Supporting Online Material for

Transition to Addiction Is Associated with a Persistent Impairment in Synaptic Plasticity

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Supporting Online Material

Material and Methods

Subjects

Male Sprague-Dawley rats weighing 280-300 g at the beginning of the experiments were used. Rats were single housed under a 12 hr reverse dark/light cycle (on 20h00, off 8h00). Temperature (22 ± 1°C) and humidity (60 ± 5%) were also controlled.

Behavioral studies

Drugs: Cocaine HCl (Coopération Pharmaceutique Française, Bordeaux, France) was dissolved in 0.9% NaCl. Ketamine (Imalgène®) and xylazine (Rompun®) were mixed for anesthesia. Gentamicine (Gentalline®) was dissolved in 0.9% NaCl.

Surgery: A silastic catheter (internal diameter = 0.28 mm; external diameter = 0.61 mm; dead volume = 12µl) was implanted in the right jugular vein under ketamine (100 mg/kg) + xylazine (1 mg/kg) anesthesia, according to a procedure previously described (*S1*). Rats were allowed to recover for 5 to 7 days after surgery. During the first 4 days following surgery, rats received an antibiotic treatment (gentamicine 1 mg/kg i.p.). After surgery, catheters were flushed daily with a saline solution containing unfractionated heparin (100 IU/ml).

Self-administration apparatus: The self-administration (SA) setup consisted of 48 SA chambers made of Plexiglas and metal (Imetronic, Pessac, France). Each chamber (40 cm long x 30 cm width x 52 cm high) was located within an opaque sound proof box equipped with exhaust fans that assured air renewal and masked background noise. Briefly, each rat was placed daily in a SA chamber and the intracardiac catheter connected to a pump-driven syringe (infusion speed: 20 µl/sec). Two holes, located at opposite sides of the SA chamber placed at 5 cm from the grid floor, were used to record instrumental responding. A white house light at the top of the chamber allowed its complete illumination. A white cue light (1.8 cm in diameter) was located 9.5 cm above the active hole. A green cue light (1.8 cm in diameter) was located 10 cm right to the white cue light. A blue cue light (1.8 cm in diameter) was located on the wall opposite to the one containing the active hole at 33 cm from the floor on the left side. Experimental contingencies were controlled and data collected with a PC Windows-compatible software (Imetronic, Pessac, France).

Self-administration procedures

Intravenous self-administration (SA): basal training protocol

The daily SA session was composed of three drug components (40 min each) separated by two 15 min periods during which responding in the active hole had no scheduled consequences (non-drug periods). Drug periods were signaled by the blue cues light, while the non-drug periods were signaled by illumination of the entire SA box and extinction of the blue cue light. During the non-drug periods, nose-pokes were without scheduled consequences. During the drug periods, introduction of the rat's nose into one hole (active device) turned on the white cue light located above it and then, 1 sec later, switched on the infusion pump. The cue light remained on for a total of 4 sec. Nose-pokes in the other hole (inactive device) had no scheduled consequences. The self-infusion volume was 40 µL (2 sec infusion) and

contained 0.8 mg/kg of cocaine. Each infusion was followed by a time-out period of 40 sec. During the first 6 days, a Fixed Ratio 3 (FR3) schedule of reinforcement (i.e. three nose-pokes resulted in an infusion of 0.8 mg/kg of cocaine) was applied. Then, the FR was increased to 5 for the rest of the experiment. Criterion for acquisition of cocaine SA was defined by a stable number of self-infusions over at least three consecutive SA sessions (± 10%).

Measurements of addiction-like behaviors

Persistence of drug seeking in the absence of cocaine: It was assessed daily by measuring the responses in the active hole during the two non-drug periods of the basal training. Active responses during the two daily non-drug periods were summed. For analysis, the mean responses over two to three consecutive sessions were used as dependent variables.

Progressive-ratio schedule: The ratio of responses per infusion was increased after each infusion according to the following progression (10, 20, 30, 45, 65, 85, 115, 145, 185, 225, 275, 325, 385, 445, 515, 585, 665, 745, 835, 925, 1025, 1125, 1235, 1345, 1465, 1585), the last ratio completed, called the breakpoint, was used to measure motivation for cocaine.

Resistance to punishment: During these sessions rats were placed for 40 min in the SA chamber. The blue cue light signaling drug availability was on. The schedule was the following: The first response led to the illumination of the green cue light signaling the presence of the shock. After 3 additional responses, rats received an electric shock (0.8 mA, 2 sec), and after the 5th response, rats received both an electric shock (0.8 mA, 2 sec) and a cocaine infusion (0.8 mg/kg), associated with the corresponding conditioned stimulus (white cue light). Then the green cue light was turned off. The schedule reinitiated at the end of the time-out period, i.e. 40 sec after the infusion. If, within a minute, rats did not complete response requirements leading to shock and shock plus infusion respectively, the green cue light turned off and the sequence was reinitiated, i.e. the following response turned on the green cue light. The number of active responses earned during this test was expressed in percentage of the number of active responses performed, over the same period of time, during the basal training session conducted the day before.

Establishment of Addict and non-Addict groups. A rat was considered positive for an addiction-like criterion when its score for this behavior was in the 35% highest percentile of the distribution. Four groups of rats were isolated depending on the number of positive criteria met (0crit, 1crit, 2crit and 3crit). 0 and 3 criteria rats were defined as non-Addict and Addict animals respectively.

Addiction score. It was calculated as the algebraic sum of standardized scores of each of the three addiction-like criteria (S*1*). Standardization consisted in subtracting the mean of the group to each individual score and then dividing this number by the standard deviation. This procedure yields scores which have a mean of 0 and a standard deviation of 1. The addiction score is thus distributed along a scale from -3 to 3.

Establishment of Addiction Vulnerable and Addiction Resistant groups. We previously showed that the pattern of use after 25 to 30 days of cocaine SA predict vulnerability to addiction (S*1*). Here our goal was to identify an earlier behavioral feature which could detect future Addict animals (3 positive criteria) as early as 17 days of SA. We focused on the addiction criterion that we measure during every basal SA session, i.e. the persistence in active

responses during the non-drug periods and performed a meta-analysis on two previous experiments conducted on 71 rats. The protocol applied in these experiments was similar to the one previously described (S*4*). Using a regression analysis we correlated the addiction score after late training (60 to 70 SA sessions) with the active responses during the non-drug periods from sessions 1 to 29. The addiction score was positively correlated with non-drug responses from sessions 13. Analyzing responding during these early non-drug periods, we identified a pattern of responses, which allows differentiating Addict from Non-Addict rats. Indeed, starting from sessions 11-13, Addict rats tended to progressively increase responding during the last part of the non-drug periods (figS4A). This pattern suggests the early development of an anticipatory response to the next drug period in future Addict rats. Then we computed several variables characterizing responding during these early non-drug periods and performed a factorial analysis which included the addiction score. This allowed isolating four variables which loaded heavily (more than 60%) on the same factor with the addiction score. These variables were: the number of active responses during the last 9 minutes averaged over sessions 15 to 17 (Last 9min J15-J17); 2. The number of active responses during the first 6 minutes averaged over sessions 15 to 17 (First 6min J15-J17); 3. The difference between the first and the second variable (Last9min J15 to J17 - First6min J15 to J17); 4. The difference between the number of active responses during the last 9 minutes averaged over sessions 15 to 17 and the number of active responses during the last 9 minutes averaged over sessions 1 to 5 (Last9min J15 to J17 - Last 9min J1 to J5). Rats were then ranked for these four variables. Rats with a score in the 20th highest percentile of the population were defined as positive for this predictive criterion. Rats with 4 positive criteria were called *Addiction Vulnerable* rats, whilst rats with 4 negative criteria were called *Addiction Resistant* rats. The addiction score of *Addiction Vulnerable* and *Addiction Resistant* rats was very different after late training [ANOVA, group effect, $F(1,17)=22.44$, $p<0.0001$] and similar respectively to the one of the Addict and non-Addict groups constituted as described in the previous paragraph on the basis of the number of positive addiction-like behaviors evaluated after two months of self-administration (FigS4B).

Electrophysiological Studies

Slice preparation: These methods were described in detail previously (S*2*). Briefly, rats were anesthetized with a mixture of ketamine (100 mg/kg) + xylacine (1 mg/kg) and decapitated. The brain was sliced (300 µm) in the coronal plane using a vibratome (Integraslice, Campden Instruments, Loughborough, UK). Slices were maintained in a sucrose based physiological solution at 4° C (in mM: 87 NaCl, 75 sucrose, 25 glucose, 5 KCl, 21 $MgCl_2$, 0.5 $CaCl_2$ and 1.25 NaH_2PO_4). Immediately after cutting, slices were stored for 40 min at 32°C in a low calcium artificial cerebrospinal fluid (low Ca - ACSF) (in mM): 130 NaCl, 11 Glucose, 2.5 KCl, 2.4 $MgCl_2$, 1.2 $CaCl_2$, 23 $NaHCO_3$, 1.2 NaH_2PO_4, and were equilibrated with 95% O_2/5% CO_2. Slices were then stored in low Ca - ACSF at room temperature until recording. For recording, slices were placed in the recording chamber and superfused (1.5 - 2 ml/min) with ACSF (same as low Ca – ACSF with the following exception: 2.4 mM $CaCl_2$ and 1.2 mM $MgCl_2$). All experiments were performed at 28-

30°C. The superfusion medium contained picrotoxin (100 µM) to block GABAA receptors. All drugs were added at the final concentration to the superfusion medium.

Drugs: Picrotoxin was from SIGMA (St. Quentin Fallavier, France). LY 379268 was from Tocris (Bristol, UK). DNQX and AP-5 were either from Tocris (Bristol, UK) or Ascent Scientific (Bristol, UK).

Patch Clamp and Extracellular Recording Procedures: Whole cell patch-clamp recordings were performed from visualized medium spiny neurons (MSN) located in the NAC core. Glass electrodes (resistance 4-6 MOhms) were filled with either Cesium Methane-Sulfonate or K+Gluconate based solutions, as follows (mM): 128 Cesium Methane-Sulfonate (CH3O3SCs) or K+Gluconate, 20 NaCl, 1 MgCl2, 1 EGTA, 0.3 CaCl2, 2 Na2+ATP, 0.3 Na+ GTP, buffered with 10 Hepes, pH 7.3, osmolarity 290-300 mOsm. In order to evaluate the access resistance (Ra), a 2 mV hyperpolarizing pulse was applied before each EPSC. Ra was not compensated and cells were rejected if Ra was >25 MOhms or changed >20% during the experiment. The potential reference of the amplifier was adjusted to zero prior to breaking into the cell. Data was recorded with an Axopatch-200B amplifier (Molecular Devices, Sunnyvale, USA), filtered at 1-2 kHz, digitized at 10 kHz on a DigiData 1332A interface (Molecular Devices, Sunnyvale, USA) and collected on a PC using Clampex 10. Analysis was performed using Clampfit 10 (Molecular Devices, Sunnyvale, USA). For field excitatory post-synaptic potential (fEPSP), extracellular recording electrodes were filled with ACSF. To evoke synaptic currents, stimuli (100 µsec duration) were delivered at 0.067 Hz with a glass electrode filled with ACSF and placed at a distance > 150 µm in the dorsomedial direction.

sEPSC recordings: Spontaneous EPSCs (sEPSC) were recorded in the whole cell voltage-clamp configuration using Axoscope 10 (Molecular Devices, Sunnyvale, USA). sEPSC recorded at -70 mV were detected using a template of sEPSC generated from averaging several typical synaptic events with Clampfit 10 (Molecular Devices, Sunnyvale, USA). The template was slid along the data trace one point at a time. At each position, this template was optimally scaled and offset to fit the data. A lower amplitude threshold of 6 pA was applied, equivalent to 2.5 times the standard deviation of baseline noise.

LTD recordings: NMDAR-dependent LTD in MSN was induced, after 10 minutes of stable baseline recording, using a pairing protocol that consisted of three trains of stimulus at 5 Hz (train duration: 3 min, 5 minutes apart) while holding the cells at – 50mV (S*3*). The magnitude of NMDAR-LTD was estimated from EPSC recorded after 20-30 minutes LTD induction as percentage of baseline EPSC amplitudes. mGluR 2/3-dependent LTD was induced, after a stable baseline recording of fEPSP, by applying the specific mGluR 2/3 agonist LY 379268 (100 nM) for 10 minutes. The magnitude of mGluR2/3-LTD was estimated from fEPSP recorded after 30-40 minutes of washing out the drug. At the end of the experiments, the glutamatergic nature of the fEPSP was confirmed from its block with the AMPA receptor antagonist DNQX (20 µM).

Experimental timeline

First experiment: *Differences between Addict and non-Addict animals in LTD (Fig.1 and 2)*. Five to 7 days after surgery, rats were either tested for

intravenous cocaine SA (N=96) or left undisturbed (Controls) in the animal house (N=11). SA sessions were performed between 10:00 and 16:00 hrs. A progressive ratio was conducted on session 40 and resistance to punishment was tested on session 45. The mean of the total "active" responses during the "non-drug" periods of sessions 37 to 39 were also considered. Addiction severity was evaluated on the bases of the scores of these three addiction-like behaviors. After five additional basal training sessions, and 24 hours after their last SA session, using *in vitro* electrophysiology, non-Addict (N=8) and Addict animals (N=8) as well as control (N=11) rats started being tested for the expression of two forms of synaptic plasticity in the NAC (NMDAR-LTD and mGluR2/3-LTD) (one rat per day). Non-Addict, Addict and control rats were alternatively tested. Basal SA training was maintained for all animals until they were tested for electrophysiology. *Self-administering rats (Addicts and Non-Addicts) were tested for LTD between the 50th and 72nd self-administration sessions.* Persistence in responding during the non-drug periods was followed on a daily basis to control for the maintenance of the non-Addict/Addict status. *Over these 22 days, 8 0crit, 8 3crit and 6 control rats were alternated. To minimize differences in cocaine exposure between the first and the last tested self-administering rats, the 5 additional controls were tested for LTD, before or after the first and last self-administering rats were tested.*

Second experiment: *Changes in LTD at different SA time points (Fig.3A)*. For this experiment the SA procedures were identical to the ones used in experiment one. This time the animals were studied for NAC NMDAR-LTD 24h after either the 7th (N=5) or the 17th (N=5) SA sessions. Rats tested for saline SA for either 7 (N=4) or 17 (N=4) SA sessions were used as control groups. For these experiments animals were trained with saline instead of being left undisturbed in the animal house in order to verify if repeated exposures to the self-administration cage could modify synaptic plasticity. Both groups showed a non-impaired NMDAR-LTD and did not differ. Thus their results were pooled. In order to obtain the same time point within groups, the start of the SA session of each animal was scheduled appropriately. The number of active responses during the non-drug periods was evaluated at days 6 and 7 for rats tested during 7 SA sessions (Cocaine 7 days) and at days 15 to 17 for rats tested during 17 SA sessions (Cocaine 17 days) (FigS3).

Third experiment: *Changes in NMDAR-LTD in Addiction Vulnerable and Addiction Resistant rats (Fig.3B)*. For this experiment, the SA procedures were identical to the ones used in experiments one and two. This time, animals (n=44) trained for cocaine SA were studied for NAC NMDAR-LTD 24 hours after the 17th SA session and after being classified as Addiction Vulnerable (N=6) or Addiction Resistant (N=7). Rats of matching age and purchase, left undisturbed in the animal house, were used as controls (N=11). In order to obtain the same time point within groups the start of the SA sessions of each animal was scheduled appropriately.

Statistics

All values are given as mean ± S.E.M. For SA data, one-way or repeated measures analysis of variance (ANOVA) was used to determine possible group effects and interactions [experimental groups (Controls, Addict, Non-Addict, Cocaine 7 days, Cocaine 17 days, Addiction Vulnerable, Addiction

Resistant) were used as between-subject factor and time as within-subject factor]. Significant main effects or interactions were explored by a pair wise comparison of means using the Newman Keuls post hoc test. For electrophysiological experiments, N corresponds to the number of individual cells analyzed, with at least 5 animals included in each condition. When comparing two groups, a t-test or the nonparametric Mann Whitney test was used. To determine whether LTD was successfully induced in any given group, a paired Wilcoxon signed rank test was performed comparing the mean EPSC or fEPSP amplitude during baseline and 20-30 minutes after LTD induction. Kolmogorov-Smirnov test was used for the statistical comparison of the sEPSC distributions. Pearson's correlation analysis was used to assess the relationship between the addiction score and the amplitude of NMDAR-LTD. Statistical tests were performed with GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) for the electrophysiological data and Statistica 6.0© (StatSoft Inc, Tulsa, OK, USA) for the behavioral data and the correlation analysis. A critical probability of $p < 0.05$ was applied.

Supporting References

S1. D. Belin, E. Balado, P. V. Piazza, V. Deroche-Gamonet, *Biol Psychiatry* **65**, 863 (May 15, 2009).

S2. F. Kasanetz, O. J. Manzoni, *J Neurophysiol* **101**, 2516 (May, 2009).

S3. M. Martin, B. T. Chen, F. W. Hopf, M. S. Bowers, A. Bonci, *Nat Neurosci* **9**, 868 (Jul, 2006).

S4. V. Deroche-Gamonet, D. Belin, P. V. Piazza, *Science* **305**, 1014 (Aug 13, 2004).

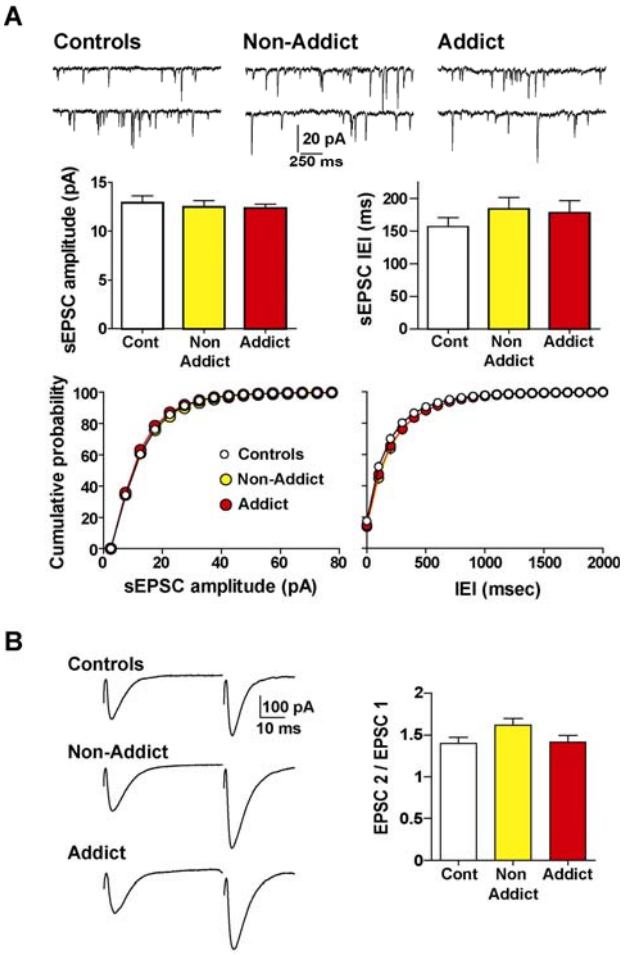

Fig. S1: Basic parameters of synaptic transmission were not changed in Addict and non-Addict rats. **A.** Spontaneous synaptic transmission in the NAC was similar between Controls (or Cont) (N=16), Non-Addict (N=11) and Addict (N=15). Top: typical traces of 4 consecutive seconds of recording are depicted (top). Middle: average data of median sEPSC amplitude and inter-event interval (IEI) showing no differences between groups (One-way ANOVA: amplitude: $F(2,39)=0.22$, $p=0.8$; IEI: $F(2,39)=0.74$, $p=0.48$). Bottom: Cumulative probability distribution of sEPSC amplitude and IEI was similar between groups. **B**. The expression of short-term synaptic facilitation was not associated with addiction. Left: two consecutive stimuli delivered at 20 Hz induced a facilitation of the second response in Controls (N=14), Non-Addict (N=17) and Addict (N=16). Typical traces of EPSC responses are depicted. Stimulation artifacts were removed. Right: Average data showing that the magnitude of paired-pulse facilitation, expressed as the ratio between the two EPSCs, was not different between groups (One Way ANOVA, $F(2,44)=2.58$, $p=0.09$).

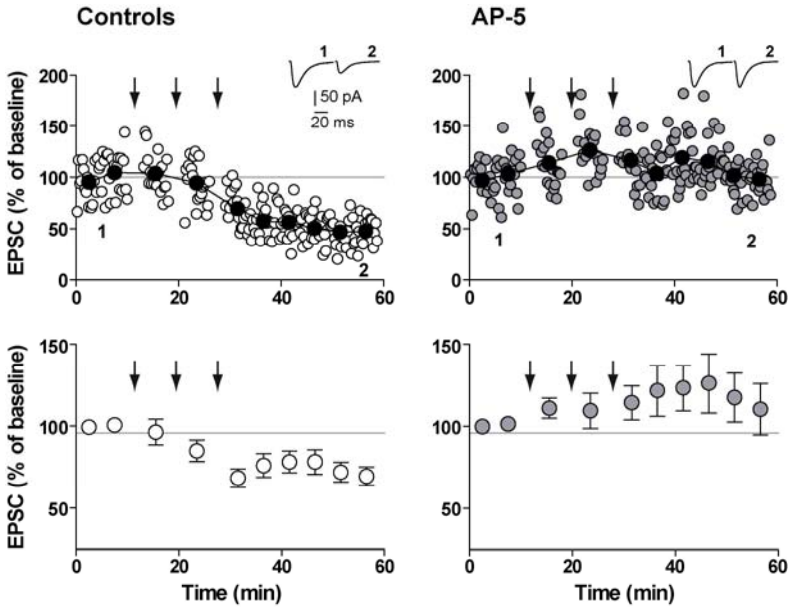

Fig. S2: LTD induced with a pairing protocol was NMDAR (NMDA receptor)- dependent. Individual experiments and average data obtained from control slices and slices preincubated with the NMDAR antagonist DL-AP5 (100 µM) are shown (LTD in % of baseline; control: 70.9 ± 5.4 [N=9, W=45, p<0.005]; AP-5: 113.9 ± 14.7 [N=7, W=-12, p=0.37]). Traces represent the average of 30-40 EPSC induced during baseline (1) and after 20-30 minutes LTD induction (2). Arrows indicate time of pairing stimulation. Stimulation artifacts were removed. Horizontal lines indicate baseline levels. This experiment was performed in adult rats of age and purchase matching with those of control rats described in the second experiment. They were left undisturbed in the animal house. Experiments with slices pre-incubated with AP-5 were interleaved with control slices.

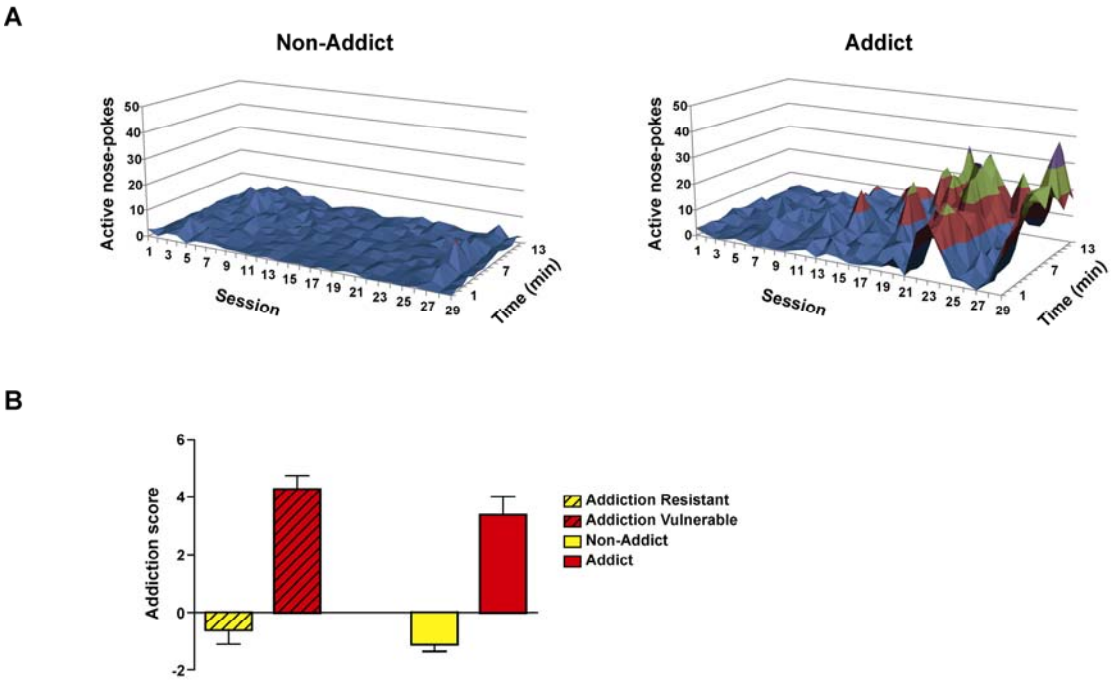

Fig. S3: Early identification of Addiction Vulnerable and Resistant rats: a metaanalysis study. A. Pattern of responding during the non-drug periods from sessions 1 to 29 in Addict (n=13) and Non-Addict rats (n=25). Results are expressed as mean responding per minute over the two daily non-drug periods. Rats were trained for cocaine SA over 60-70 SA sessions and classified as Addict and Non-Addicts. Persistence in responding was retrospectively analyzed. After early training (starting around sessions 11-13), future Addicts tend to progressively increase responding during the last part of the non-drug periods. Animals were then classified in *Addiction Vulnerable* and *Addiction Resistant* on the basis of four variables measuring the pattern of responding during the non-drug periods between day 15 and day 17 of SA. **B.** Addiction score calculated after 50-70 cocaine SA sessions in Addiction Vulnerable and Addiction Resistant rats and in rats classified as Addict and Non- Addict at the end of the SA experiments. The addiction scores of the two predicted groups were very different after 50-70 days of cocaine SA [ANOVA, group effect, $F(1,17)=22.44$, $p<0.0001$]. Similarly the addiction scores of the Addict and Non-Addict rats were statistically different [ANOVA, $F(1,39)=64.17$, $p<0.0001$]. However, the addiction score of Addiction Resistant and Non-Addict as well as the one of Addiction Vulnerable and Addicts did not differ.

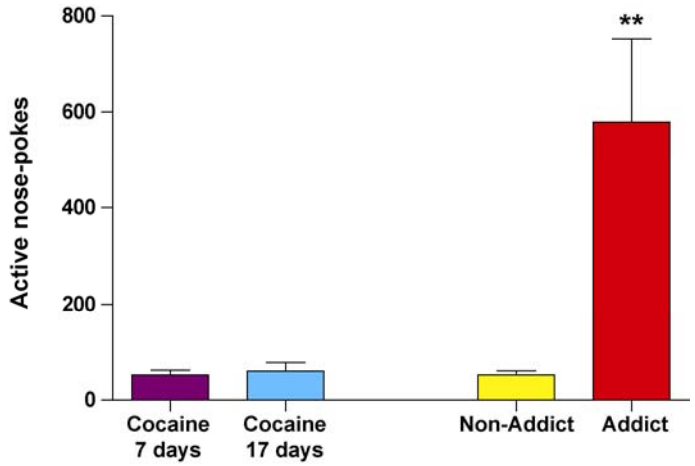

Fig. S4: Addiction-like behavior was not developed after early cocaine training. Difficulty to limit drug seeking was evaluated by the persistence in responding (active nose-pokes) during the non-drug periods measured during the daily basal training protocol. Persistence in responding (mean active nose-pokes during the non drug periods over sessions 5-7, 15-17 and 37-39 respectively) after 7 days of cocaine SA (Cocaine 7 days), after 17 days of cocaine SA, in Non-Addict and in Addict rats after approximately 40 days of cocaine SA. After early training, rats show a low level of responding comparable to the one exhibited by Non-Addict rats after 40 cocaine SA sessions and lower than the one exhibited by Addict rats [ANOVA, group effect, $F(3,22)=6.57$, $p<0.005$]. ** $p<0.01$, Addict vs. all other groups (NK post-hoc test).