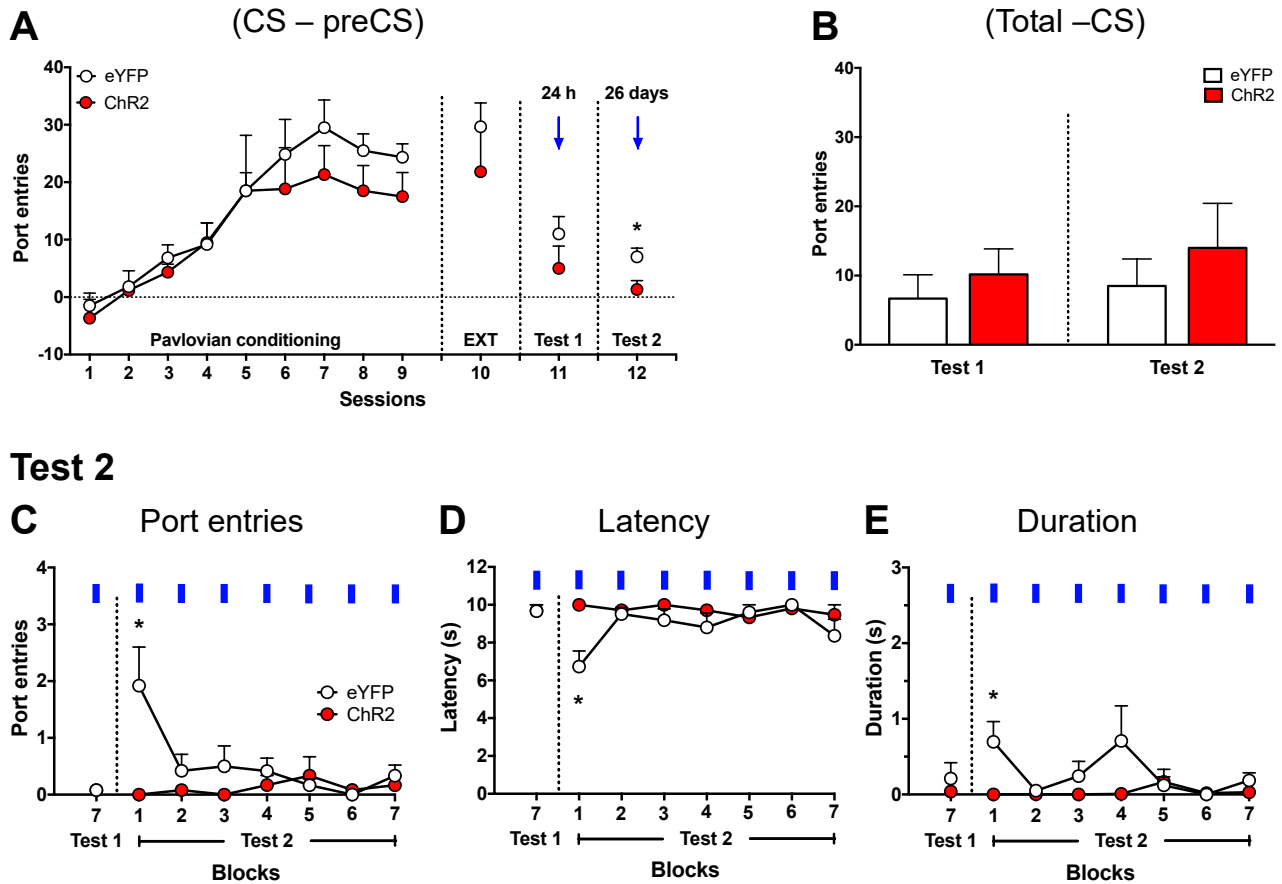


Figure 3. Optogenetic activation of IL-PFC neurons during CS trials reduces spontaneous recovery of Pavlovian sucrose-seeking. **A** Average (\pm SEM) number of normalized port entries during the CS across Pavlovian training, extinction (EXT) and spontaneous recovery tests sessions for eYFP (white circles) and ChR2 (red circles) groups. **B** Average number of port entries made outside of CS trials during both tests. **C** Number of CS-elicited port entries, **D** latency to the first CS-elicited port entry, and **E** duration of CS-elicited port entries averaged across blocks of two CS trials in eYFP and ChR2 groups during spontaneous recovery test 2. For an indication of spontaneous recovery at test 2, the average (\pm SEM) number of CS port entries made during the last CS trial block (block 7) of test 1 is also shown for each measure. $*p < .05$, comparison between the eYFP and ChR2 groups. Blue arrows indicate sessions in which optical

stimulation was delivered, and blue rectangles indicate that optical stimulation was delivered during the corresponding trials. Some error bars are not visible because they are hidden by the symbols.

Optogenetic activation of the IL-PFC during CS trials reduces reinstatement

The number of CS-elicited port entries increased across Pavlovian training sessions (Fig. 4A) [Session, $F(8, 136) = 10.462, p < .001$], with no difference in responding between the ChR2 and eYFP groups [Group, $F(1, 17) = 1.773, p = .201$; Session x Group, $F(8, 136) = .456, p = .695$], and decreased across extinction sessions (Fig. 4A) [Session, $F(5, 85) = 48.206, p < .001$] equivalently in both groups [Group, $F(1, 17) = 47.458, p = .359$; Session x Group, $F(5, 85) = 1.186, p = .322$].

Reinstatement was triggered by delivering a 0.2 ml of 10% sucrose into the fluid port at the start of an extinction session (Fig. 4A). This manipulation increased CS-elicited port entries compared to the preceding extinction session [Phase, $F(1, 17) = 14.351, p = .001$] to a greater degree in the eYFP group than the ChR2 group [Phase x Group, $F(1, 17) = 6.630, p = .020$], with no difference in responding between groups when collapsed across both phases [Group, $F(1, 17) = 1.895, p = .186$]. Follow-up independent samples *t*-tests revealed that there was no difference between groups in CS-elicited port entries during the extinction session preceding the first reinstatement test, [$t(17) = -1.184, p = .253$], but that responding was lower in the ChR2 group than the eYFP group at test [$t(17) = 2.183, p = .043$]. Moreover, paired-samples *t*-tests showed that eYFP rats made more CS-elicited port entries at test when compared to the last extinction session [$t(10) = -4.201, p = .002$], whereas this was not the case for ChR2 rats [$t(7) = -1.148, p = .289$].

CS-elicited port entries increased across three Pavlovian retraining sessions (Fig. 4A), [Session, $F(2, 32) = 9.682, p = .001$], and did not differ overall between the ChR2 and eYFP groups [Group, $F(1, 16) = .950, p = .344$]. There was, however, a Session x Group interaction [Session x Group, $F(2, 32) = 12.523, p < .001$], suggesting that the pattern of reacquisition

differed between groups. Independent samples *t*-tests indicated the number of CS-elicited port entries was greater in the Chr2 group than the eYFP group during the third reacquisition session [$t(16) = -2.909, p = .010$].

The number of CS-elicited port entries decreased across three subsequent extinction sessions (Fig. 4A) [Session, $F(2, 32) = 13.344, p < .001$] in an equivalent manner for both the eYFP control and Chr2 groups [Group, $F(1, 16) = .130, p = .723$; Session x Group, $F(2, 32) = .749, p = .441$].

During the second reinstatement test, 0.2 ml of 10% sucrose was delivered during the delay period before the start of the session and also during the first CS trial (Fig. 4A). There was an overall increase in CS responding when compared to the preceding extinction session [Phase, $F(1, 16) = 13.589, p = .002$], but there was no difference in CS port entries between the Chr2 and eYFP groups [Group, $F(1, 16) = 2.404, p = .141$; Phase x Group, $F(1, 16) = 2.984, p = .103$].

Port entries made outside of CS trials (Fig. 4B) also increased during the first [Phase, $F(1, 17) = 8.851, p = .008$] and second [Phase, $F(1, 16) = 20.466, p < .001$] reinstatement tests when compared to their respective preceding extinction session. During both tests, there were no differences in this measure between the Chr2 and eYFP groups [Test 1, Group, $F(1, 17) = 1.374, p = .257$, Phase x Group, $F(1, 17) = 1.350, p = .261$; Test 2, Group, $F(1, 16) = .009, p = .924$; Phase x Group, $F(1, 16) = .017, p = .899$].

The number of port entries per CS trial averaged over blocks of two trials in the first reinstatement test (Fig 4C) was lower in the Chr2 group than the eYFP control group [Group, $F(1, 17) = 5.181, p = .036$]. Overall the number of CS-elicited port entries remained the same across blocks [Block, $F(6, 102) = 1.750, p = .117$] with no Group x Block interaction [$F(6, 102) = .930, p = .450$].

Latency to the first CS-elicited port entry (Fig. 4D) increased across blocks during the first reinstatement test [Block, $F(6, 102) = 2.326, p = .038$], with no differences between the ChR2 and eYFP groups [Group, $F(1, 17) = 2.924, p = .105$; Group x Block, $F(6, 102) = .553, p = .767$]. Duration of port entries (Fig 4E) decreased across the first reinstatement test [Block, $F(6, 102) = 5.811, p = .002$] with no differences between the ChR2 and eYFP groups [Group, $F(6, 102) = 2.870, p = .109$; Group x Block, $F(6, 102) = .993, p = .399$].

During the second reinstatement test, CS-elicited port entries decreased across blocks (Fig 4F) [Block, $F(6, 96) = 13.019, p < .001$], with no overall difference in responding between the ChR2 and eYFP groups [Group, $F(1, 16) = 2.569, p = .129$] and no Block x Group interaction [$F(6, 96) = 1.605, p = .198$]. Latency (Fig 4G) was overall longer in the ChR2 than in the eYFP control group [Group, $F(1, 16) = 5.646, p = .030$] and increased across blocks [Block, $F(6, 96) = 13.447, p < .001$] to the same degree in both groups [Group x Block, $F(6, 96) = .492, p = .813$]. Port entries duration (Fig. 4H) during the second test was overall shorter in the ChR2 group than the eYFP control group [Group, $F(1, 16) = 5.175, p = .037$] and decreased across blocks [Block, $F(6, 96) = 20.459, p < .001$] equivalently in both groups [Group x Block, $F(6, 96) = 1.207, p = .315$].

Pavlovian conditioning, Extinction and Test Extinction and Test

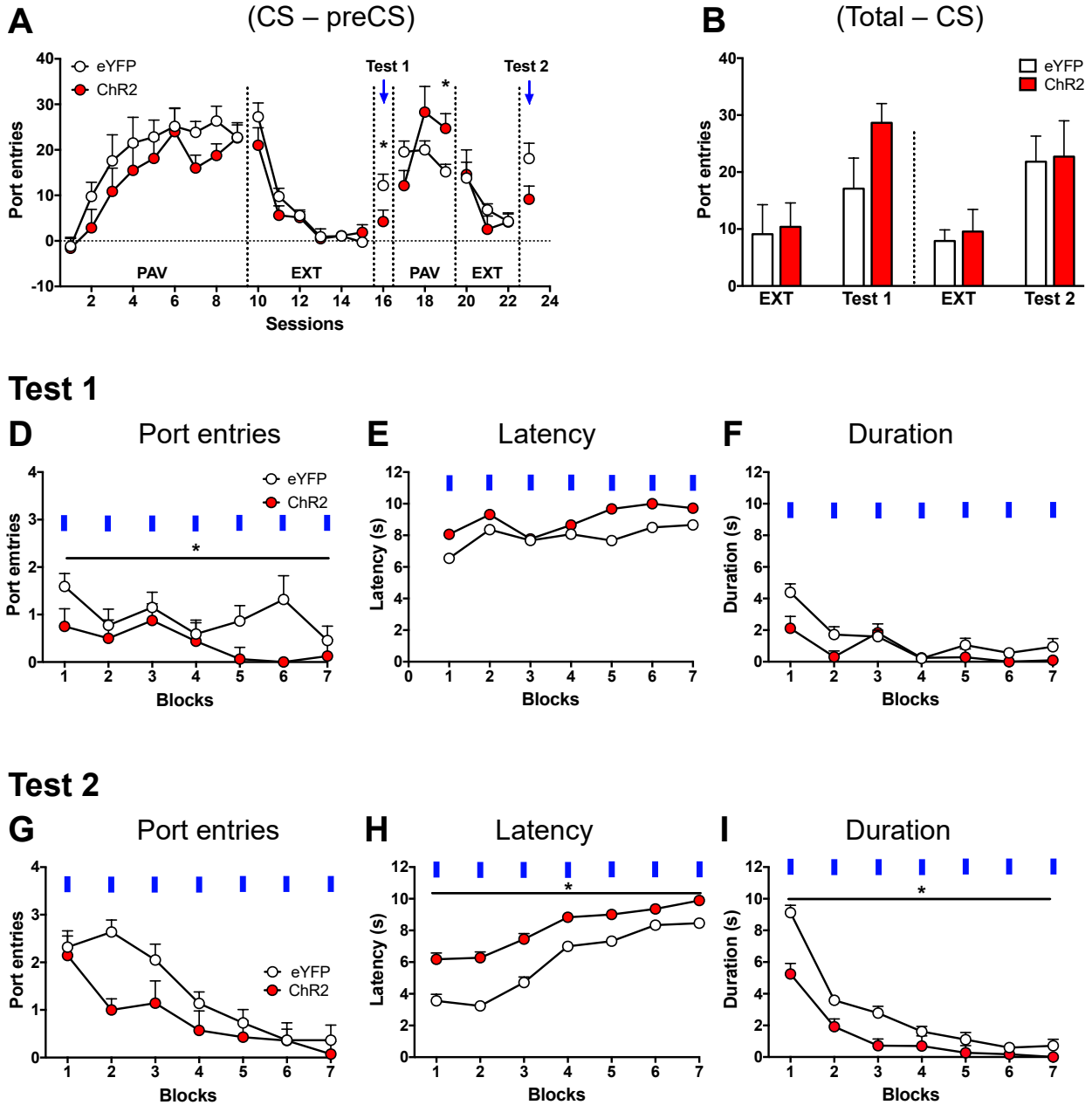


Figure 4. Optogenetic activation of IL-PFC neurons during CS trials diminished sucrose-induced reinstatement of Pavlovian sucrose-seeking. **A** Average (\pm SEM) number of normalized port entries during the CS across Pavlovian training (PAV), extinction (EXT) and two reinstatement tests for the eYFP and ChR2 groups. Before test 1 a single 0.2 ml drop of 10% sucrose was delivered during a 2 min delay before the first CS. Before test 2, this step was repeated and in

addition a 0.2 ml drop of 10% sucrose was delivered during the first CS trial. **B** Average number of port entries made outside of CS trials during both reinstatement tests. **C** Number of port entries, **D** latency to first CS-elicited port entry, and **E** duration of port entries averaged across blocks of two CS trials in eYFP and ChR2 groups during reinstatement test 1. **F-H** The same measures for reinstatement test 2. $*p < .05$, comparison between the eYFP and ChR2 groups. Blue arrows indicate sessions in which optical stimulation was delivered, and blue rectangles indicate that optical stimulation was delivered during the corresponding trials. Some error bars are not visible because they are hidden by the symbols.

Optogenetic activation of Chr2 in the IL-PFC increases Fos expression

Optogenetic stimulation of IL-PFC neurons induced higher Fos positive cell counts in Chr2 than in eYFP rats (Fig. 5A and 5B) [Group, $F(1, 5) = 13.666, p = .014$]. Figure 5A shows representative micrographs of eYFP and Fos expression in IL-PFC brain sections from eYFP and Chr2 rats. Overall expression of Fos was greater in the hemisphere containing the virus infusion that received light stimulation than in the opposite, control hemisphere [Hemisphere, $F(1, 5) = 105.211, p < .001$]. Importantly, Fos counts increased in the stimulated hemisphere when compared to the control hemisphere to a greater extent than in the in the Chr2 group than the eYFP control group [Hemisphere x Group, $F(1, 5) = 31.597, p = .002$]. Independent samples t -tests confirmed that in the stimulated hemisphere, Fos expression was greater in Chr2 than in eYFP control rats [$t(5) = -5.123, p = .004$], whereas in the control hemisphere there was no difference between groups [$t(5) = -.206, p = .845$]. Fos counts in Chr2 rats were higher in the stimulated than in the control hemisphere [$t(2) = -13.404, p = .006$]. There was also a trend for optical stimulation to increase Fos counts in eYFP control rats, relative to the control hemisphere [$t(3) = -3.158, p = .051$].

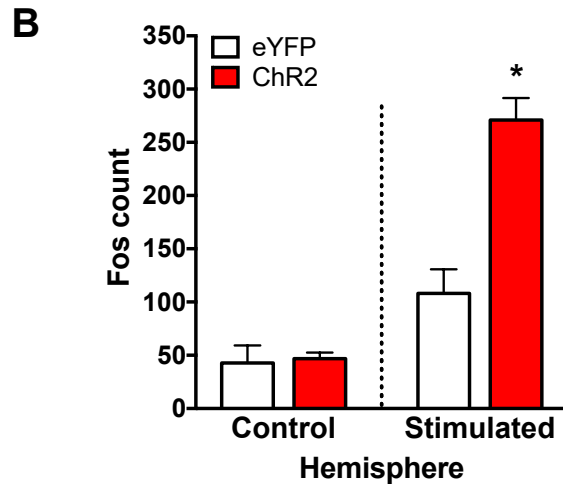
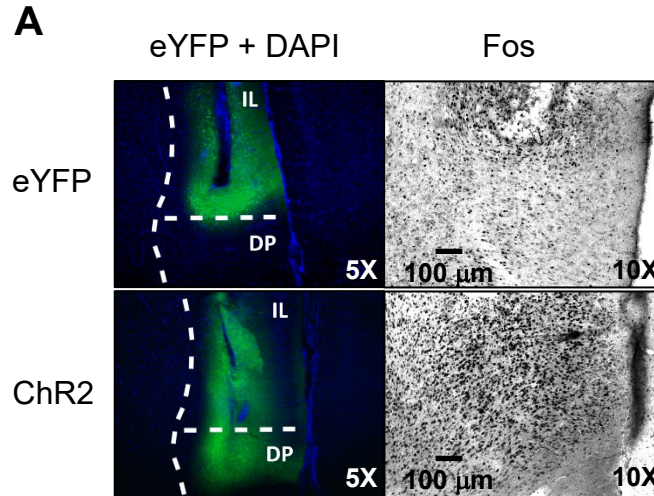


Figure 5. Optogenetic activation of IL-PFC neurons increased Fos expression. **A** Micrograph showing eYFP and Fos expression in representative IL-PFC sections from an eYFP and a ChR2 rat. **B** Average (\pm SEM) number of Fos-positive neurons in the control and stimulated hemispheres in eYFP and ChR2 groups. * $p < .05$, comparison between the eYFP and ChR2 groups.

Discussion

We investigated the effect of IL-PFC activation on the reinstatement and spontaneous recovery of Pavlovian sucrose-seeking behaviour, as well as the effect of optically stimulating IL-PFC neurons on Fos expression. Following Pavlovian training and a varying number of extinction sessions, bilateral activation of the IL-PFC with AMPA diminished sucrose-induced reinstatement of sucrose-seeking during CS trials. We then replicated this effect by optogenetically activating the IL-PFC during CS trials, which blocked sucrose-induced reinstatement of CS-elicited port entries after the first round of Pavlovian training and extinction, but not after a round of Pavlovian retraining and re-extinction. However, an increase in latency to first CS-elicited port entries and a decrease in duration of CS-elicited port entries, which were not present in the first test, emerged during the second test as a result of increasing IL-PFC activity. Additionally, IL-PFC optical stimulation blocked spontaneous recovery 26 days, but not 24 h following the last extinction session. These reductions in behaviour were specific to conditioned responding and did not affect port-entries made outside of CS trials. Finally, light stimulation of IL-PFC neurons expressing ChR2, but not eYFP alone, increased the expression of Fos. However, there was also a trend for light stimulation to increase Fos counts in IL-PFC neurons infected with the control eYFP virus when compared to the non-infected brain hemisphere, suggesting that light itself might contribute to activating neurons in the absence of ChR2. These findings provide support for the hypothesis that IL-PFC activity promotes the inhibition of appetitive, Pavlovian conditioned behaviour following extinction. This conclusion was strengthened by our ability to replicate these results using both pharmacological and optogenetic IL-PFC activation, and following two different behavioural paradigms.

IL-PFC activation reduces the reinstatement of appetitive Pavlovian conditioned behaviour following extinction

We tested the effect of stimulating IL-PFC neurons on sucrose-induced reinstatement of Pavlovian conditioned sucrose-seeking behaviour using two different methods. In Experiment 1, we infused AMPA bilaterally into the IL-PFC before test, and in Experiment 3, we optically stimulated IL-PFC neurons expressing ChR2 during CS trials. In both experiments, CS-elicited port entries increased across Pavlovian training sessions, suggesting that rats established an association between an auditory CS and sucrose delivery. Prior unpublished data from our laboratory indicates that if the CS and US are unpaired this increase in CS-elicited port entries does not occur (Sciascia & Chaudhri, unpublished data). Across the extinction phase, CS-elicited port entries decreased in the absence of sucrose, indicative of the extinction of conditioned behaviour. At test, a drop of sucrose was delivered before the start of the session to trigger the reinstatement of sucrose-seeking. In control groups, this manipulation produced an increase in CS-elicited port entries, which is characteristic of reinstatement. We found that reinstatement was blocked by increasing IL-PFC activity using either bilateral AMPA microinfusions before the test or by unilateral optical stimulation of IL-PFC neurons during CS trials. The reduction of behaviour was specific to CS-elicited port entries, as indicated by similar levels of responding between groups receiving IL-PFC activation and control groups during intertrial intervals. Moreover, as there were no differences between the AMPA and saline groups in Experiment 1, and between the ChR2 and eYFP groups in Experiment 3 in the number of CS-elicited port entries during Pavlovian conditioning and extinction, these differences at test were not a result of pre-existing differences in conditioned responding between the groups.

Bilateral intra-IL-PFC AMPA microinfusions prevented sucrose-induced reinstatement of sucrose-seeking, which is in agreement with Peters et al. (2008) who found that AMPA microinfusion into the IL-PFC reduced the reinstatement of extinguished lever-pressing for cocaine following the administration of a non-contingent cocaine prime. Pre-session, intra-IL-PFC administration of PEPA, an AMPA glutamatergic receptor potentiator also blocked cue-induced reinstatement of lever-pressing for heroin (Chen et al., 2016). Thus the IL-PFC might be part of a common pathway involved in the suppression of reinstatement of both drugs and food, in both instrumental and Pavlovian conditioning paradigms. This effect could be driven endogenously by glutamatergic activity within this region.

We replicated these results using optogenetics to activate IL-PFC neurons during CS trials only. Optical stimulation of IL-PFC neurons decreased the overall number of CS-elicited port-entries at test 1, although this effect did not reach statistical significance in a second test. This difference could be explained by the fact that the two reinstatement tests utilized slightly different procedures. Whereas in test 1 a single drop of sucrose at the beginning of the session was used to trigger reinstatement, in test 2 an additional, second drop was paired with the first CS trial. The latter manipulation was conducted in order to trigger a more robust reinstatement effect at the beginning of the second test session. However, preliminary data from our laboratory (Lacroix et al., 2015) indicate that optical stimulation of IL-PFC neurons expressing ChR2 either has no effect or augments responding to a CS when it is paired with sucrose during Pavlovian reacquisition. It is therefore possible that pairing a CS trial with a drop of sucrose in test 2 made the second test similar to a regular Pavlovian training session, which could in turn have affected responding during subsequent extinction trials by recruiting neuronal structures involved in reacquisition. Another possibility is that reinstatement may recruit different neural structures and

processes following reacquisition and re-extinction. This hypothesis is supported by data showing that inactivation of projections from the ventral subiculum to the nucleus accumbens shell (NAshell) reduced context-induced renewal of alcohol lever-pressing during a first, but not second test conducted following phases of retraining and extinction (Marchant et al., 2016). Thus, the control of behaviour after one relapse test could be taken over by different brain regions. Moreover, from a neurological perspective initial training and extinction phases might be fundamentally different from subsequent reacquisition and re-extinction phases. These possibilities warrant further research.

Nonetheless, although the difference in CS-elicited port entries between the ChR2 and eYFP groups was not statistically significant at test 2, differences were observed in other variables. The ChR2 group had longer latencies to the first CS-elicited port entries and shorter port entry durations during CS trials relative to the eYFP group, suggesting that different aspects of conditioned behaviour were affected at test 2. Interestingly, such differences in latency and duration were not observed during test 1. Latency and duration are unfortunately rarely reported in the literature. Taking a closer look at these variables might give a more complete picture of the effects of IL-PFC manipulations on conditioned behaviour during CS trials.

Although conditioned behaviour was the same between the ChR2 and eYFP groups during the initial Pavlovian conditioning and extinction phases, differences emerged during the reacquisition of Pavlovian training after test 1. During the last session of the retraining phase, the ChR2 group made more CS-elicited port entries than the eYFP control group. Since IL-PFC activity is involved in consolidation of extinction memory (Do-Monte et al., 2015; LaLumiere et al., 2010; Sierra-Mercado et al., 2011), it is possible that this increase in conditioned responding was the result of long-term effects of optically stimulating IL-PFC neurons during the first

reinstatement test. However, because responding was equivalent between groups during extinction, we do not think that this difference impacted results during the second reinstatement test.

IL-PFC activation reduces spontaneous recovery after a long, but not a short delay

In Experiment 2, the number of CS-elicited port entries was equivalent during Pavlovian training and extinction in both the Chr2 and eYFP groups. Contrary to our expectations, optical activation of IL-PFC neurons 24 h after a single extinction session did not reduce CS-elicited port entries in the Chr2 group, compared to the eYFP control group. However, the same manipulation blocked spontaneous recovery 26 days later. This finding is in contrast with aversive Pavlovian conditioning studies that have shown that IL-PFC activation during CS trials inhibits conditioned responding in a test conducted 24 h after the first extinction session (Domonte et al., 2015; Milad et al., 2004; Milad & Quirk, 2002). These results might point to a different mechanism of inhibition between aversive and appetitive conditioning. The effectiveness of IL-PFC activity in inhibiting appetitive conditioned behaviour might depend as well on the length of the delay intervening between conditioning and test. For example, in a cocaine conditioned place preference (CPP) assay, activating the IL-PFC during tests in the absence of cocaine reduced the time spent in the cocaine context when the tests occurred after a prolonged, but not a short interval following CPP training (Van den Oever et al., 2013). The authors proposed that increasing IL-PFC activity may have preferentially inhibited “remote,” rather than “recent” memories. Thus, it is possible that in the present experiment, the longer interval before the spontaneous recovery test conducted 26 days after the last extinction session

contributed to establishing Pavlovian conditioning as a “remote” memory and to making IL-PFC activation more effective in inhibiting CS-elicited port entries.

Furthermore, because test 1 was essentially a second extinction session, it is possible that IL-PFC activation during test 1 affected extinction consolidation and strengthened extinction recall during test 2. Pre- and within-extinction session IL-PFC stimulation leads to greater recall during subsequent extinction sessions (Do-Monte et al., 2015; LaLumiere et al., 2010; Milad et al., 2004; Milad & Quirk, 2002). Optical stimulation during test 1 might have enhanced the consolidation of extinction memory and produced a stronger inhibition of behaviour during test 2. It would be interesting to see if these results replicate after omitting light stimulation during the first test.

Another concern inherent to our methods is that ChR2 expression might have been lower during test 1 than test 2. The 26 day delay preceding test 2 might have allowed for greater ChR2 expression to occur, therefore strengthening the effect of optical stimulation. While the interval of 4-5 weeks following the last virus infusion surgery before test 1 is consistent with what other researchers have used and is generally sufficient for robust infection of cell bodies (Do-Monte et al., 2015; Smith et al., 2012), it might have been sub-optimal in our case.

Overall, we established that IL-PFC activation restricted to CS trials maintains the extinction of Pavlovian conditioned behaviour during sucrose-induced reinstatement and spontaneous recovery procedures. Despite some differences, our main findings were generally consistent with those from the aversive conditioning literature that used pharmacological compounds (Sierra-Mercado et al., 2011), electrical stimulation (Milad & Quirk, 2002; Milad et al., 2004) and optogenetics (Do-Monte et al., 2015; Kim, Cho, Augustine, & Han, 2016) to activate or inactivate IL-PFC neurons during extinction sessions. These results show that IL-PFC

activity is involved in maintaining the inhibition of both aversive and appetitive conditioned behaviour after extinction has occurred. It extends previous results by demonstrating that the IL-PFC is involved in controlling extinguished behaviour elicited by a discrete appetitive CS.

Our results are also coherent with studies that used instrumental conditioning (LaLumiere et al., 2010; Peters et al., 2008) and suggest that the IL-PFC is a common locus for the inhibition of both instrumental and Pavlovian conditioned behaviours. This hypothesis is partly corroborated by an earlier study from our laboratory (Mendoza et al., 2015) in which rats received Pavlovian and instrumental conditioning sessions with sucrose as the US or reinforcer. Bilateral IL-PFC inactivation using microinfusions of M/B before a first extinction session enhanced within-session extinction of Pavlovian conditioning; however, this manipulation had no impact on within-session extinction of instrumental behaviour. Thus, differences in the recruitment of certain pathways during extinction of Pavlovian and instrumental conditioning could emerge in specific procedures.

Optical stimulation of IL-PFC neurons increases Fos positive cell counts

To assess whether our optogenetic manipulation produced physiologically measurable neuronal activation, we used immunohistochemistry to observe Fos expression in IL-PFC neurons following optical stimulation. To replicate conditions experienced during test sessions of Experiments 2 and 3, rats were administered optical stimulation following the same schedule, but in the absence of auditory cues and sucrose delivery. This manipulation induced greater Fos expression in ChR2 expressing rats than in eYFP rats. Positive cell counts were also compared within-subjects across both hemispheres, and in the ChR2 group were greater in the infected, stimulated than in the non-infected, control hemisphere. This finding is consistent with

electrophysiology data that measured action potentials evoked by optical stimulation of glutamatergic neurons expressing ChR2 (Lacroix et al., 2015; Ji & Neugebauer, 2012; Do-Monte et al., 2015), and with other studies that have used Fos as a marker of neuronal activation in response to optical stimulation (Do-Monte et al., 2015; Van den Oever et al., 2013). Thus, optogenetic stimulation as used in the present experiments produced physiologically relevant activation of neurons within the IL-PFC.

There was also a trend for Fos counts to be greater in the infected, stimulated hemisphere compared to the non-infected, control hemisphere in the eYFP group, although this effect did not reach statistical significance. While optical stimulation of IL-PFC neurons induced greater Fos positive cell counts in ChR2 expressing neurons than in eYFP only neurons, observations from the eYFP group suggest that light itself might produce small increases in cell activity. Previous reports have indicated that light delivery in the brain via an optical fiber could lead to local increases in temperature, which could in turn affect the integrity of the neurons (Han, 2012; Yizhar et al., 2011). Thus, the lowest effective power output should be chosen when using optical manipulations in the brain to avoid damage to the cells caused by excessive heat.

Future directions

IL-PFC to nucleus accumbens shell projection. Pyramidal neurons in the IL-PFC project to brain regions that are involved in appetitive conditioned behaviour. For example, the nucleus accumbens shell (NAshell) receives glutamatergic input from the IL-PFC (Sesack, Deutch, Roth, & Bunney, 1989), and this pathway might be involved in modulating the maintenance of extinction. Glutamate levels in the NAshell measured using microdialysis increase during the first 40 min of extinction of lever-pressing for cocaine, suggesting that glutamatergic input is

involved in the initial acquisition of extinction (Suto, Ecke, You, & Wise, 2010). Accordingly, bilateral inactivation of the NAshell or disconnection of IL-PFC to NAshell projections reinstates lever-pressing on a cocaine-associated lever following extinction (Peters et al., 2008). This evidence indicates that the NAshell itself supports the inhibition of appetitive conditioned behaviour and that this role might be mediated by the IL-PFC, more specifically by its glutamatergic input. Indeed, the reinstatement of lever-pressing for heroin is diminished by pre-test, intra-IL-PFC administration of PEPA and this effect is related to an increase in the AMPA receptor subunit GluR1 in the NAshell (Chen et al., 2016). Another possibility is that the IL-PFC to NAshell projection suppresses behaviour during unrewarded events. Inactivation of the IL-PFC did not affect phasic firing in the NAshell during a rewarded CS, but it increased phasic firing during a neutral stimulus that had never been paired with an appetitive US (Ghazizadeh, Ambroggi, Odean, & Fields, 2012). This change in firing pattern was in turn related to increases in responding during the neutral stimulus. The IL-PFC to NAshell pathway might therefore be involved in modulating the extinction of appetitive conditioned behaviour, a hypothesis that could be investigated further by using viral vectors to target glutamatergic projections from the IL-PFC to the NAshell.

Optical inhibition using halorhodopsin. Studies that have observed the effects of both stimulation and inhibition of the same region on conditioned behaviour found that these two manipulations might not have equivalent, opposite effects on conditioned behaviour. Different patterns of results were obtained by Do-Monte et al. (2015) between studies using optogenetic activation and inactivation of the IL-PFC. Whereas IL-PFC activation during CS trials caused an immediate reduction in freezing behaviour, IL-PFC inhibition only resulted in extinction deficits during a recall session, in the absence of optical inhibition. Moreover, enhancement and

inhibition of IL-PFC activity might differently influence memories of associations that are recent or remote (Van den Oever et al., 2013). Using cocaine CPP, the authors demonstrated that optical stimulation of the IL-PFC using ChR2 reduced CPP after a protracted period of abstinence, but had no effect after a shorter period. The reverse pattern was observed with optical inhibition of the IL-PFC using halorhodopsin. It is not clear why activation and inhibition of neurons in the same region affects conditioned behaviour in such different ways. These differences may be related to the timing of when IL-PFC activity is manipulated. Because different studies used a different number of training and extinction sessions before testing the effect of manipulating IL-PFC activity on behaviour, it might partly explain some discrepancies found in the literature.

Role of IL-PFC during the acquisition and expression of conditioning. Our results show that activation of IL-PFC neurons enhances response inhibition after extinction has already occurred. Few studies have investigated the effect of IL-PFC activation during the extinction phase itself. Pre-extinction session microinfusions of PEPA in the IL-PFC enhance the acquisition of extinction (Chen et al., 2016) and electrical stimulation of the IL-PFC during extinction trials facilitates the extinction of aversive Pavlovian conditioning (Milad et al., 2004; Milad & Quirk, 2002). To our knowledge, however, very few studies have investigated the effect of augmenting IL-PFC activity during acquisition. IL-PFC lesions, compared to sham lesions, do not affect appetitive Pavlovian conditioning (Rhodes & Killcross, 2004, 2007). However, lesion studies do not allow the investigation of a normal, intact brain, and this technique might result in the use of alternative mechanisms during conditioning to compensate for the loss of a relevant brain region. Indeed, pharmacological inactivation of the IL-PFC with M/B before a Pavlovian conditioning session had no effect on Pavlovian conditioned CS responding, but increased

responding outside of CS trials (Mendoza et al., 2015), suggesting that the IL-PFC plays a role in restricting behaviour to meaningful events during acquisition. Therefore, it would be advantageous to use reversible methods to study how IL-PFC activity modulates the acquisition of a conditioned response on a session by session, trial-by-trial basis. Assessing the impact of IL-PFC activity during conditioning might also help clarify its role during extinction in a more comprehensive fashion.

Final comments

We demonstrated across three different studies that increasing the activity of IL-PFC neurons promotes the maintenance of extinguished behaviour. Pharmacological activation of the IL-PFC prevented sucrose-induced reinstatement of Pavlovian conditioned sucrose-seeking. These results were replicated using optogenetics to limit the activation of IL-PFC neurons to CS trials. Increasing activity of IL-PFC neurons also prevented the spontaneous recovery of sucrose-seeking after a prolonged period of time. Together, these results suggest that IL-PFC activity maintains the inhibition of behaviour, but that this role might not be stable across time. Thus, the IL-PFC seems to be intricately involved in the control of conditioned behaviour and our understanding of its function still needs to be refined. Taking advantage of the variety of neuroscientific techniques and behavioural assays currently available would help obtain a more complete picture of this role.

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