Title

Neurobehavioral precursors of compulsive cocaine-seeking in dual fronto-striatal circuits

Short title

Neural antecedents of cocaine addiction

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Abstract

Only some individuals using drugs recreationally eventually become addicted, and persist in drug seeking and taking despite adverse consequences. The neurobehavioral determinants of this individual vulnerability have not been fully elucidated. We report that in drug naïve rats the future tendency to develop compulsive cocaine seeking is characterised by behavioral stickiness-related functional hypoconnectivity between the prefrontal cortex and posterior dorsomedial striatum in combination with impulsivity-related structural alterations in the infralimbic cortex, anterior insula and nucleus accumbens. These findings show that the vulnerability to develop compulsive cocaine seeking behavior stems from pre-existing structural or functional changes in two distinct cortico-striatal systems that underlie deficits in impulse control and goal-directed behavior.

One sentence summary

Distinct neurobehavioral correlates of impulsivity and behavioral inflexibility together predispose to compulsive cocaine seeking in rats.

Introduction

Compulsion is a core component of addiction, defined by drug seeking and taking that persists despite personal harm (*1, 2*). Vulnerability to compulsion is hypothesized to result from an interaction between pre-existing individual differences in traits, environmental and experiential factors and long-term drug exposure (*3-5*). Thus, human stimulant addiction has been associated with novelty seeking (*6*), impulsivity (*7-9*) and impaired cognitive control (*10*) alongside alterations in both ventral and dorsal cortico-striatal circuits (*9, 11, 12*). However, the causal significance of such neurobehavioral vulnerability markers is unclear because they can be affected by drug exposure (*7, 13-16*).

Studies in rodents have been able to implicate causal neural substrates of impulsivity and novelty preference in vulnerability to compulsivity (*3, 17-19*). By contrast, novelty reactivity and sign-tracking (ST, the tendency to attribute incentive salience to Pavlovian cues) (*20*) confer an increased susceptibility to the acquisition of cocaine self-administration and sensitivity to the associative properties of cocaine-associated cues, respectively, rather than vulnerability to addiction (*3, 18, 21*). Nevertheless, none of these studies has acknowledged that compulsive drug use conflates phases of drug 'foraging' or anticipation, which actually occupies the majority of the time spent in waking activity of addicted individuals, prior to actual drug taking. Thus, they have principally investigated neurobehavioral circuitries implicated in compulsive stimulant drug-taking (*22-24*) following drug exposure thereby limiting our understanding of the neuropsychological basis of vulnerability to the compulsive tendency to seek drugs.

Compulsive drug use in preclinical models has generally been assessed in terms of the resistance to punishment of drug self-administration (*25*). Such self-administration behaviors exemplify instrumental (goal-directed) responding governed by the principles of reinforcement learning. However, the propensity for compulsive drug seeking in terms of possible predisposing differences in reinforcement sensitivity and the balance between goal-directed and habitual control over behavior (*26, 27*), has not yet been compared in conjunction with Pavlovian or neurobehavioral vulnerability markers.

Consequently, we combined a multidimensional behavioral approach to vulnerability with magnetic resonance imaging (MRI) to identify in drug naive male rats the structural and functional brain correlates of the later emergence of compulsive drug seeking. We therefore developed a novel behavioral procedure to investigate in individuals with a prolonged history of cue-controlled cocaine seeking (*28*) the individual vulnerability to persistent drug seeking in the face of punishment (**Fig. S1**).

Results

Following acquisition of intravenous cocaine self-administration, rats were progressively trained to seek cocaine daily for long (15 min) periods of time under the control of the conditioned reinforcing properties of the response-produced drug-paired conditioned stimulus (CS) under a second-order schedule of reinforcement (SOR) (*29*). The compulsive nature of cocaine seeking was then measured individually as the tendency to persistent cocaine seeking, in a drug free state, in the face of response-contingent mild foot-shock punishment.

High-compulsive (HC), low compulsive (LC), and intermediate compulsive (IC) rats identified according to an unbiased cluster analysis of the punishment resistance of cocaine seeking (*30*), represented 18%, 41%, and 41% of the cohort, respectively (**Fig. 1A**). The incidence of HC rats was similar to that previously reported for compulsive cocaine taking (*3, 31*) as well as the prevalence of addiction in human cocaine users (*32*). HC rats received more shocks than the other groups during the first 15 min drug-free period (**Fig. 1B**) and during the entire daily session (**Fig. 1C).** This reflected the tendency to persist in cocaine seeking, as HC rats maintained higher levels of responding on the active lever during the first drug-free seeking period (**Fig. 1D**) and throughout the entirety of each punished session than the other groups (**Fig. 1E).** Consequently, HC rats continued to obtain the maximum number of cocaine infusions available daily whereas NC rats decreased significantly their cocaine intake across punished sessions (**Fig. 1F).** The development of punishment-resistant cocaine seeking shown by HC rats was not due to differences in their acquisition of intravenous drug self-administration or drug seeking compared with LC rats (**Fig. S2**), nor was it attributable to a differential sensitivity to pain, as assessed using a hotplate test (**Fig. S3**) (*3*).

Factorial analysis building on a dimension reduction strategy (**see SOM**) was used retrospectively on the putative behavioral markers of vulnerability (**Fig. S4**) to identify the detailed phenotype of drug-naïve rats later developing compulsive drug seeking (**Fig. 2**). These markers were premature responses in the 5-choice serial reaction time task (*33*), indexing impulsivity, the locomotor response to novelty (*18*), indexing novelty reactivity, conditioned approach to a food-related CS, indexing sign-tracking (*3*); reinforcement learning parameters (*34*) derived from a serial reversal learning task reflecting reward-based learning (α), reinforcement sensitivity (β) and 'response stickiness' (κ) (*15*). The latter parameter is the likelihood of the same response being repeated regardless of its reinforcing outcome and is thus a putative measure of value-free habitual responding (*35*). Importantly, this 'stickiness' is elevated in humans with stimulant use disorder (*36*) but it is unknown whether it is pre-disposing to addiction.

Impulsivity and stickiness loaded on Factor 1 of the analysis (**Fig. 2A**, **Table S3**) while sign-tracking and novelty reactivity loaded on Factor 2, independent of Factor 3 which accounted for α and β (see **SOM** for more details). This multidimensional behavioral structure shows that more than half the overall model variance is explained by Factor 1 (vulnerability to compulsivity) and Factor 2 (resilience to compulsivity). Further factorial analysis incorporating compulsive cocaine seeking into this model (**Fig. 2B**, **Table S4**) revealed a shared construct of impulsivity, stickiness and compulsive cocaine seeking, loading on Factor 1, whereas Factors 2 and 3 accounted for resilience and reinforcement learning parameters, as in the initial analysis.

Subsequently, impulsivity was revealed to correlate with cue-controlled cocaine seeking only when rats had learnt that persisting in responding resulted in punishment, i.e. from the second punished session onwards (**Fig. 2C**). By contrast, stickiness not only correlated with cocaine seeking under punishment, albeit less systematically and robustly, but was also marginally related to baseline levels of cue-controlled cocaine seeking (**Fig. 2C**). Further analysis revealed that the co-occurrence of the behavioral traits of impulsivity and stickiness results in an increased vulnerability to develop compulsive cocaine seeking, the product of their variance being systematically a better predictor of compulsivity than that of each alone (**Fig. 2D**).

We next sought to define the neural basis of the complex endophenotype predicting compulsive cocaine seeking and the extent to which it mapped onto recent structural and functional neuroimaging studies of the propensity to stimulant use disorder in humans (*9, 12, 37*). Voxel-based morphometry analysis of scans carried out after behavioral screening and before any cocaine self-administration (~post-natal day 228; **(Fig. S1)**) (**Fig. 3, S12**) revealed that, compared to LC, rats destined to compulsively seek cocaine (HC) had lower grey matter density in the infralimbic (IL) cortex (**Fig. 3A**) and left ventral striatum (**Fig. 3B**), but greater grey matter density in the left caudal anterior insula (AI) (**Fig. 3C**). This structural signature was specific to the tendency compulsively to seek cocaine as no such structural differences were observed in relation to baseline cue-controlled cocaine seeking (**Fig. S10**), and overlapped with the neural signature of impulsivity. Hence, as compared to Low Impulsive (LI) rats, High Impulsive (HI) rats showed lower grey matter density in the IL cortex (**Fig. 3D**), the right ventral striatum (**Fig. 3E**) and the left rostral AI (**Fig. 3F**), a neural profile consistent with earlier findings (*38, 39*). Together, these data suggest a rostro-caudal functional gradient in the AI mapping onto two different, yet interacting, behavioral manifestations of impulse control deficit, namely impulsivity and compulsivity, respectively (*18, 38, 40-43*).

The functional coupling signature (**Fig. 4A, Fig. S11C, D**) of compulsivity revealed a specific association with corticostriatal networks involved in goal-directed instrumental responding (*44, 45*). The coherence of coupling strength of the blood-oxygen-level-dependent (BOLD) response between either the prelimbic cortex (PrL) or the anterior cingulate cortex (ACC) with the posterior dorsomedial striatum (pDMS) was lower in HC compared to NC rats (**Fig. 4C**) but was not related to baseline cue-controlled cocaine seeking (**Fig. 4B**). Importantly, this hypofunctionality of the PrL→ pDMS and ACC→ pDMS that characterises compulsive cocaine seeking was also shown to underlie stickiness (**Fig. 4D**), but not impulsivity (**Fig. S11A, B**), a possible reflection of impaired goal-directed behavioral circuitry.

Discussion

Our findings demonstrate the co-occurrence of high impulsivity and high stickiness traits increases the vulnerability to develop compulsive cocaine seeking in a large cohort of rats. These traits are related to structural and functional connectivity

changes in dual neural systems that predict, prior to any drug exposure, the future transition to compulsive cocaine seeking. At the neural systems level, whereas impulsivity was accompanied by structural changes in the IL cortex, AI and ventral striatum, consistent with the recognised role of these regions in impulse control (*38, 39, 46*), the associated loss of control of cocaine intake (*33, 47*) or inflexible behaviors (*48*), stickiness, as indexed by κ, was associated with reduced connectivity strength between dorsomedial areas of the prefrontal cortex (PFC) (ACC/ PrL) and pDMS. This finding resonates with recent work showing increased stickiness in human stimulant users (*36*). Collectively, our results show that the vulnerability to develop compulsive cocaine seeking is predicted by abnormalities in two distinct neural circuitries, one involving decreased functional connectivity between the ACC/PrL and pDMS, associated with increased stickiness, and a second, centred on structural abnormalities in the IL cortex and ventral striatum, two key nodes of an impulsivity network arising from a loss of top-down cognitive control (*49*).

Reduced connectivity between the medial PFC (mPFC) and pDMS, a region implicated in goal-directed behavior including drug seeking (*50*), is consonant with recent findings of a neurobehavioral endophenotype in this system in humans with stimulant use disorder and their unaffected relatives (*37*). It invites the hypothesis that pre-existing impairments in the goal-directed system lead to inflexible ('sticky'), habit-prone behavior, which is expressed in compulsive drug seeking (*27*). This tendency to behavioral repetition independent of outcome may reflect the default operation of a value-free habit system that complements value-based reinforcement learning (*35*) and may underlie the profound perseverative tendencies of those addicted to cocaine, exacerbated by further failures of cognitive control (*7, 51, 52*). Pre-existing deficits in different circuits contributing to impulse control and flexible, goal-directed behavior may thus be joint precursors for the emergence of compulsive cocaine seeking.

This identification of a complex neurobehavioral endophenotype for stimulant use disorder has implications for how we approach related psychiatric disorders, including addictions, as a necessary prelude for determining how the development of these fundamental behavioral systems is subject to polygenic and early experiential influences. This knowledge may ultimately help us appreciate how such putative

genetic and environmental factors lead to the evidently profound individual differences in susceptibility to compulsive drug-seeking behavior that remain central to understanding and preventing substance use disorders. A first step will be to determine whether the neurobehavioral signature of vulnerability identified here in males is shared by females.

The multi-modal approach we have adopted (with its neuroimaging and computational, as well as behavioral components) may also represent a more general strategy for modelling the etiology of other neuropsychiatric disorders.

Materials and Methods

Subjects

Pregnant dams purchased from Charles River, UK (n=13) were initially housed in temperature and humidity controlled individually ventilated cages upon arrival. On the birth of each litter, female rats were culled. Each litter contained four male offspring with excess males culled. Rat offspring were MRI scanned on post-natal day (PND) 21. On completion of this scan, offspring were weaned. All animals were kept on a reverse light/dark cycle with red light on between 07:30am and 19:30pm and white light on between 19:30pm and 07:30am. All animals were allowed *ad libitum* access to food and water during this period. Prior to behavioral phenotyping, rats were also MRI scanned at PND 35 and 63. Following the PND 63 scan, all animals were kept under food restriction to 85% of their free feeding weight during behavioral phenotyping. Upon completion of behavioral phenotyping, at PND 212 - 244, rats were MRI scanned prior to intravenous catheter surgery. Post-surgery animals were singly housed for the duration of the experiment. All experiments were carried out in accordance with the (U.K Animals) Scientific Procedures Act (1986) under the UK Home Office project licenses (PPL 70/7587 & PPL 70/8072) held by BJ and DB, respectively and were approved by the University of Cambridge Ethics Committee. The number of animals used during each stage of this longitudinal study are summarized in supplementary **Tables S1** and **S2**.

Drugs

Cocaine hydrochloride (kindly supplied by the NIDA Drug Supply Programme to DB) was dissolved in sterile 0.9% saline. Drug doses are reported as the salt form.

Behavioral phenotyping

Beginning at PND 64, rats were screened for behavioral endophenotypes of vulnerability or resilience to cocaine addiction *(3, 53)*, as summarised in **Fig.S1** below. Rats were first screened for the trait of sign-tracking (ST) (*20*), then impulsivity *(18)*, reversal learning (*15*), followed by locomotor reactivity to novelty *(54)*. Individual differences in approach responses to conditioned stimuli (e.g., sign- or goal-tracking trait) were assessed in an autoshaping task *(3, 20)*. Impulsivity was measured in the 5-Choice Serial reaction Time task (5-CSRTT) (*55*); reinforcement learning and stickiness (*56*) were measured in a spatial reversal learning task *(15)*, while locomotor reactivity to novelty was assessed using four open fields and a video tracking system (ViewPoint Behavior Technology®, Lyon, France) (*3*).

Sign and Goal Tracking

The ST trait, which has been shown to be associated with the resilience to develop compulsive cocaine self-administration (*3*), was identified using a procedure based on our earlier work *(3)*. Two days prior to the beginning of autoshaping training for food (i.e., 45 mg food pellets, Bio-Serv, USA), food-restricted rats received 20 pellets in their home cage to minimise food neophobia. The experimental sessions were conducted for 6 consecutive days in 12 operant chambers (31.8 cm x 25.4 cm x 26.7 cm, Med Associates, St. Albans, USA) located within a ventilated sound-attenuating cubicle. Front and back panels of the test chambers were made of aluminium, while the right and left walls and the roof were transparent acrylic plastic with a stainless grid floor. A pellet dispenser was installed behind the front wall of each chamber, supplying food pellets to a food magazine located 2 cm above the grid floor. Each test chamber was illuminated by one 3-Watt light bulb during the experimental session. The first day of training was a habituation session in which rats were placed in the operant chamber (house light and fan turned on) for 30 min. The second day of training consisted of a single session of magazine training for the rats to learn where food pellets were delivered, 50 pellets being delivered under a 30 s-variable time (VT) schedule. Rats were then trained over 6 daily sessions to associate the presentation of a compound conditioned stimulus (CS) comprising a cue-light and a lever, deemed active in that it enabled the measurement of Pavlovian approach and contacts with the compound CS. Responding on that lever had no programmed consequences to the subsequent delivery of a food pellet (unconditioned stimulus: US). CS-US presentations were delivered according to a VT-90 schedule such that

on termination of each variable time, the cue light was illuminated and the active lever was inserted for an 8 sec period. At the end of this period, the CS was turned off, the active lever was retracted, and one pellet was delivered in the food magazine. During the 8 sec interval, contacts with the compound CS measured as active lever depressions, were recorded as sign-tracking events while head entries into the food magazine were recorded as goal-tracking events. Inactive lever presses (uncoupled from the CS) were also recorded as an indicator of general activity. Individuals within the upper and lower quartiles of the population stratified on their level of sign-tracking (lever presses) over the last three sessions, were considered sign-trackers (ST) and goal-trackers (GT), respectively *(3)*.

Impulsivity

Impulsivity, as measured in the 5-CSRTT, is an endophenotype of vulnerability to develop compulsive behavior (*18, 38, 57*). The 5-CSRTT has been described previously (*55*). After one habituation session during which 5 pellets were placed in each of the five apertures and 5 pellets into the magazine in order to facilitate exploration of the chamber and nose-poking behavior, rats were trained to acquire the 5-CSRTT. Each session began with illumination of the house light and the delivery of a food pellet in the magazine. Collecting this pellet initiated the first trial. After a fixed inter-trial interval (ITI) of 5 sec, a light at the rear of one of the response apertures was briefly illuminated. Responses in this aperture within a limited hold period (5 sec) were reinforced by the delivery of a food pellet in the magazine (correct responses). Responses in a non-illuminated aperture were recorded as incorrect responses and were punished by a 5 sec time-out period. A failure to respond within the limited-hold period was deemed an omission and was punished by the house light being extinguished for 5 sec and no delivery of food reward. Additional responses in any aperture prior to food collection (perseverative responses) were recorded but not punished. Responses made in any aperture before the onset of the target stimulus or ‘premature’ responses, were punished by a 5 sec time-out period. Subjects were considered to have acquired the task (i.e., with a stimulus duration of 0.7 sec and an ITI of 5 sec) when their accuracy was greater than 80% and omissions were fewer than 20%. Rats then underwent three 60 min challenge sessions with a 7 sec long ITI (LITI), separated by two baseline 5 sec ITI sessions. Increasing the ITI results in a marked increase in premature responding

and therefore facilitates the identification of inter-individual differences in impulsivity. HI and LI rats were selected in the upper and lower quartile of the population based on the average of the premature responses expressed during the last two LITI sessions (*38*).

Reversal learning

Spatial-discrimination reversal learning was assessed using twelve 5-choice operant chambers placed in ventilated, sound-attenuating cubicles (Med Associates, Georgia, VT), as previously described (*15*). Subjects were initially habituated to the apparatus over two days, with each session lasting 20 min. They were then trained to enter the magazine to trigger the illumination of a single stimulus light (left or right) and to respond in the illuminated aperture for food delivery under a fixed-ratio (FR) 1 schedule of reinforcement. Once rats had achieved 50 correct responses, food reward was successively delivered under FR2 and FR3 schedules to the same criterion within a 30 sec limited hold period. A failure to respond within the 30 sec period resulted in a 5 sec time-out. Once animals achieved criterion under a 5 sec inter-trial interval, they were tested for spatial discrimination followed the next day by a reversal of the stimulus-reward contingency. Rats were given 1 h to complete the discrimination task by achieving 9 correct trials across the previous 10 trials. During the 1-hour within-session reversal test, responses in the previously incorrect aperture were signalled as correct (and reinforced with a food pellet) whereas responses in the previously correct aperture were signalled as incorrect (and not reinforced with food) (**Fig. S5**).

A Q-learning model was used to simulate the reversal learning data as previously described (*15, 58*). The model, defined below, included three parameters: α, β, and κ. Model parameters were fitted to each animal's reversal data individually and then compared using analysis of variance (ANOVA). The learning rate α determines how quickly the agent adjusts the expected value of a response following positive or negative feedback. High α values allow the agent to increase (or decrease) the expected Q-value for that response after the response is followed by a reward (or not). The inverse temperature parameter β regulates how much an agent explores by responding randomly or exploits what the agent learned about the responses to date. A low β value would lead an agent to rely on the expected Q-values of the responses and hence exploit what they have learnt about the responses already. A

high β value would lead to exploration that under some circumstances may lead to higher rewarded outcomes. However, in the present reversal task, with deterministic outcomes, a high β value would result in fewer rewards. Finally, the choice autocorrelation parameter κ is a measure of stickiness, or how likely an agent will perform the same response again regardless of reward outcome. Values of κ close to 1 reflect an agent "sticking" to the previous response while κ values close to −1 reflect choice alternation. In the present task, high stickiness is not advantageous since it leads to a reduction in rewarded outcomes following contingency reversals.

The model used to generate α, β, and κ parameters for subsequent analyses is summarized as follows:

$$P(c_t = L|Q_t(L), Q_t(R), L_{t-1}, R_{t-1}) = \frac{exp\ (Q_t(L)/\beta + \kappa * L_{t-1})}{exp\ exp\ (Q_t(L)/\beta + \kappa * L_{t-1})\ +\ exp\ (Q_t(R)/\beta + \kappa * R_{t-1})}$$

whereby a larger κ results in greater probability of the choice c_t at trial t being the same as the choice c_t at trial t−1.

Locomotor reactivity to novelty

High locomotor reactivity to novelty predicts an increased tendency to acquire drug self-administration (*18*) but resilience to switch from controlled to compulsive drug intake (*3, 17, 18*). The locomotor reactivity to novelty test was conducted as previously described (*3*) in four infra-red illuminated open fields (50 x 50 x 50 cm) under a light intensity similar to that of the holding rooms (~ 550 Lux at the centre of the open field). Rats were placed in the open fields for two hours and their locomotor activity recorded throughout. Individuals whose total distance travelled was in the upper and lower quartiles of the population were considered High Responders (HR) and Low Responders (LR) rats, respectively (*3*).

Drug self-administration

Apparatus

Experiments were conducted using thirty-six standard operant conditioning chambers (Med Associates, St. Albans, VT, USA) enclosed within a sound-attenuating box containing a fan to eliminate background noise. Each chamber was

equipped with two retractable levers (4 cm wide, 12 cm apart, and 8 cm from the grid floor), a cue light (2.5 W, 24 V) above each lever, and a white house light (2.5 W, 24 V) at the back of the chamber, in front of the levers. Silastic tubing shielded with a metal spring extended from each animal's IV catheter to a liquid swivel (Stoelting, Wood Dale, IL, USA) mounted on an arm fixed outside of the operant chamber. Tygon tubing extended from the swivel to a Razel infusion pump (Semat Technical, Herts, UK) located adjacent to the external chamber. Lever presses, presentation of light stimuli, reward delivery, and data collection were controlled by a PC running MED-PC IV.

Intra-jugular surgery

Rats were implanted with a custom-made indwelling catheter into their right jugular vein under isoflurane anaesthesia (O_2 carrier gas; 2 L/min; 5% for induction and 2-3% for maintenance and analgesia (Metacam, 1mg/kg, sc., Boehringer Ingelheim) as previously described (*59*). Following surgery, rats received daily oral treatment with the analgesic for three days and an antibiotic (Baytril, 10mg/kg, Bayer) for one week. Catheters were flushed with 0.1 ml of heparinized saline (50 U/ml, Wockhardt®) in sterile 0.9% NaCl every other day after surgery and then before and after each daily self-administration session.

Self-administration training

Rats were trained to acquire cocaine self-administration (0.25 mg/100µl/5.7sec/infusion) under continuous reinforcement over 4 daily, 2-hour sessions. Under this schedule, each active lever press resulted in drug infusion initiated concurrently with a 20 sec time out that included onset of a 20 s illumination of cue light positioned above the active lever (conditioned stimulus; CS), offset of the house light and retraction of both levers. Inactive lever pressing was recorded but had no scheduled consequence. Active and inactive lever assignment was counterbalanced, and a maximum of 30 infusions was available for this stage. Following these 4 daily sessions under continuous reinforcement, the daily schedule of reinforcement was changed to fixed intervals, increasing across daily training sessions from 1 min (fixed interval 1 min, FI1) to FI2, FI4, FI8, FI10 and eventually FI15 min (*60*). After three sessions under an FI15 schedule of reinforcement rats

were trained to seek cocaine under the control of the drug-paired CS for 30 sessions under a FI15(FR10:S) second order schedule of reinforcement (SOR) (*28, 61*). Under this schedule of reinforcement, a CS is response-produced every tenth lever press while the drug is self-administered upon the tenth lever press after a 15 min interval has elapsed.

Compulsive cocaine seeking

For five daily sessions under SOR (SOR sessions 21-25), drug seeking behavior was punished when rats were actively engaged in responding for the drug, namely during the last 7 minutes of each 15-minute interval. Thus, during the last 7 min of each interval, mild electric foot-shocks (1 sec duration, 0.25-0.45 mA) dispensed by a scrambler (Med Associate St. Albans, USA) connected to the grid floor of the operant boxes were delivered on every 16th lever press. Punishing responding only during the last 7 minutes of each interval offered the advantage that animals did not receive an electric foot-shock immediately after a drug infusion, which could result in counterconditioning and a decrease in the aversiveness of the shock (*62*). Each aversive stimulus was paired with a cue light located on the top middle region of the wall (independent from those paired with drug infusions) and rats received one shock every 16th lever press to minimise the probability of co-occurrence of CSs and shocks and to avoid extinction of their drug seeking behavior. The shock intensity was increased from 0.25mA on the first punishment session to 0.35mA on sessions two and three to 0.45 mA on sessions four and five, as previously described (*63*).

Following the punished sessions, rats were re-exposed to five baseline SOR sessions to investigate their ability to recover their initial drug seeking behavior, or to display long-term behavioral adaptations to successive punishment sessions. A K-means cluster analysis *(64, 65)* was carried out on the number of shocks rats were willing to receive during the first, drug-free, interval of the last two punishment sessions, when shock intensity was 0.45mA and a significant and stable suppression of seeking and taking was observed in non-compulsive individuals, as shown in **Figure 1C** and **1E**. This approach identified non-overlapping populations stratified according to their propensity to persist in seeking cocaine in the face of adverse consequences.

Pain sensitivity measurement

To ensure that potential differences in resistance to punishment were not attributable to a differential sensitivity to a nociceptive ('pain') stimulus, high and low compulsive rats from an independent cohort were subjected to a hot plate test prior to punishment sessions *(3)*. Six hours following the 17th SOR session, rats were placed on a hot plate (Ugo Basile, Gemonio, Italy), calibrated to remain at a stable temperature of 52ºC. The time elapsed before the appearance of pain-associated behaviors, including paw-licking and jumping, was measured and considered a direct indicator of pain threshold *(66)*. Rats were then immediately removed from the hot plate and returned to their home cage.

Magnetic resonance imaging

Imaging acquisition

High resolution MRI was performed on a 9.4 T horizontal bore MRI system (Bruker BioSpec 94/20 Bruker Ltd. Coventry UK). Images were acquired using the manufacturer-supplied rat brain array coil with the rat in a prone position. Structural images were obtained based on a three-dimensional multi-gradient echo sequence (TR/TE 25/2.4 ms with 6 echo images spaced by 2.1 ms, flip angle 6° with RF spoiling of 117°). The field of view was 30.72 × 25.6 × 20.48 mm^3 with a matrix of 192 × 160 × 160 yielding isotropic resolution of 160 µm with a total scan time of 6 min 36 sec with zero-filling acceleration (25% in the readout direction; 20% in each phase encoding direction). Magnetisation transfer pulses (10 µT, 2 kHz off-resonance) were applied within each repetition to enhance grey-white matter contrast. Post-reconstruction, images from each echo were averaged after weighting each by its mean signal. A series of resting state fMRI (rs-fMRI) scans were carried out using a multi-echo planar imaging (EPI) sequence, with zero-filling (factor 1.33) used for readout and a partial-FT of 1.5 in the phase-encoding direction with an acceleration factor of 1.5. Scan parameters were: field of view = 28.8 x 21.6 mm^2, matrix size = 64 x 48 x 40, echo time = 15 ms, repetition time = 1.832 s, 450 frames with a thickness of 0.5 mm. A total of 450 frames were collected, with sequential acquisition, and three echoes acquired for each frame. A total of 171 scan sessions occurred across 52 animals. Throughout all scans, rats were anaesthetised with isoflurane (1-2% in 1L /min O_2:air 1:4). During the acquisition of rs-fMRI, isoflurane was reduced to 0.8% in 1L /min O_2:air 1:4. Respiratory rate and pulse oximetry (SA

instruments; Stony Brook, NY) were measured with anaesthetic dose rates adjusted to ensure readings remained within a physiological range. Body temperature was measured and regulated with a rectal probe and heated water system to 36-37°C.

Voxel based morphometry

Voxel-based morphometry (VBM) was performed to assess morphological correlates of impulsivity, stickiness and compulsivity. An *unbiased* whole-brain analysis approach was adopted to capture brain-wide significant differences in grey matter. Although ROI-based methods have potentially greater statistical power due to averaging over whole anatomical areas they assume that the underlying signal is consistent within that area. Voxel based methods do not rely on anatomical areas having a uniform signal throughout a given area and thus are better suited to detect effects that are more heterogeneous in nature. Nevertheless, in practice, with spatial smoothing included in the VBM procedure, and anatomical information used to interpret the findings, there is considerable overlap between these methods. Images were first manually reoriented (pitch, yaw, roll, x, y, z) to match the orientation of a reference template image using the bulk manual registration tool in the Statistical Parametric Mapping (SPM) 8 (Wellcome Trust Centre for Neuroimaging, University College London, UK) toolbox SPMMouse (SPMMouse, Wolfson Brain Imaging Centre, University of Cambridge (*67*)). After correspondence was achieved, structural images were bias corrected and segmented into three different tissue classes (grey, white and cerebrospinal fluid). Tissue class images were then rigidly co-registered to a reference template image. Non-linear registration was achieved through the use of the diffeomorphic anatomical through exponentiated algebra (DARTEL) procedure (*68*). Using the DARTEL warp fields, images were then warped and modulated to match the newly generated template images (**Fig. S8**). All images were manually checked for accurate registration and segmentation. Modulated grey matter maps were smoothed with an isotropic Gaussian kernel of 0.45 mm to promote normality of the data and mitigate imperfections in image registration. A general linear model was used for voxelwise analysis on the smoothed maps. Three independent models were created with block designs to assess main effects of impulsivity (HI, n = 12; LI, n = 12), compulsivity (HC, n = 7; LC, n = 7) and stickiness (HK, n = 14; LK, n = 14). Two independent linear regression models were created to

assess compulsive cocaine seeking (n = 39) and cocaine seeking under no punishment (n = 39).

Prior to any formal analysis, total intracranial volume (TIV) was correlated with each variable of interest. A correlation analysis between TIV and compulsivity, stickiness and cocaine seeking under no punishment showed no relationship with TIV ($p > 0.3$), whilst impulsivity was somewhat related to TIV ($p = 0.065$). A *two-way* t-test showed that HI rats showed lower TIV than LI animals ($t = 2.271$, $p = 0.03$), while no other behavioral measures showed group differences in TIV. As a result, TIV was not included in the impulsivity model (above). Although the removal of variables such as TIV can increase sensitivity by accounting for known variance in the data, if the confounding variable correlates with important modelling factors, effects that are accounted for by that variable will also be removed. Thus, although the evidence ($p = 0.065$) is not definitive for a linear relationship between TIV and impulsivity, there is stronger evidence ($p < 0.05$) of a group difference. Therefore, the benefits of including the confounding variable are unjustified by the resulting risk of losing sensitivity and that is why we did not include it in the model. Moreover, it is difficult to account for such a specific effect of the TIV variable in areas of the brain shown previously using a range of convergent techniques to relate to impulsivity (*49*). Nevertheless, TIV was included as a covariate in all other models to remove differences related to brain size among the rats. Main effects were assessed with contrasts based on Student's *t,* with a restricted number of assessments made to avoid type I errors due to multiple comparisons: i) HI < LI; ii) HC < LC; ii) HC > LC; iv) HK < LK; v) compulsive cocaine seeking (negative correlation); vi) cocaine seeking under no punishment (negative correlation), among others (**Fig. S12**). These were selected based on our previous findings that high impulsivity is related to reduced grey matter volume in the ventral striatum (*39*), thinning of the insula cortex (*38*) and evidence of grey matter abnormalities in stimulant-dependent individuals (*69, 70*). To control for multiple comparisons across voxels, cluster statistics were used. A cluster-forming threshold of $p < 0.005$ was used to generate clusters which were then considered further where $p < 0.05$ (*uncorrected* cluster-level significance). Small clusters (smaller than a 0.5 mm sphere equivalent, c. 130 voxels) were ignored in further mitigation of type I errors.

Laterality effects were included in our MRI analysis. As shown in Fig.3, HI and HC rats showed apparent differences in the laterality of grey matter changes in the nucleus accumbens. However, although there was some overlap in the membership of these two groups, these were not necessarily the same animals. Furthermore, we previously reported using combined VBM and ex-vivo protein analysis that the HI phenotype implicates dendritic spine abnormalities in *both* the left and right nucleus accumbens (*39*). However, in this study, presumed structural abnormalities in the right nucleus accumbens were evidently of an insufficient magnitude to cross the threshold for detection by MRI. We interpret these findings to suggest that impulsive and future compulsive behaviors are shaped by abnormalities in *both* the left and right nucleus accumbens but in an asymmetric way that may vary from one batch of animals to the next.

Functional connectivity analysis

Prior to functional connectivity analysis, voxel dimensions in the header files for both the structural (MT-weighted) and functional images were scaled by a factor of 10 to facilitate processing with software designed for human brain images. Pre-processing steps included 1) averaging of echo times, 2) removal of the first ten volumes, 3) brain extraction, 4) registration, 5) despiking, 6) motion correction, 7) spatial smoothing, and 8) bandpass filtering (*71*). Images were first manually reoriented to match a reference template, as described above. For each volume of the rs-fMRI time-series, the three echo times were averaged (3dcalc, Analysis of Functional Neuroimages, AFNI_16.2.07, https://afni.nimh.nih.gov) (*72*) and the first ten volumes discarded, to reach steady state (fslmaths). To improve registration, non-brain voxels were removed from both structural and functional images. Individually-registered grey, white, and cerebrospinal fluid tissue prior outputs from the VBM pre-processing procedure (above) were combined to obtain a mask excluding non-brain voxels. Following brain extraction, registration of functional images to a reference template was performed using a two-step registration approach. First, the brain-extracted functional image was registered linearly (6 DOF) to the brain-extracted structural (MT-weighted) image (FLIRT (FMRIB Linear Image Registration Tool (*73, 74*). Subsequently, the structural image was registered non-linearly to the template image (FNIRT (FMRIB Nonlinear Image Registration Tool (*31*)). Both linear transformation matrix and nonlinear warp-fields were combined to transform the functional image

into template space to minimise interpolation effects. Registration accuracy was manually checked for each image (**Fig. S7**). Temporal spikes were then removed (3dDespike) followed by motion correction (3dvolreg). Excess motion was calculated through relative framewise displacement (FWD) *via* the methods of Power et al., using a rat brain radius of 8 mm, as previously described (*75, 76*). All animals displayed minimal FWD and therefore none were removed from any formal statistical analysis. Motion parameter outputs from the motion correction procedure were subsequently regressed from the functional images (fsl_regfilt). Spatial smoothing was then applied using an isotropic 4.5 mm kernel (3dBlurInMask) and bandpass filtering was applied (0.01 - 0.1 HZ) (3dBandpass). To assess any residual motion effects, average FWD was regressed against global connectivity (calculated as the average correlation value for each subjects' correlation matrix), regional connectivity (average row-wise correlation value for each subjects' correlation matrix), and edgewise connectivity (correlation between each region-to-region value for each subject). No connectivity measure was related to average FWD (**Fig. S9**). Signal to noise ratio (SNR) and temporal SNR (tSNR) was also calculated for each image, with SNR and tSNR well in line with previously published reports (*71*) (**Fig. S9**). Following pre-processing, region-to-region functional analysis was carried out. The first eigenvariate of the BOLD timeseries was extracted for each region of interest (**Fig. S6**) (fslmeants) and the Spearman's rho correlation coefficient was calculated pairwise between each region of interest (ROI) as a measure of functional connectivity (Fig. S12). Correlation matrices were subsequently used to investigate the relationship between connectivity and behavior. All correlations between ROI BOLD signals were corrected for multiple comparisons with a false discovery rate set at q = 0.05 (*77*).

Data and statistical analyses

Data are presented as means ± 1 SEM, box plots (medians ± 25% and min/max as whiskers) or individual datapoints, and analysed using STATISCA 10 (Statsoft, Palo Alto, USA). Assumptions for normal distribution, homogeneity of variance and sphericity were verified using the Shapiro–Wilk, Levene, and Mauchly sphericity tests, respectively. When these assumptions were significantly violated, the data were log-transformed. Behavioral data were subjected to repeated measures analysis of variance (ANOVA) with time (daily sessions or time bins) or blocks of

sessions (baseline vs punished sessions vs post-punishment recovery for cocaine seeking) and levers (active vs inactive) or response (CS vs magazine approach) as within-subject factor, while groups (ST vs GT, HI vs LI, HK vs LK, HR vs LR and HC vs LC) were used as between-subject factors.

Significant interactions were analysed further using the Newman Keuls *post hoc* test or hypothesis-driven planned comparisons wherever appropriate. For all analyses, significance was set at $\alpha = 0.05$. Effect sizes are reported as partial eta squared values (ηp^2).

Dimensional relationships between behavioral variables were analysed using Pearson's correlations. Descriptive statistical analyses (Factorial analyses) were carried out on variables with inherent theoretical construct such as premature responses during LITI sessions in the 5-CSRTT *(55)*, CS contacts in the autoshaping task *(3, 20)*, the computationally derived variable κ that represents stickiness in the reversal learning task task (*78*) and the total distance travelled in the locomotor reactivity to novelty task *(79)* (**see Table S1 and S2**).

When several variables measured within the same procedure had potentially overlapping variance, they were subjected to dimension reduction. This was the case of the various variables and associated parameters measured in the reversal learning task, namely win-stay, lose-shift, α, β, κ, trials to criterion, perseverative errors, % correct trials and errors to criterion. Here, a first dimensional approach was carried out to identify variables that heavily correlated with one another ($R > 0.5$, $p < 0.05$ as a threshold value) so that only one representative variable was subsequently used in a following factorial analysis. In doing so, α, β, marginally related to one another ($R = 0.42$), but not to κ, were positively correlated with win-stay and lose-shift, respectively. α and β were both negatively correlated with the level of perseverative errors while the former was also negatively correlated with trials to criterion and errors to criterion. Thus, a factorial analysis was carried out on α, β, κ that yielded two factors, one that accounted for α, β (-0.84 each) and one for κ (-0.99), accounting for 47% and 33% of the total variance overall. The outcome of this analysis confirmed that κ can be used as a behavioral measure of stickiness while the individual projection on factor 1 ($\alpha\beta$) was used as the behavioral measure of reinforcement learning in subsequent analyses

All factorial analyses were carried out using a principal component extraction method, with a maximum number of factors set at n-1 where n refers to the number of variables used in the analysis, with a minimum eigenvalue of 1 and varimax rotation.

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Author contributions:

J.W.D, D.B, J.A.J, A.B.R, T.W.R, B.J.E and B.J contributed to the experimental design. J.A.J, L.H, B.J, C.V.S and P.Z contributed to behavioral screening. J.A.J, B.J, P.Z and S.J.S performed the imaging experiments. J.A.J, C.V.S and S.J.S analysed the structural imaging data. J.A.J analysed the functional imaging data. J.A.J, D.B, K.Z, P.Z, A.B.R and M.F performed the analysis of the behavioral data. D.B performed the intravenous catheter surgeries. A.B.R performed self-

administration training with support from J.A.J and B.J. M.F and A.B.R analysed the cocaine self-administration data. M.F and D.B. designed and validated the compulsive drug seeking procedure. M.F. carried out the pain sensitivity experiment on an independent cohort. J.A.J, J.W.D, D.B, T.W.R and B.J.E wrote the manuscript. All authors contributed to editing the manuscript.

Competing interests:

T.W.R is a consultant for Cambridge Cognition, has received grants from GlaxoSmithKline (GSK) and Shionogi Inc; and royalties from Cambridge Cognition. J.W.D has received research grants from GSK and Boehringer Ingelheim Pharma GmbH. D.B has received research funding from Shionogi Inc. B.J.E has received research funding from GSK. The remaining authors declare no competing interest.

Data and materials availability:

All data needed to evaluate the conclusions in the paper can be found at: Zenodo. DOI: 10.5281/zenodo.7118542

Figures

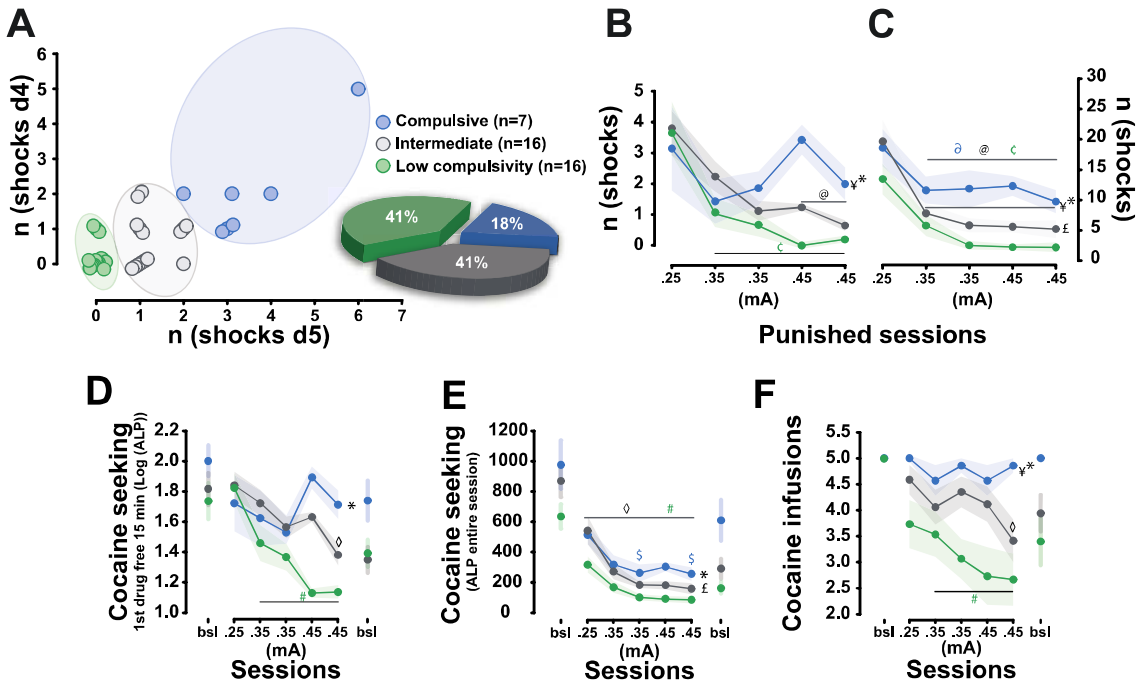


Figure 1. Emergence of a compulsive foraging phenotype in a subpopulation of rats with a long history of cue-controlled cocaine seeking.

(**A**) Rats with a long history of cocaine seeking (n = 39) were identified by clustering as high compulsive (HC), intermediate (int) and low compulsivity rats (LC) based on the number of shocks they tolerated to pursue cocaine seeking over successive daily 15 min drug-free periods. (**B**) Under the threat of punishment, HC rats received more foot-shocks than Int and LC rats during the drug-free seeking periods **(B)** and the total session (**C**) [effect of compulsivity: $F_{2,36} = 3.31$, $p = 0.047$, $\eta p^2 = 0.15$ and $F_{2,36} = 7.59$, $p = 0.0018$, $\eta p^2 = 0.29$, respectively; compulsivity x session interaction: $F_{8,144} = 2.15$, $p = 0.035$, $\eta p^2 = 0.11$ and $F_{8,144} < 1$, respectively]. This willingness to persist in seeking cocaine in the face of punishment was reflected in higher levels of responding on the active lever in HC than LC rats during the first drug-free intervals [$F_{2,36} = 3.29$, $p = 0.048$, $\eta p^2 = 0.15$] (**D**) and the entire sessions (**E**) [$F_{2,36} = 4.18$, $p = 0.023$, $\eta p^2 = 0.19$]. (**F**) HC rats did not decrease their cocaine intake in the face of punishment as LC and Int rats [effect of compulsivity: $F_{2,36} = 4.10$, $p = 0.025$, $\eta p^2 = 0.18$].

n(shocks d4), n(shocks d5): number of shocks on the 4th and 5th punishment session; n(shocks), total number of shocks; mA, milliamperes; ALP, active lever

presses; bsl, baseline session. Newman Keuls post-hoc tests, #, $, ◊: LC, HC, Int different from baseline, $p < 0.05$; ¢; ∂, @: LC; HC, Int different from d1, $p < 0.05$; *, ¥: HC different from LC and Int, $p < 0.05$; £: Int different from LC, $p < 0.05$.

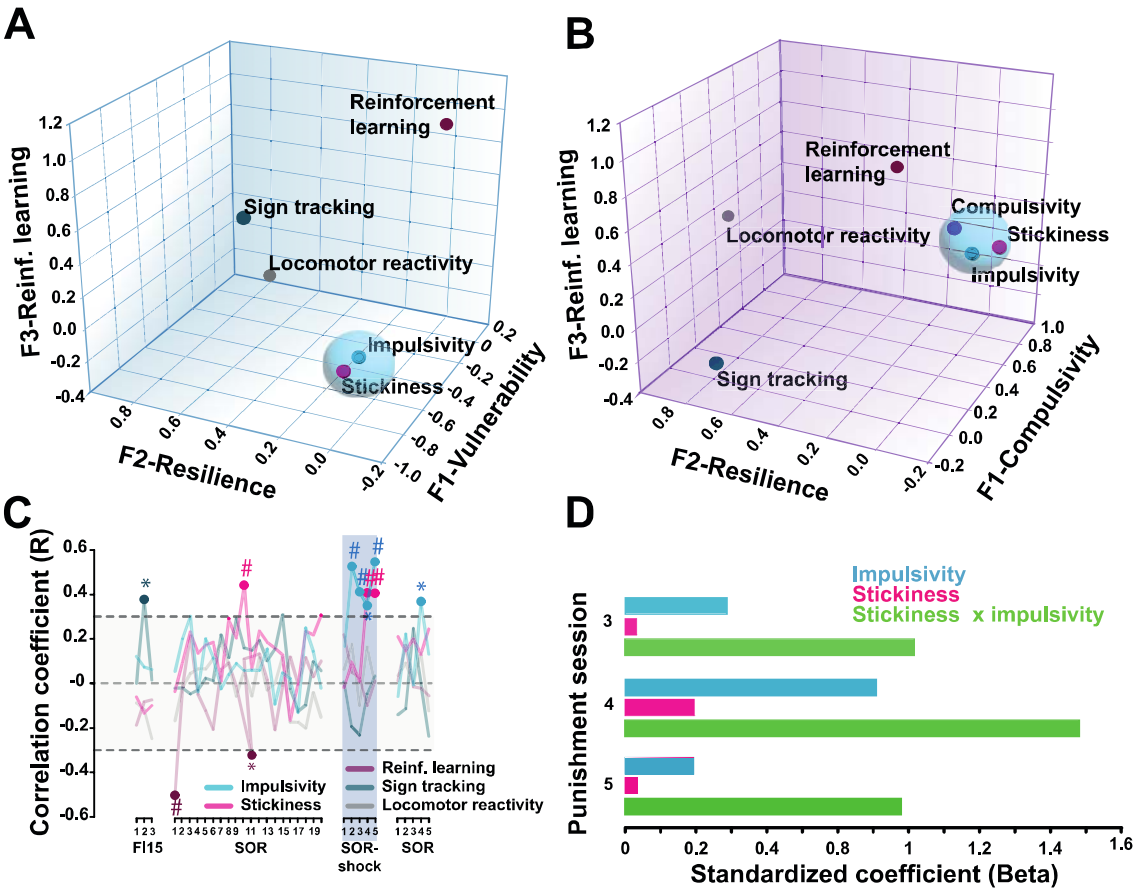


Figure 2. Impulsivity and stickiness interact to confer increased vulnerability to develop compulsive cocaine seeking.

(A) A principal component analysis using locomotor reactivity to novelty, sign-tracking, the factor of reinforcement learning αβ, the stickiness factor κ, as well as impulsivity revealed three overarching factors accounting for more than 72.9% of the total variance (see **SM Table 1** for more details). Impulsivity and stickiness loaded on to factor 1, which represents vulnerability to compulsivity and was orthogonal to factor 2, which, by accounting for sign-tracking and locomotor reactivity-to-novelty, represents resilience to compulsion. Finally, reinforcement learning (αβ) loaded on the third, independent factor. **(B)** When compulsive cocaine seeking was added to the model, it loaded on the same factor as impulsivity and stickiness, while novelty reactivity and sign-tracking remained clustered on an orthogonal factorial capturing resilience, and reinforcement learning (αβ) was accounted for by a third independent factor. (**C**) Further dimensional analyses revealed that impulsivity and stickiness only correlated with cocaine seeking when under punishment, even though κ tended to correlate with cue-controlled cocaine seeking at baseline prior to the introduction of

punishment. This suggests a relationship between stickiness and the tendency to engage in cue-controlled cocaine seeking. Grey area reflects the -.25 - +.25 marginal R value range. **(D)** Factorial regression analysis demonstrated that stickiness (κ) interacted with impulsivity in better predicting compulsive cocaine seeking on each of the last three days of punishment. *#: p < 0.01; *: p < 0.05.* FI15, fixed-interval 15 minutes; SOR, second order schedule of reinforcement.

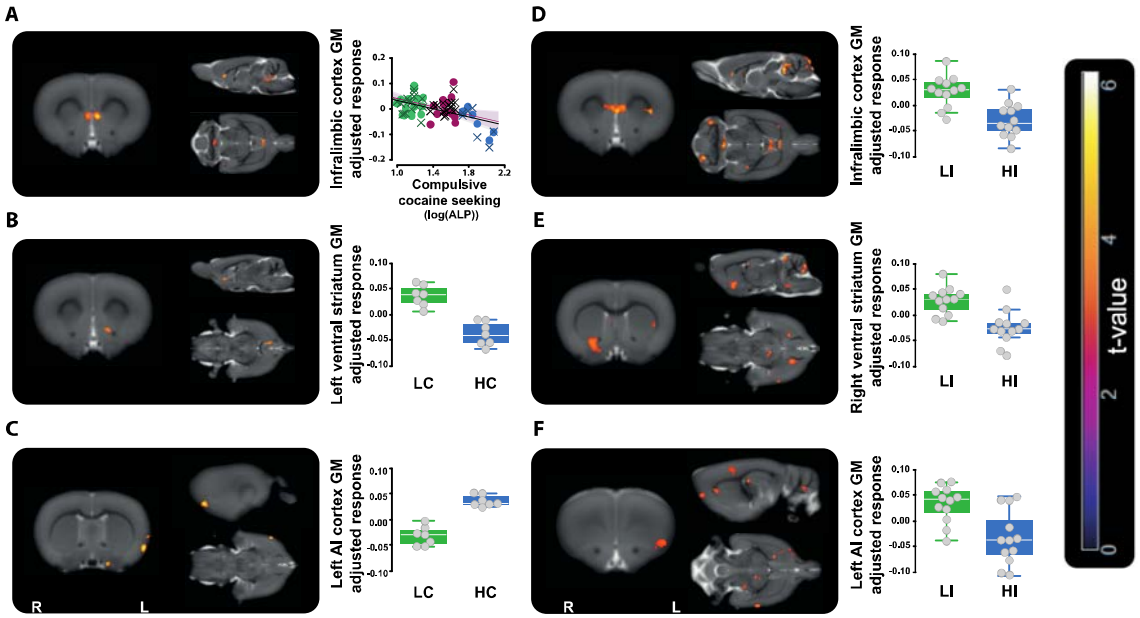


Figure 3. Grey matter alterations in the prefrontal cortex, the insula and the ventral striatum that underlie impulsivity predict compulsive cocaine seeking. Rats that will later compulsively seek cocaine (High-compulsive (HC) rats), showed prior to any drug exposure, lower grey matter density bilaterally in the infralimbic (IL) cortex **(A**), revealed by a negative correlation between compulsive cocaine seeking and the IL cortex density, (IL cortex, (left (•)) rho= -0.386, voxels = 13, cluster-level p = 0.013, (right (**X**)) rho= -0.356, voxels = 130, cluster-level p = 0.014), accompanied by a clear asymmetric distribution of HC and LC rats in the lower and upper terciles of the population ranked on the IL density [4 out of 7 HC rats were in the lower tercile as opposed to 3 LC which instead were predominantly represented in the upper tercile (10/16) which only contained 1 HC, Chi^2: p = 0.0265] and a difference between groups in the left ventral striatum **(B)** [HC *versus* Low-compulsivity (LC) rats, voxels = 135, p = 0.003]. (**C**) Conversely, HC rats show higher grey matter density in the left caudal anterior insula (AI) (HC *versus* LC rats: voxels = 76, p =

0.019). This structural signature overlapped with that of High impulsive rats (HI) rats which, as compared to Low impulsive (LI) rats, showed lower grey matter density bilaterally in the IL cortex **(D)**, in the right ventral striatum **(E)** and the left rostral AI **(F)** [HI *versus* LI: voxels=176, p = 0.005, voxels = 397, p < 0.0001, and voxels = 154, p = 0.008, respectively]. Scatter plot and bar chart represent mean adjusted grey matter density from the significant cluster of interest. R = right hemisphere; L = left hemisphere. GM, grey matter; ALP, active lever presses

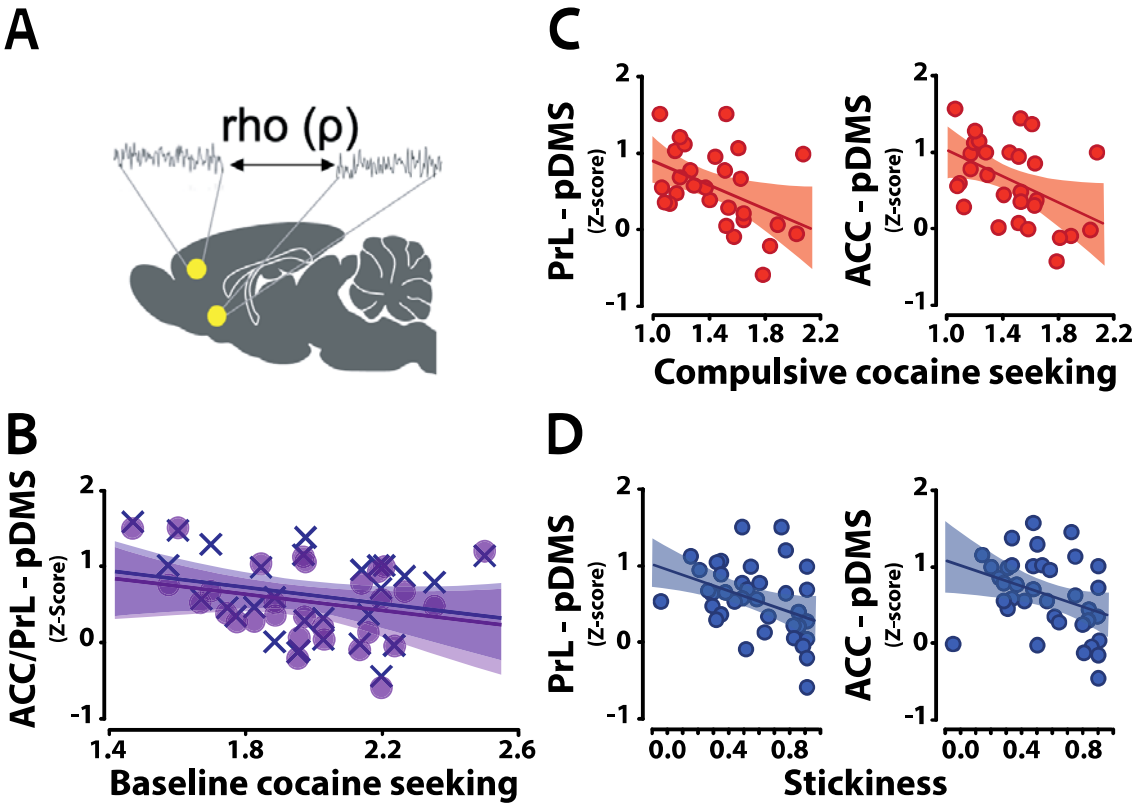
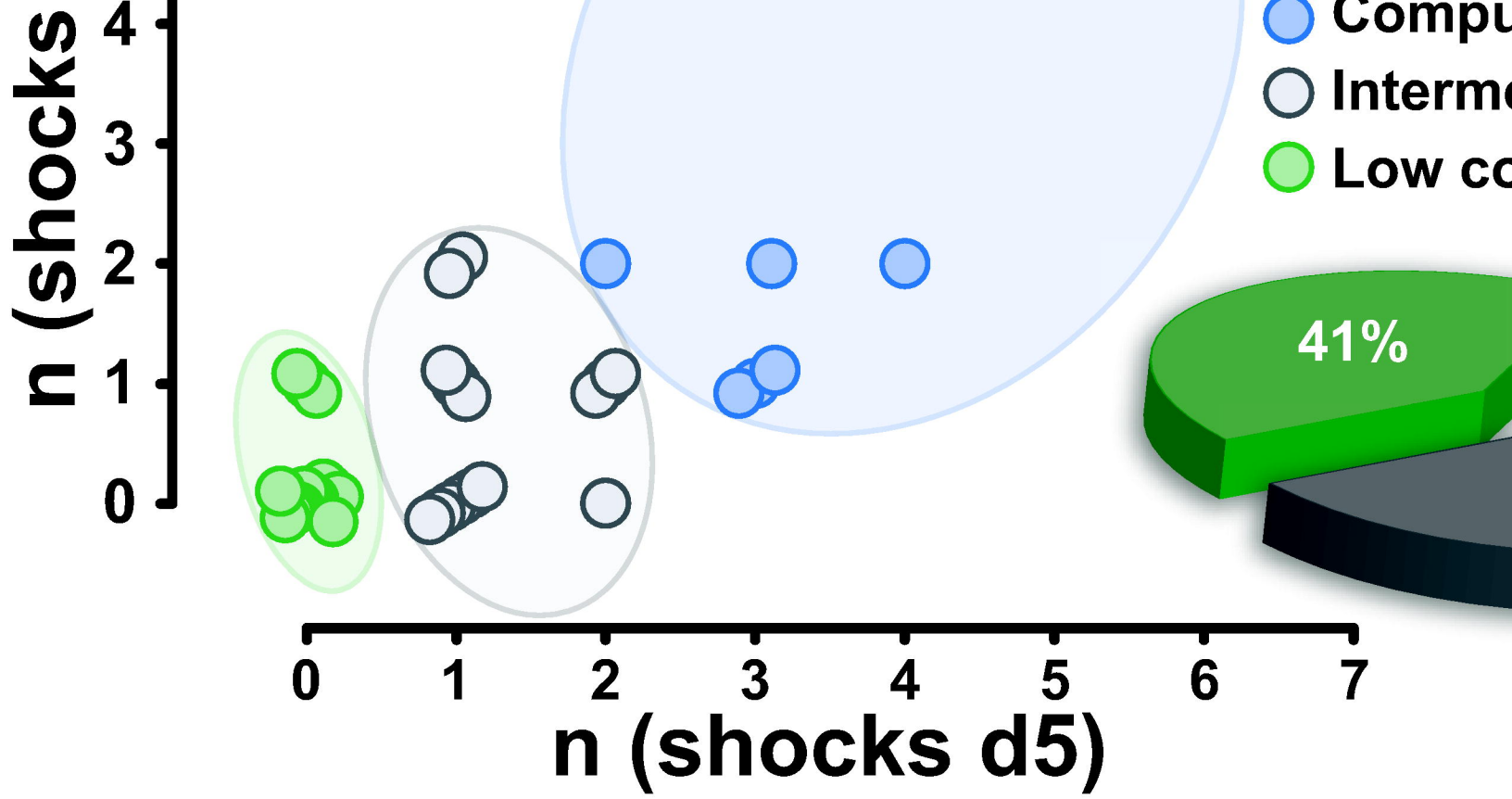


Figure 4. The functional hypoconnectivity of fronto-striatal regions that underlies stickiness predicts the vulnerability to develop compulsive cocaine seeking.

(**A**) Several prefrontal-striatal regions of interest were assessed for their functional connectivity using Spearman's Rho correlation coefficient. The functional connectivity between the anterior cingulate cortex (ACC) (**X**) and prelimbic cortex (PrL) (•) to the posterior dorsomedial striatum (pDMS), which was otherwise not significantly associated with cocaine seeking at baseline (**B**), was in drug naïve rats inversely proportional to the level of subsequent compulsive cocaine seeking [rho = -

0.441, p = 0.038 fdr-corrected and rho = -0.457, p = 0.030 fdr-corrected, respectively] (**C**). The hypofunctionality of these two systems in relation to compulsivity was similar to that observed in relation to stickiness (κ) prior to any drug exposure [rho = -0.463, p = 0.027, fdr-corrected and rho = -0.425, p = 0.048, fdr-corrected, respectively] (**D**). *(80)*


n (shocks
0
1
2
3
4
Compu
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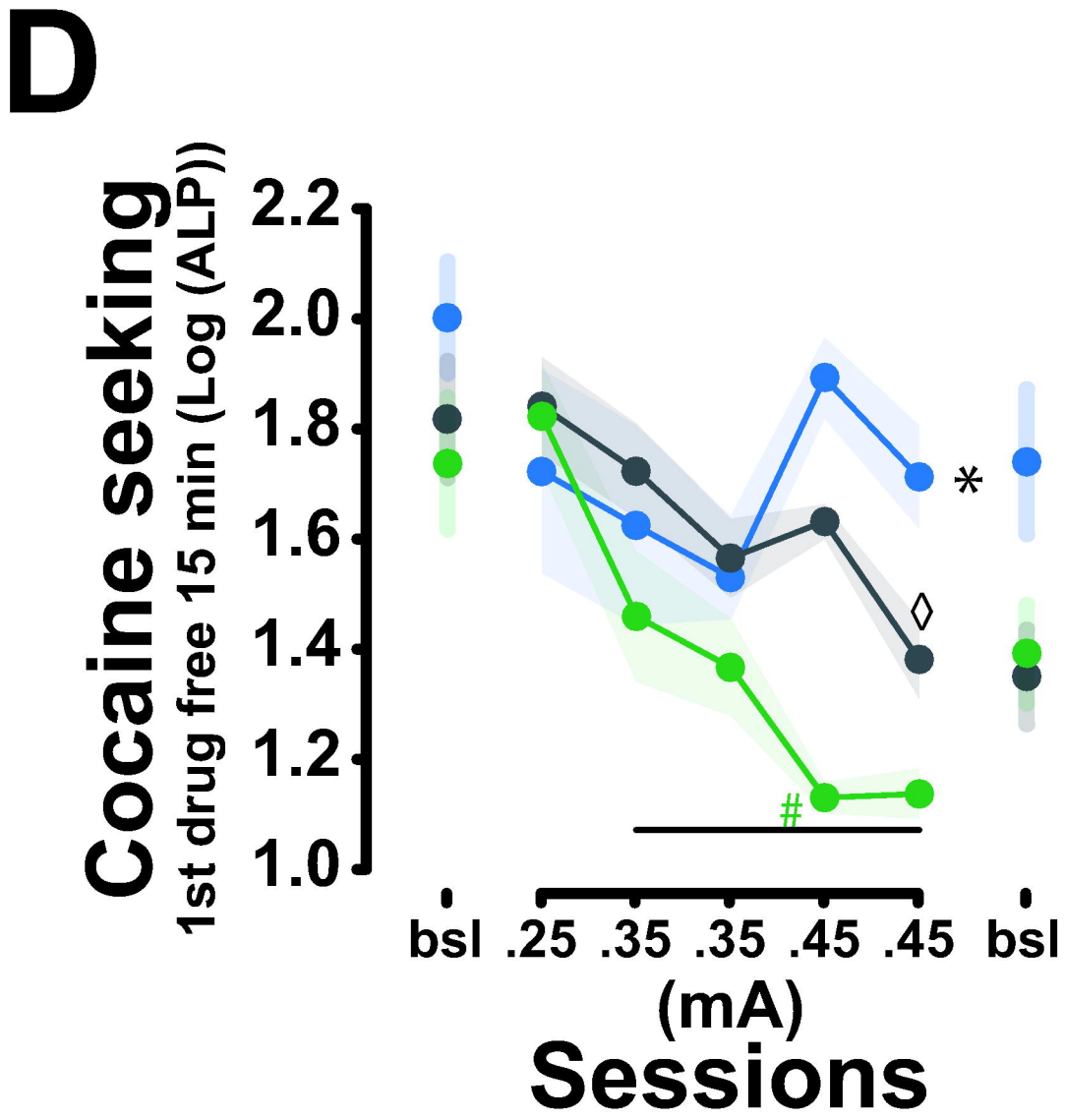
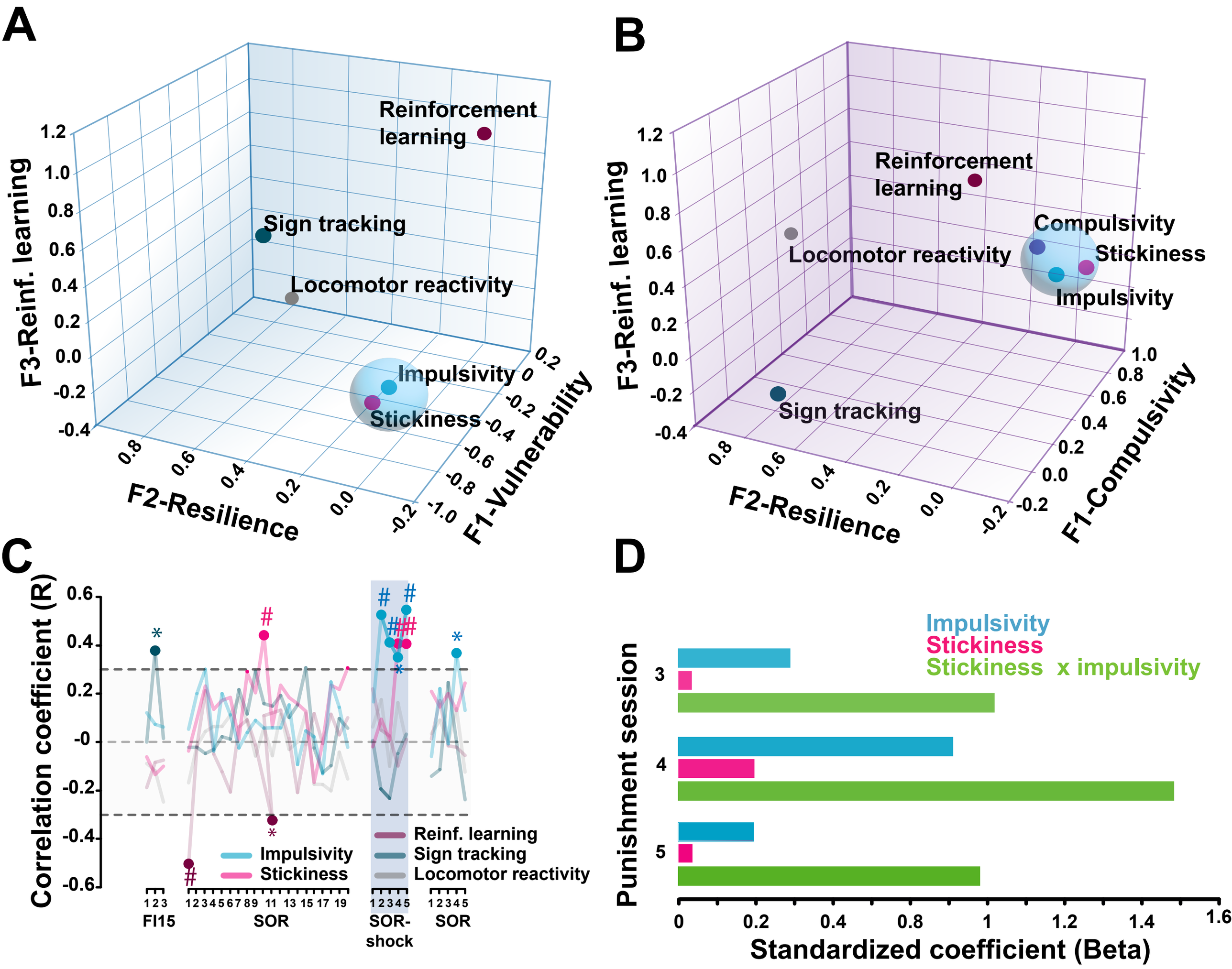
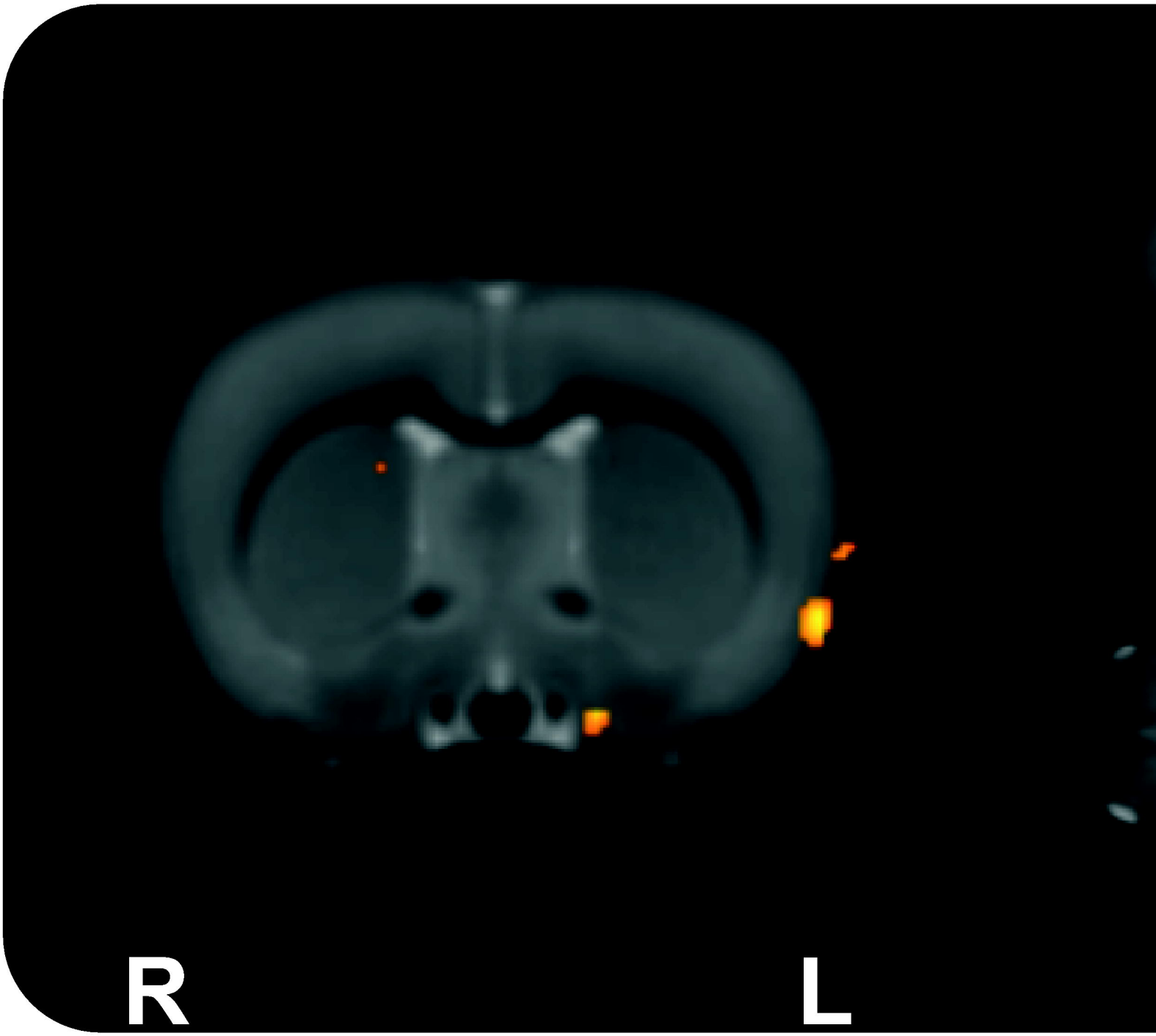
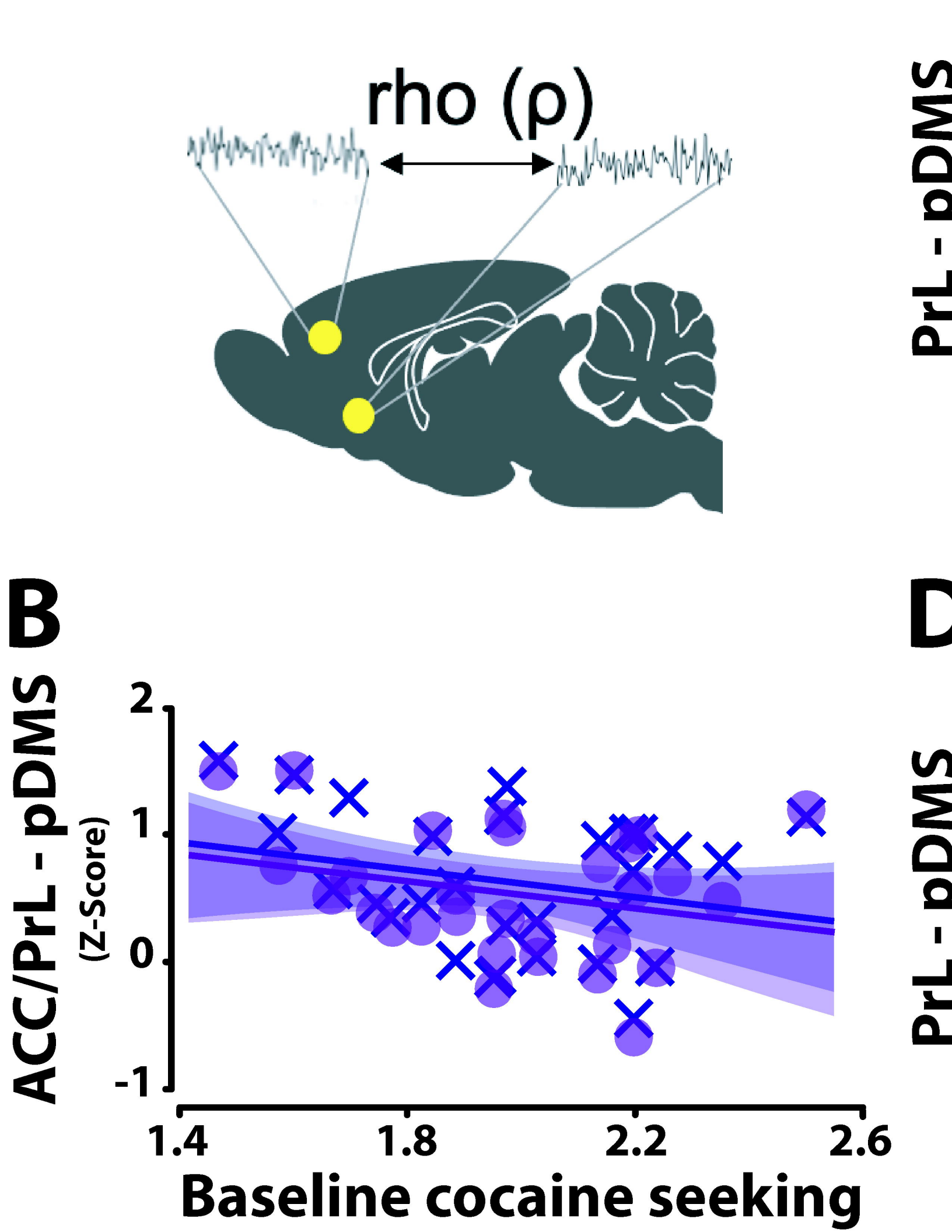

D
Cocaine seeking
1st drug free 15 min (Log (ALP))
2.2
2.0
1.8
1.6
1.4
1.2
1.0
*
◊
#
bsl
.25
.35
.35
.45
.45
bsl
(mA)
Sessions


A
Reinforcement learning
Sign tracking
Locomotor reactivity
Impulsivity
Stickiness
F3-Reinf. learning
F2-Resilience
F1-Vulnerability
B
Reinforcement learning
Compulsivity
Locomotor reactivity
Stickiness
Impulsivity
Sign tracking
F3-Reinf. learning
F2-Resilience
F1-Compulsivity
C
Correlation coefficient (R)
Impulsivity
Stickiness
Reinf. learning
Sign tracking
Locomotor reactivity
FI15
SOR
SOR-shock
SOR
D
Impulsivity
Stickiness
Stickiness x impulsivity
Punishment session
Standardized coefficient (Beta)

C





rho (ρ)
PrL - pDMS
B
D
ACC/PrL - pDMS
(Z-Score)
2
1
0
-1
1.4
1.8
2.2
2.6
Baseline cocaine seeking
PrL - pDMS