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Cocaine-induced structural plasticity in frontal cortex correlates with conditioned place preference

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Abstract

Contextual cues associated with previous drug exposure can trigger drug craving and seeking, and form a significant obstacle in substance use recovery. Using in vivo imaging in mice, we found that cocaine administration induced a rapid increase in the formation and accumulation of new dendritic spines, and that measures of new persistent spine gain correlated with cocaine conditioned place preference. Our data suggest new persistent spine formation in the frontal cortex may play a role in stimulant-related learning driving appetitive behavior.

The rodent dorsomedial prefrontal cortex (dmPFC) has previously been shown to support appetitive responses to cocaine-paired stimuli and behavioral sensitization to cocaine[1,2]. Anatomically, the dmPFC is particularly well-poised to integrate information from sensory and memory systems and modulate behavior. Changes in the connectivity of layer 5 neurons in this area could directly impact the output of the frontal cortex to the basal ganglia and other downstream subcortical circuits known to play a role in action selection[3,4].

At a structural level, repeated daily exposure to stimulants has been shown to increase dendritic spine density in layer 5 pyramidal neurons of rodent dmPFC after weeks of withdrawal[5–7]. However, little is known about the timing and dynamics of these plastic events and their potential relationship with behavioral changes induced by stimulant exposure. More detailed knowledge of cocaine-induced structural plasticity could enhance understanding of the mechanisms supporting drug associations that fuel substance use disorders.

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AUTHOR CONTRIBUTIONS

F.J.M.-C., J.A. and D.P. performed the experiments. L.W. and F.J.M.-C. designed the experiments, analyzed the data and wrote the paper.

To address these questions, we used *in vivo* 2-photon imaging via a cranial window in *Thy1* YFP-H transgenic mice[8] to follow structural changes in dendritic spines in the apical dendrites of layer 5 neurons in the dmPFC before and after cocaine exposure. In our first experiment, we imaged neurons in the dmPFC every 48 hours for up to 32 days (Fig.1a). After a baseline period, i.p injections of either cocaine (15mg/kg) or saline were given daily for 12 days and the imaging continued for a 2 week withdrawal period. Additionally, we also measured locomotor behavior in response to drug-administration on the days that animals were not being imaged (Fig. 1a). We found that cocaine-treated mice showed significantly greater spine gains than saline-treated controls ($p<0.001$), most notable after the first day of treatment (Fig. 1d). In contrast, there was no effect of treatment on spine loss ($p>0.6$; Supp. Fig. 1b). During the withdrawal phase, we observed no significant effect of prior cocaine treatment on the fraction of spines gained or lost ($p>0.7$; Fig. 1e and Supp. Fig. 1c).

Over the course of the treatment and withdrawal period, we found that spine density diverged between saline- and cocaine-treated groups (Fig. 1f). This divergence was explained by the greater accumulation of new spines in cocaine-treated mice (Fig. 1g) and enhanced survival of stable spines present before the treatment onset in the cocaine group (Supp. Fig. 1e). Cocaine had no effect on average survival of new spines measured every 48 hours after treatment onset (Supp. Fig. 1f,g). Declines in spine density in 2–3 month old mice, similar to that observed in our saline control mice (Fig. 1f), have been observed in other *in vivo* imaging studies in various cortical regions[9–11]. Naïve tissue studies suggest these declines represent late developmental spine pruning and not an imaging-induced decrease in total spine density (Supp. Fig. 2a).

Animals receiving 12 daily cocaine injections showed an expected increase in cocaine-induced locomotion, known as locomotor sensitization (Fig. 1h). To test if changes in locomotion were related to the effects of cocaine on spines, we performed correlation analyses between spine dynamics and changes in locomotion (Fig. 1i,j). When the measurements of spine dynamics and locomotion change of all the animals were pooled and standardized (z-norm), no correlations were found with net spine change (Fig. 1j) or other measures (Supp. Fig. 3,4). Furthermore, a cohort of animals allowed to run on a running wheel (Supp. Fig. 5,6) showed no correlation between spine gains and locomotion (Supp. Fig. 5e) and running wheel experience did not occlude the effect of cocaine on spine gain (Supp. Fig. 6c).

Using a new cohort of mice, we next measured spine gains after acute cocaine treatment with greater temporal resolution (Fig. 2a). We found that a single cocaine injection enhanced spine gains (Fig. 2b, $p<0.01$) and spine density (Fig. 2c, $p<0.01$) as early as 2h after drug administration. We found no significant effect of cocaine on spine loss (Fig. 2d). We replicated previous observations of gains and loss made 24h after treatment (Supp. Fig. 7b,c).The day after treatment, new spines observed just 2 hours after cocaine injection, made up a >3 fold larger fraction of the total spines (20–24 hours later) in cocaine-treated mice compared to saline ($p<0.01$; Fig. 2e).

Increases in spine gain immediately after cocaine exposure (Fig. 2b) and accumulation of these new spines (Fig. 1g) could represent a mechanism by which cocaine facilitates new associations made between drug experience and predictive contextual cues. To test the relationship between cocaine exposure and associative learning we performed a final experiment measuring spine dynamics in a cocaine conditioned place preference (CPP) paradigm (Fig. 3). Mice underwent two sessions of CPP training with either saline or 30 mg/kg cocaine paired with a conditioning chamber (Fig. 3a). Preference for the cocaine vs. saline paired context was measured 24 hours after cocaine-pairing. We found that mice gained more new spines on the day when they received cocaine than on the day when they received saline ($p=0.002$, Fig. 3b). New spines observed after cocaine-conditioning showed shorter length-to-width ratios than new spines gained after saline-conditioning ($p=0.006$, Supp. Fig. 8d) suggesting they were more likely to persist and potentially form synapses[12–16]. When we isolated the population of new spines that persist 96h or more (suggesting they form synapses[16]), we found that new persistent spines gained after cocaine-conditioning represented a significantly greater fraction of total spines than those gained after saline-conditioning (Fig. 3d, $p=0.007$). The percentage of new 96h persistent spines that were gained on cocaine-pairing day strongly correlated ($r=0.76$, $p=0.017$) with the magnitude of the change in preference for the cocaine-paired side (Fig. 3e) suggesting a relationship between the two variables. There was no correlation between CPP preference measures and spine loss or density (Supp. Fig. 8f,h). Control experiments in which only saline was given on both conditioning days showed no correlation between new 96h persistent spine gains and CPP preference measures ($r=–0.22$, $p>0.54$, Supp. Fig. 9c).

The correlation that we observed between new persistent spine gains and CPP preference score (Fig. 3e) suggests that new persistent spine gain may support learning about drug context cues or appetitive expression of these associations. Associative or reconsolidation processes involved in CPP might also play a role in stabilizing new spines formed after cocaine exposure. Future studies investigating mechanisms underlying cocaine-induced spine formation and persistence should shed further light on their role in learning, substance use and abuse and potentially aid the development of therapeutic interventions for addiction.

ONLINE METHODS

Animals

Male C57BL/6J transgenic mice (n=77) expressing YFP (line H; Jackson labs 00378)[8] were housed on a 12h/12h reverse light-dark cycle (lights off at 10AM). Mice were weaned at P21 and housed with siblings (2–5 mice per cage), nesting material, and a round plastic hut. In experiments where cocaine and saline treatment was required, littermates were evenly distributed to each group (except within-animal design experiments shown in Supp. Fig. 9a&d where the experiments were performed several months apart). At the first imaging session, mice were P58-112. All procedures were approved by the Ernest Gallo Clinic and Research Center Animal Care and Use Committee.

Surgery

Under isoflurane anesthesia, we made a ~3mm diameter craniotomy over the dorsomedial frontal cortex of both hemispheres. Surgical procedures used were described in detail in Holtmaat et al. (2009)[17]. Mice were left to recover a minimum of 7 days for the repeated-treatment and withdrawal (Fig. 1, Supp. Fig 1–4), running wheel (Supp. Fig. 5–6) and conditioned place-preference (CPP) experiments (Fig. 3, Supp. Fig. 8–9). For the acute-treatment experiments (Fig. 2, Supp. Fig. 7) all the animals were imaged 1–3 days after surgery except for one animal belonging to the saline group that was imaged after >7 days of recovery. We found no significant differences in the fraction of spines gained or lost between these two recovery time groups (see Supp. Fig. 10). Similar results have been described previously[17].

Drug Treatment and Behavior Measurement

Repeated cocaine and withdrawal experiment (Fig. 1 and Supplementary Fig. 1–4)—During the last days of recovery from surgery, mice were handled to habituate them to restraint for *i.p.* injections and they were habituated to locomotor chambers for at least 2 days for 3 hours (Fig. 1a). After this habituation period, the animals entered the baseline phase in which we performed all the same procedures as in the treatment phase but mice received only sham *i.p.* injections. To measure cocaine sensitization, locomotor test sessions were performed every second day during the baseline and treatment phase on days when animals were not imaged. For locomotor testing, mice were introduced into a clear chamber (7.5”× 7.5”×7.5”) with an IR monitoring system (MED Associates, St. Albans, VT) for 60 minutes (pre-test period), then removed to receive the *i.p* injection (sham for the baseline; or saline or cocaine 15mg/kg (Sigma) for the treatment phase). After injection, mice were immediately placed back in the chamber for 2 hours (test period). Locomotor data on four saline-treated mice were lost during a computer upgrade and they could not be included for analysis. Mice were imaged on alternate days in the morning under isoflurane anesthesia. The time at which each mouse was imaged was held constant to eliminate any potential effects of sleep wake cycle on spine dynamics. Mice were scheduled for treatment injections three hours after the imaging session was concluded. Throughout the treatment phase, mice received 12 daily injections of either saline or cocaine (15mg/kg). During the withdrawal phase, mice were imaged every other day for 2 weeks (7 sessions) and were returned to their home cage after recovery from anesthesia. In 12 mice (6 per group), we were able to image the same dendrites through the entire baseline, treatment, and withdrawal protocol without bone growth obscuring the window. A subset of animals in both groups was imaged only for the treatment or withdrawal portion of the protocol. Values for measures of spine density and spine dynamics were normalized to baseline for analysis of treatment and to the final session of treatment for withdrawal specific analysis.

Running wheel experiment (Supplementary Fig. 5–6)—Pre-habituation: Animals were handled and habituated to restraint *i.p*. procedures and running wheel for 5 sessions in which the wheel was unlocked for ~30 minutes (animal was allowed to run in the wheel) and ~30 minutes where the wheel was locked (the wheel was blocked so the animal could not run). Imaging phase (Supp. Fig. 5a): Imaging was then started and mice were imaged during an additional habituation session in which they were in the wheel for 20 minutes with

the wheel unlocked for 5 of the 20 minutes (day 1: 5'). The next day the wheel was unlocked for 15 of the 20 minutes to allow more running (day 2: 15'). On day 3 the wheel was unlocked for only 5 minutes of the 20 minute session (day 3: 5'). The rationale behind this unlocked-locked running wheel protocol was to standardize the amount of time in the chamber (20 minutes) and to regulate the amount of running within that time. Mice were imaged daily in the morning and were allowed at least 3h of recovery from anesthesia before running wheel exposure. Between day 1 (5' running) and day 2 (15' running), mice ran a proportional distance similar to that observed between the last session of the baseline and first day of treatment in the repeat cocaine treatment experiment (Supp. Fig. 5c). This allowed us to test the effect of increased locomotion on dorsomedial frontal cortex spine dynamics in the absence of cocaine.

Animals running in the running wheel for 15 minutes traveled longer distances than those measured in the open field over the same time frame (running 5'=3792 ± 453 cm vs. pre-treatment locomotion=1026 ± 107 cm; running 15'= 10824 ± 1972 cm vs. treatment day 1 locomotion: 2973 ± 1013 cm). Given the large amount of locomotion on the running wheel in even 5', it was possible there was a "saturation" effect on the first measure of the fraction of spines gained. Under this saturation scenario, any further manipulation would not have any impact on spine gain due to occlusion. To test this possibility, we included an extra running wheel session (day 4) in which mice were allowed to run for 5 minutes on the running wheel after a cocaine (15mg/kg) *i.p* injection (Supp. Fig. 6). The results showed that there was room for further increases in spine gains illustrated by the significant increase in the fraction of spines gained after the cocaine+5 minutes running protocol when compared to the value obtained after the 15 minutes running-session (without drug) (Supp. Fig. 6c).

Acute treatment experiment (Fig. 2 and Supplementary Fig. 7)—Mice were handled and habituated to restraint *i.p*. procedures before cranial window implant surgery and recovered 1–3 days afterwards. Usually, saline and cocaine groups were formed by littermates for whom the surgery-recovery times were identical. Mice were then imaged twice daily (starting in the morning) with a 3h interval between imaging sessions. Mice were scheduled for treatment injections when one hour had passed since the conclusion of isoflurane anesthesia and were imaged again in the afternoon starting 2 hours after injection of either saline or cocaine (15mg/kg) (Fig. 2a).

Cocaine conditioned-place preference (CPP) experiment (Fig. 3 and Supplementary Fig.8–9)—Animals were handled and habituated to restraint *i.p*. procedures after cranial window implant surgery. All animals were imaged daily in the morning and allowed to recover for at least 3h from anesthesia before any behavioral manipulation was performed (see Fig.3a). CPP place preference conditioning was performed in the afternoon. The CPP chamber (MED associates) consisted of 2 compartments separated by a black (infrared transparent) wall. On the habituation and test sessions (days 2 and 5 of the schedule, see Fig. 3a), the wall had an opening that allowed the animals to freely explore both chambers. Each chamber contained a set of three different contextual cues (see Supp. Fig. 8b):1) visual cue (horizontal vs. vertical lines) on the chamber walls; 2) texture cues (square vs. random textures) on the chamber floor; and 3) odor cues (vanilla vs.

cinnamon extract, 200 µl on filter paper positioned on opposite corners of the chambers). On experimental days, when two conditioning sessions were performed, 100 µl of odor was added before the second session to prevent loss of odorant intensity. The combination of sensory cues for each chamber was counterbalanced between animals.

After a habituation session where the animals were allowed to explore both chambers for 30 minutes, an initial measurement of baseline preference was taken (CPP_{hab}, defined as the time difference between the time spent in the two chambers). The next day (Fig. 3a) mice underwent saline paired conditioning (2 sessions of 15 minutes separated by 2 hours) in the chamber for which they showed preference on the habituation day (designated chamber "A"). The following day, the animals were conditioned to cocaine (30mg/kg, *i.p.*, Fig 3 and Supp. Fig. 8, 9d–f) or saline (Supp. Fig. 9 a–c) in the opposite, non-preferred chamber (designated chamber "B") for 2 sessions of 15 minutes separated by 2 h. During the given days, the animals were restricted to one side of the chamber exclusively. On the CPP test day, the animals were introduced into the CPP chamber with the open gate configuration (Fig. 3a) for 30 minutes and preference for chamber A vs. B was again measured (CPP_{test}). CPP was defined as the extent of the preference shift after cocaine, $CPP = CPP_{hab} - CPP_{test}$ (Supp. Fig. 9b,e).

To measure the persistence of the new spines gained after the saline- and cocaine-conditioning sessions, we performed two more imaging sessions 96h after the session 4 and session 5 (Fig. 3a). In two mice from the saline-only treated group (Supp. Fig. 9a–c) some regions of interest (ROIs) were obscured by bone growth between the CPP test day and the 96h persistent imaging sessions (see Supp. Fig. 9a for reference). On day 9, we imaged visible ROIs in vivo and then perfused with mice with fixative. For these two special cases, some images were then obtained in a whole mount preparation. Previous studies have found that majority of spines observed after fixation correspond to spines observed in vivo in a session just before fixation[16].

Imaging procedure and analysis

Our procedures for *in vivo* imaging have been previously described[17]. Briefly, we imaged the apical dendrites of YFP expressing pyramidal neurons using a Mai Tai HP laser (920nm, Spectra-physics, Santa Clara, CA), Ultima IV *in vivo* laser scanning microscope (Prairie Technologies, Middleton, WI) and a 40× 0.8 NA objective (Olympus). 40 micron segments of 3rd order (and higher) dendrites were imaged with high resolution (0.085–0.17 µm/pixel). Branches were located within 100 µm from the surface (Layer I). In about 50% of the dendrites imaged, we could follow the dendritic arbor until its main bifurcation. In these neurons we found that the dura–initial apical bifurcation was 314.8 ± 17.63 in cocaine-treated mice (n=21 neurons) and 277.9 ± 23.16 (n=17 neurons) in saline-treated mice (P=0.45, Supp. Fig. 2b).

To control for the impact of surgery and anesthesia, we repeated the same schedule of handling and injection in a subset of mice that did not receive a craniotomy or repeated anesthesia. We sacrificed these animals 2 weeks after the last injection of saline and compared their dendritic spine density with that of the animals subject to *in vivo* imaging. There were no significant differences (saline *in vivo* 0.401 ± 0.02 spines/µm, n=6 mice, vs

saline *fixed*: 0.373 ± 0.02 spines/um, n=6 mice, *U*=12, p>0.39, Mann-Whitney U-test, Supp. Fig. 2a).

We used Matlab (Mathworks, MA) and custom SpineAnalysis image software to manually score the spines using guidelines for scoring from Holtmaat et al (2009)[17]. On average, we analyzed 101.6± 1.8spines/mice (n=77 mice) measured on the first imaging session. There were no differences in sampling between the cocaine and saline groups used in this study (p>0.69). Animals for which we could not analyze a minimum of 80 spines at day one of the experiment were excluded from analysis. All images were scored by an observer blind to the animal's treatment.

Spine gain fraction as defined in Figure 1d is obtained from the formula:

$$FG_{ab}=(NG_{ab}|TS_a),$$

where FG_{ab} represents the number of new spines gained (NG_{ab}) between two consecutive sessions *a* and *b* divided by the total number of spines (TS_a) present on session *a*. This value is then normalized to the averaged fraction gain between baseline sessions:

$$normFG_{ab}=(FG_{ab}|\overline{FGbase}) \times 100$$

Where $\overline{FGbase}$ is defined as the average of the fraction of spines gained ($FG_{(i-1)\rightarrow i}$) between consecutive baseline sessions *i −1* and *i*, where *j* represents the total number of baseline gain sessions.

$$\overline{FGbase}=\frac{\Sigma_{i=1}^{j} FG_{(i-1)\rightarrow i}}{j}$$

Identical calculations were made for spine loss as for gain using loss data.

To understand how the accumulation of new spines (SA) after cocaine treatment could alter spine density we created a measure of normalized spine accumulation (Fig. 1g). We defined normalized spine accumulation ($normSA_{p\rightarrow n}$) between the last pre-treatment session (*p*) and a treatment session (*n*) as,

$$normSA_{p\rightarrow n}=\left(\frac{SA_{p\rightarrow n}}{\overline{FGbase}}\right) \times 100;$$

Where $SA_{p\rightarrow n}$ is the number of spines present in session *n* that were not present on the last session of the pretreatment baseline *p*, and $\overline{FGbase}$ is defined as the average of the fraction of spines gained ($FG_{(i-1)\rightarrow i}$) between consecutive baseline sessions *i −1* and *i*, where *j* represents the total number of baseline gain sessions. In the theoretical case where an animal would always gain the same amount of spines between any given two consecutive sessions, and all those spines gained would be transient (not present on the next imaging session);

then the value for $SA_{p\rightarrow n}$ would be the same than the value of the baseline ($\overline{FGbase}$) and the percentage would be 100% (where 100% means no accumulation).

For the morphological analysis of new spines (Supp. Fig. 8c,d), we measured the length of the dendritic spine from the base of the dendrite to the furthest tip of the spine and divided this value by the maximal head width to obtain the length-width ratio.

Statistics

Two-way mixed (Within-Between) ANOVAs for groups of different sizes were performed with Matlab (http://phy.ucsf.edu/~loren/NS248/Matlab/BetweenWithinAnova/). Comparisons of two groups at a single time point were performed after testing for normality by using the D'Agostino-Pearson omnibus test (Graphpad Prism 5). Whenever the distribution was considered normal, paired or unpaired Student *t*-tests were performed. For unpaired *t*-tests, homoscedasticity (equal variances) was tested with a F-test (Graphpad Prism 5). Non-parametric tests were used to compare non-normal distributions and experiments with small samples. Wilcoxon rank test was used for paired groups and Mann-Whitney test for unpaired groups (Graphpad Prism 5). One-Way repeated measures ANOVA was used to analyze the effects of conditioning session on spine dynamics in the CPP experiments (Graphpad Prism 5). Whenever the distribution of these values was not normal, a Friedman's test for repeated measures was used (Graphpad Prism 5). To analyze the potential differences between cumulative histogram distributions, a two-sample Kolmogorov-Smirnov (KS) test was used (Matlab). Pearson's *r* values were used to measure correlations between parameters (Graphpad Prism 5). No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to those reported in previous publications[9–11,16].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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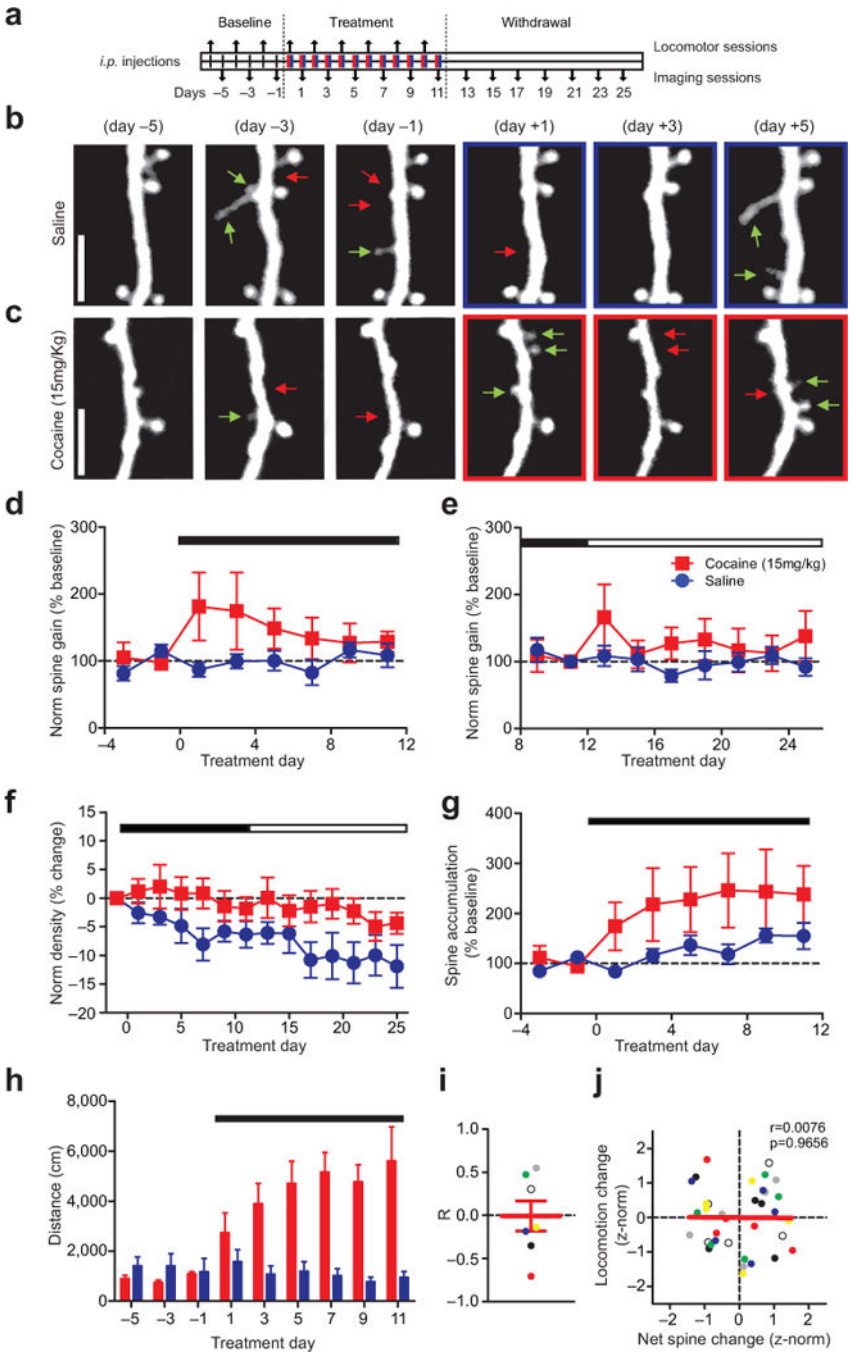

Figure 1. Cocaine exposure increases spine gains in the frontal cortex

a) Schematic of the protocol schedule (see also Online Methods). **b–c**) Repeated imaging of dendrites from *Thy1* YFP-H mice. Green arrows indicate spines gained; red arrows indicate spines lost. Scale bars=5μm. **d**) Fraction of spines gained between imaging sessions normalized to the gains measured during the baseline period (*group*: $F(1,16)=3.72$, $p=0.001$; *time*: $F(6,96)=1.22$, $p>0.86$; *Interaction*: $F(6,96)=2.94$, $p<0.0001$, cocaine n=7 vs. saline n=11 mice; Two-way mixed ANOVA). Solid black line represents the treatment phase. **e**) Normalized spine gain during the withdrawal period (*group*: $F(1,16)=0.78$, $p>0.72$; *time*:

F(7,112)=1.15, p>0.8; *Interaction*: F(7,112)=1.16, p>0.8, cocaine n=10 vs. saline n=8 mice; Two-way Mixed ANOVA). Hollow line represents the withdrawal phase. **f**) Normalized spine density (*group*: F(1,10)=1.84, p>0.6; *time*: F(13,130)=6.11, p<0.0001; *Interaction*: F(13,130)=1.64, p<0.001, cocaine n=6 vs. saline n=6 mice; Two-way Mixed ANOVA). **g**) Summary plot showing spine accumulation (see Online Methods for details) during the treatment phase (*group*: F(1,16)=3.29, p<0.002; *time*: F(6,96)=6.09, p<0.0001; *Interaction*: F(6,96)=2.92, p<0.0001, cocaine n=7 vs. saline n=11 mice; Two-way mixed ANOVA). **h**) Average plot of the locomotor activity measured during 120 minutes after i.p injection (*group*: F(1,12)=14.278, p<0.0001; *time*: F(6,72)=3.722, p<0.0001; *Interaction*: F(6,72)=5.172, p<0.0001, cocaine n=7 vs. saline n=7 mice; Two-way Mixed ANOVA). **i–j**) Summary plot of the correlation between the net change in spine number between consecutive imaging sessions and the changes in distance traveled between consecutive treatment sessions (see Supplementary Fig. 3a–c for details). **i**) Each dot represents correlations obtained from individual mice during treatment. **j**) Each dot represents a z-normalized plot of net spine change and the subsequent locomotor change on a given day of the treatment. Each individual color represents an animal. Bars are means and error bars are s.e.m.

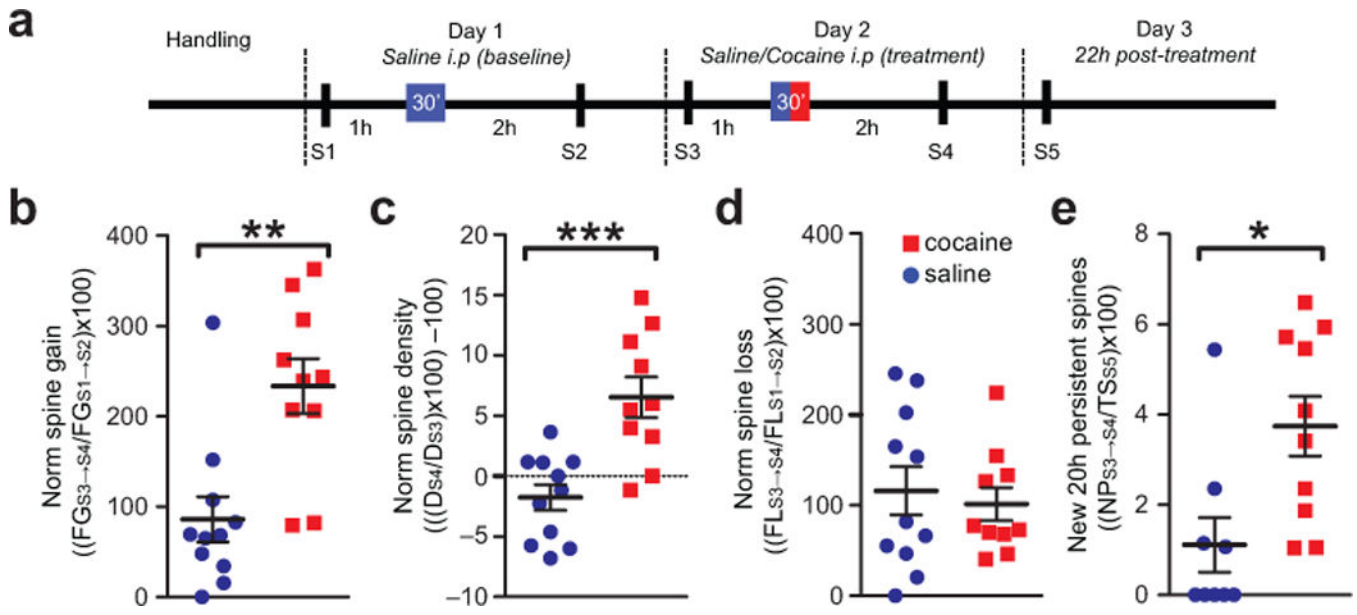

Figure 2. Cocaine increases spine gains in the frontal cortex within 2 hours of injection

a) Schematic of the imaging and treatment schedule. S1 to S5 refers to the imaging session number. **b**) Fraction of spines gained (FG) 2 hours after injection ($FG_{S3\rightarrow S4}$) normalized to the baseline ($FG_{S1\rightarrow S2}$) (*U*=13, p<0.004; cocaine n=10 vs. saline n=11 mice; Mann-Whitney U-test). **c**) Normalized spine density observed 2 hours after cocaine treatment (*t*=4.25, p<0.001; cocaine n=10 vs. saline n=11 mice; unpaired Student *t*-test). **d**) Fraction of spines lost (FL) 2 hours after injection (*U*=52, p>0.8, cocaine n=10 vs. saline n=11 mice; Mann-Whitney U-test). **e**) New spines that were first observed 2 hours after cocaine-injection ($NP_{S3\rightarrow S4}$) and persisted 20 hours later (S5), made up a greater percentage of the total number of spines (TS_{S5}) (*U*=10, p=0.011; cocaine n=10 vs. saline n=9 mice; Mann-Whitney U-test). Each symbol represents one mouse. Bars are means and error bars are s.e.m. *,** and *** represents p<0.05, p<0.01 and p<0.001 respectively.

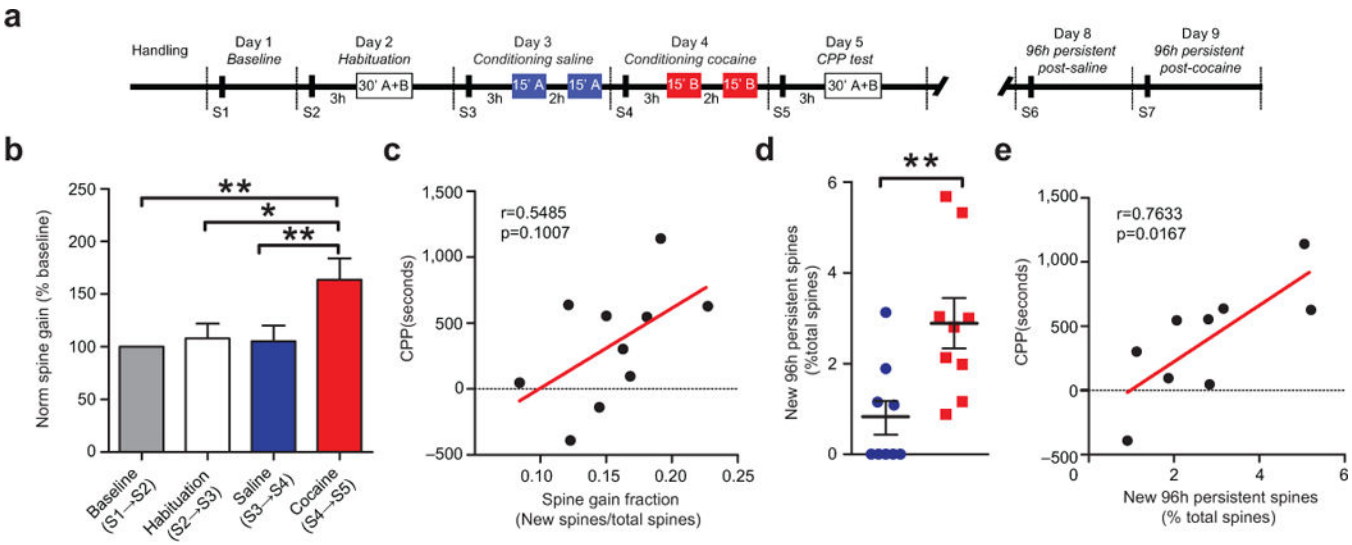

Figure 3. New persistent spine gains after cocaine conditioned place preference (CPP) training correlate with the magnitude of gain in preference for the cocaine-paired context

a) Schematic of the imaging and CPP protocol (see also Online Methods for details). S1 to S7 refers to the imaging session. **b**) Fraction of spines gained between consecutive imaging sessions. A One-way repeated measures ANOVA showed significant differences between sessions ($F(3,27)=6.84$, $p=0.001$, n=10 mice). A Bonferroni post-hoc analysis showed that more spines were gained during the cocaine treatment (S4→S5) than during previous sessions (*cocaine vs. baseline*: $p<0.01$; *cocaine vs. habituation*, $p<0.05$; *cocaine vs. saline*, $p=0.01$, n=10 mice). **c**) Correlation between the fraction of spines gained after cocaine treatment (S4→S5) and preference for the cocaine-paired chamber. **d**) New persistent spines (present for >96h) gained during cocaine conditioning (S4→S5), accounted for a greater percentage of total spines (at S7) than after saline at (at S6) ($t=3.56$, $p=0.007$; n=9 mice; paired Student *t*-test). **e**) New persistent spines first gained during cocaine-conditioning (S4→S5) showed a significant positive correlation with the change in magnitude of preference for the cocaine-paired chamber between habituation and test day. Bars are means and error bars are s.e.m.