Yohimbine increases impulsivity through activation of CREB in the orbitofrontal cortex. *(Sun et al)*

SUPPLEMENTAL INFORMATION

Materials and Methods

All experiments were carried out in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at UT Southwestern and the Animal Care Committee at the University of British Columbia.

Subjects:

Subjects were male Lister Hooded rats (Charles River, Kingston, NY) weighing 300-320g at the start of each experiment. Animals used in behavioral testing experiments (n = 56), were food restricted and maintained at 85% of their free-feeding weight through feeding them 14g of standard rat chow per day. Animals used for *ex vivo* determination of CREB levels following yohimbine administration (n = 12) had free access to food. Water was freely available for all animals. Animals were pair-housed under a reverse light cycle (lights on from 21.00-09.00). Testing took place between 09.00 and 19.00 five to six days per week.

Experiment 1: Determining the effects of yohimbine on 5CSRT performance

Testing took place in 8 standard five-hole operant chambers (Med Associates, Roanoke, VA). Each chamber was fitted with an array of five evenly-spaced response

apertures (2.5 x 2.5 x 2.2 cm). The five-hole array was positioned 2 cm above a bar floor, and each aperture contained a stimulus light. Nosepoke responses into these apertures could be detected by a horizontally-positioned infra-red beam located 1cm from the entrance to each hole. Each operant chamber was also fitted with a houselight, a food tray, a traylight and an external pellet dispenser capable of delivering 45 mg pellets (Noyes dustless pellets, Bioserv, San Diego, CA) to the food tray. Each box was contained within a ventilated and sound-attenuating chamber. The boxes were controlled by software written in Med PC (Med Associates, Vermont, USA) by TAG and CAW running on an IBM compatible computer.

16 rats were trained to perform the 5CSRT as described in detail in previously published reports (1; 2). In brief, animals were trained to respond in one of the five holes when the stimulus light located in the back of the response aperture was briefly illuminated (0.5s). The stimulus light could appear in any of the five holes, and the spatial location of the target was varied randomly from trial to trial. Each session consisted of 100 trials and lasted approximately 30 min. Animals initiated each trial by making a nosepoke response at the food tray. There was then a 5 s ITI during which animals had to withhold from making a response at the array before the stimulus light was presented in one of the holes. Premature or impulsive responses made at the array during this time period were punished by a 5 s time-out period during which the houselight was turned on and no further trials could be initiated. A correct response at the illuminated hole was rewarded with delivery of one food pellet in the food tray. Food delivery was signaled by onset of the traylight which remained on until the animal collected its reward. An incorrect or lack of response (omission) was not rewarded and

punished in the same manner as premature responses. Repeated responding at the correct hole was classified as perseverative responding and, whilst monitored, was not punished. Animals received 5-6 sessions per week until a high level of stable performance was reached (≥80% accuracy, ≤ 20% omissions).

The effects of yohimbine (0, 1, 2, 5 mg/kg) were then assessed on task performance. The drug was administered via the intraperitoneal route 10 mins prior to the start of the task. The order in which animals received different doses of drug was counterbalanced using a Latin square drug design. Each drug injection day was preceded by a drug-free baseline day and followed by a day during which animals remained in their home cages and were not tested. Care was taken to ensure that behavior on these baseline days was not significantly different from that observed prior to administering yohimbine. This design greatly reduces the risk of any carry-over effects caused by repeated drug administration.

Experiment 2: Determining the effects of yohimbine on CREB signaling within brain areas implicated in impulse control

12 rats were injected with either a dose of yohimbine which increased impulsive responding (2 mg/kg, n = 6) or vehicle (n = 6). Animals were sacrificed by live decapitation within 30 mins of receiving the injection i.e. within the time frame in which yohimbine affected impulsivity. The tissue samples were frozen on dry ice and processed for western blotting. Each sample was thawed on ice and briefly sonicated until a homogenous suspension was formed. Overall protein levels were determined by the Lowry method. Protein samples were then separated by sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% Tris-HCl gel and transferred to a PVDF membrane (BioRad, Hercules, CA). 50ug of protein was loaded per sample. After washing in TBST and blocking in 5% dry milk powder in TBST, the membrane was incubated in a solution of primary antibody (e.g. rabbit anti-pERK 1: 1000, Cell Signaling, Beverly, MA) at 4°C overnight. After thorough washing with TBST, the membrane was incubated with the secondary antibody (e.g. HRP-conjugated anti-rabbit IgG, 1: 40000, Vector, Burlingame, CA) for 45 mins at room temperature. Immunoreaction was detected using an enhanced chemiluminescence system (Pierce Biotechniology- Thermo Fisher Scientific, Rockford, IL) and quantified by densitometry using the NIH Image analysis program. The amount of protein blotted onto each lane was normalized to levels of GAPDH (1: 40000 mouse-anti rabbit GAPDH, Research Diagnostics, Concord, MA).

Experiment 3 Determining the effects of modulating CREB activity on impulsive responding.

40 rats were trained to perform a simplified version of the 5CSRT (the 1CSRT) in which only 1 hole- the central stimulus aperture- was ever illuminated. The level of premature responding observed in the 1CSRT is typically higher than in the 5CSRT, allowing for both increases and decreases in impulsive responding to be readily detected. Reducing the attentional load of the task also decreases behavioral variability within the cohort of subjects, facilitating the division of subjects into multiple groups matched for baseline behavior. The 1CSRT was therefore judged to be a better behavioral test for this

phase of the experiment, particularly as yohimbine did not affect the attentional component of the 5CSRT.

Once stable behavior had been achieved, animals were matched for baseline performance and divided into 5 groups (n = 8). Adeno-associated viruses (AAVs) designed to over-express either CREB or a mutant version of CREB (mCREB) were infused into the OFC of two different groups using standard stereotaxic techniques. mCREB acts as a dominant negative protein to CREB which prevents CREB from activating gene transcription: mCREB will bind to CREB but the resulting dimer does not form an active transcription factor complex. Separate groups also received infusions of these AAVs into the NAC, an area in which CREB's activity has been shown to modulate the response to affective stimuli, and which has been implicated in regulating impulsivity, but in which no changes were observed in response to yohimbine. These data therefore acted as a positive control. The final group received infusions of an AAV designed to express green fluorescent protein (GFP) in order to control for any effects of viral infection and non-specific protein over-expression (OFC: n = 4; NAC: n = 4). It should be noted that the AAV-2 serotype used in these experiments does not cause any neuronal damage or irregularities (3) and no behavioral consequences have been observed following its infusion intra-cerebrally as compared to vehicle infusions (e.g. (4). After surgery, animals remained in their home cages with free access to food and water for 8 days in order to allow AAV expression to peak prior to re-training on the 1CSRT. After 3 weeks of behavioral testing, a stable post-operative baseline had been established in the 1CSRT in all groups (15 sessions). Animals were then challenged again with yohimbine according to a Latin square design as in experiment 1.

Viral –mediated gene transfer surgery

Rats were anaesthetised with ketamine (Ketaset, 100 mg/kg intramuscular (i.m.) injection) and xylazine (10 mg/kg i.m.; both drugs from Henry Schein, Melville, NY). A herpes-simplex virus (HSV) designed to over-express ΔFosB, FosB or ΔJunD was infused into either the OFC or PrLC using a 31 gauge stainless steel injector (Small Parts, Miami Lakes, FL) attached to a Hamilton microinfusion pump by polyethylene tubing (Instech Solomon, Plymouth Meeting, PA). The viral vectors were infused at a rate of 0.1 μl/min according to the following coordinates taken from a stereotaxic atlas (5): OFC- site 1, AP +4.0, L ± 0.8, DV –3.4, 0.4 μl; site 2, AP +3.7, L ± 2.0, DV –3.6, 0.6 μl; site 3, AP +3.2, L ± 2.6, DV –4.4, 0.6 μl; NAc: injector set 10° off vertical, AP +1.7, L ± 2.4, DV –6.7, 1 μl. Once each infusion had been completed, the injector remained in place at the infusion site for the same amount of time each infusion had taken in order to allow the virus to diffuse into the surrounding tissue.

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