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Opiate dependence induces network state shifts in the limbic system

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ABSTRACT

Among current theories of addiction, hedonic homeostasis dysregulation predicts that the brain reward systems, particularly the mesolimbic dopamine system, switch from a physiological state to a new "set point." In opiate addiction, evidence show that the dopamine system principal targets, prefrontal cortex (PFC), nucleus accumbens (NAC) and basolateral amygdala complex (BLA) also adapt to repeated drug stimulation. Here we investigated the impact of chronic morphine on the dynamics of the network of these three interconnected structures. For that purpose we performed simultaneous electrophysiological recordings in freely-moving rats subcutaneously implanted with continuous-release morphine pellets. Chronic morphine produced a shift in the network state underpinned by changes in Delta and Gamma oscillations in the LFP of PFC, NAC and BLA, in correlation to behavioral changes. However despite continuous stimulation by the drug, an apparent normalization of the network activity and state occurred after 2 days indicating large scale adaptations. Blockade of μ opioid receptors was nonetheless sufficient to disrupt this acquired new stability in morphine-dependent animals. In line with the homeostatic dysregulation theory of addiction, our study provides original direct evidence that the PFC–NAC–BLA network of the dependent brain is characterized by a *de novo* balance for which the drug of abuse becomes the main contributor.

Introduction

Opiate use (heroin or morphine) induces a fast and powerful dependence and the prevalence of opiate use and abuse has increased in the past few years with a major impact on public health (http://www.who.int/substance_abuse/facts/opiates/en/). Among current theories of addiction, hedonic homeostasis dysregulation predicts that the brain reward systems, particularly the mesolimbic dopaminergic circuit, switch from a physiological state to a new "set point" (also named allostatic state) as dependence develops (Ahmed and Koob, 1998; Koob and Le Moal, 1997). The prefrontal cortex (PFC), nucleus accumbens (NAC) and basolateral amygdala (BLA) are main targets of the dopamine system and represent core structures at the interface of drug-reinforcement and drug and cue-reinstatement circuits which are crucial features of addiction (for review, Le Moal and Koob, 2007). These regions are the site of severe functional adaptations and homeostatic impairments following chronic drug exposure (Christie, 2008; Kalivas, 2009; Luscher and Malenka, 2011).

PFC, NAC and BLA form an interconnected limbic network; in which PFC and BLA are reciprocally connected and both project to the NAC (Cardinal et al., 2002). It is widely accepted that brain functions are distributed processes and that distant structures associated in a functional network interact through oscillatory and phase synchronization of neuronal activity (Fell and Axmacher, 2011). In line with this idea several studies have demonstrated that rhythmic interactions between PFC, NAC and/or BLA in theta (5–10 Hz) and gamma (40–100 Hz) oscillations are central to cognitive functions such as learning and memory (Berke, 2009; Popa et al., 2010; Popescu et al., 2009). In opiate addiction a few pioneer studies have investigated the impact of the drug on oscillatory processes in these structures. In both opiate dependent patients and rats, repeated morphine treatment alters the EEG in the prefrontal cortex which is subject to a marked increase in delta range oscillations (1–4 Hz) correlated with drug intake (Greenwald and Roehrs, 2005; Sun et al., 2006). Surprisingly, while a few studies have documented the effect of psychostimulants on deep structure dynamics, such as the NAC (Berke, 2009) and the hippocampus (Liu et al., 2010), the effect of opiates on NAC and BLA oscillatory activity as well as on the synchronization between PFC, NAC and BLA remains largely unexplored. Such

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functional connectivity has been partially investigated in a recent *in vitro* study which revealed that long-term depression and potentiation were both impaired at the PFC to NAC synapses in rat self-administrating heroin (Shen and Kalivas, 2012). This result shows that functional interactions are modified for at least one node of the PFC, NAC and BLA ensemble; however, to date no study has gathered the experimental conditions required to investigate neuronal dynamics at the whole network level.

In healthy subjects physiological behaviors present distinct network stable states in the forebrain characterized by specific oscillatory and synchronization profiles (Gervasoni et al., 2004). Abnormal oscillatory signatures observed in the PFC under opiates indicate that chronic drug administration is able to challenge local network stability (Greenwald and Roehrs, 2005; Sun et al., 2006). Considering that PFC, NAC and BLA are functionally bound through oscillatory synchronization, this strongly suggests that the dynamics of the entire network could be modified in opiate dependence and give rise to novel drug-related states and equilibrium. In line with this idea and current theories of addiction, we postulated that chronic morphine stimulation impairs PFC–NAC–BLA functional equilibrium by promoting the emergence of a drug related *de novo* network state. While studies so far have focused on single structures, testing this hypothesis requires an original and global approach. For that purpose we used simultaneous electrophysiological recordings in the PFC NAC and BLA in freely-moving rats combined with multivariate analysis methods to characterize network states under physiological conditions and after chronic morphine.

Materials and methods

Animals

Thirteen male Sprague–Dawley rats (Charles River Laboratories, France) were individually housed under an inverted 12-h light/dark cycle (lights off at 8:00 h) at 21 ± 2 °C with food and water available *ad libitum*. Weight varied from 250 to 300 g at the beginning of the study to 300–350 g at the time of the last recording. Surgical and experimental procedures were performed in accordance with the European Community's Council Directive (EU Directive 2010/63/EU86) and the National Institute of Health guide for the care and use of laboratory animals. The present experiment was approved by the local Animal care and Use committee (approval #5012049-A).

Electrode implantation surgery

Rats were implanted with 12 independently moveable tetrodes in PFC (2 tetrodes), NAC (4 tetrodes) and BLA (6 tetrodes). Surgery took place after at least 7 days of daily handling habituation, under full general gas anesthesia (isoflurane 1.5–2%) with local anesthetic (xylocaine 0.5%) at the incision site and under prophylactic antibiotic (ampicillin, 7 mg/kg). The recording electrodes were lowered to position through small craniotomies above each structure. Six stainless-steel skull screws were inserted and the implant was affixed to the skull with super bond and dental acrylic. Two of the skull screws were positioned over the cerebellum approximately 3 mm caudal to lambda and used for animal grounding and electrode referencing. After the surgery animals were injected with carboprofen (2 mg/kg, s.c.) for pain management and allowed to recover from anesthesia before being returned to the animal housing facilities.

Induction of morphine dependence and withdrawal

Dependence was induced by the subcutaneous implantation of two morphine pellets. Compared to repeated injections or intermittent self-administration protocols this approach allows to reach a drug-dependent state without the animal being exposed to repeated withdrawal experiences. Indeed this has been shown to modify neuronal activity and plasticity in our structures of interest (Lucas et al., 2008) and may therefore interfere with the sole effect of chronic morphine analyzed here. Moreover, this model allows the use of naloxone to precipitate and exactly time the onset of withdrawal. Thus after 5–7 days of recovery from the first surgery, animals were implanted with subcutaneous pellets. The experimental group ($n = 8$) received two morphine pellets (2 × 75 mg of morphine base; NIDA, USA) and a control group received two placebo pellets ($n = 5$). Under general anesthesia (isoflurane 3%) a small incision was made on the animal's back and two pellets were placed in the lumbar region, one on each side of the medial line. Two stitches were set at the incision site before the animal was returned to the housing facility.

Under morphine, pellet drug dependence is classically obtained after 24 hours and lasts for at least 12 days (Gold et al., 1994). Behavioral and electrophysiological activities were monitored in drug free conditions (Baseline) and for four consecutive days starting 24 hours after pellets implantation (days 1, 2, 3 and 4). Morphine and Placebo groups were then both subjected to an injection of saline (s.c.) on day 5 and of the μ opioid receptor antagonist naloxone on day 6 (Sigma, 15 μg/kg s.c.) and were monitored 24 h after that last injection (day 7).

Recordings

For all electrophysiological and behavioral recordings animals were placed for 20 minutes in a cylindrical box. Behavior was monitored with a camera placed over the box and connected to a Cineplex video tracking system (Plexon, TX, USA). In the same sessions we recorded single unit activity and LFP in the PFC, the NAC and the BLA simultaneously using Multichannel Acquisition Processor (MCP, Plexon). The wide band signal (0.1–9000 Hz) collected by the electrodes was pre-amplified (20×) and amplified (50×) before being digitized (40,000 Hz sampling rate for single unit activity and 1000 Hz for LFP) and stored for further analysis. Single units were manually sorted and cluster isolation was tested for significance in Offline sorter (Plexon). Units were then classified as putative projection neurons or interneurons (Fig. S1) using combined spike width and firing rate methods as previously described (Bartho et al., 2004; Berke et al., 2004; Dejean et al., 2012).

Behavioral data analysis

All analyses were performed while the animals were awake. All time intervals during which the animals were sleeping (i.e. displaying immobility with closed eyes) were filtered out and excluded from further analysis. Morphine pellet implantation induces characteristic episodes of a stupor state during which animals display a lack of responsiveness, immobility, a prone position and exophthalmos. These events were monitored offline by carefully inspecting video recordings of experimental sessions and isolated in specific time intervals for stupor related electrophysiological signals to be further analyzed separately for alert behavior epochs.

In morphine-treated rats the behavioral correlates of a 15 μg/kg injection of the μ opioid receptor antagonist naloxone are typical mild drug withdrawal signs, and this dose has no significant effect in placebo controls (Frenois et al., 2002). In the present study we focused on three withdrawal-related parameters that are enhanced defecation, wet dog shakes and teeth grinding. The occurrence of those signs where analyzed offline by a visual inspection of the video recording and an additional investigation of the presence of mechanical artifacts on electrophysiological traces. Indeed both wet dog shakes and teeth grinding presented a characteristic discrete noise signature that allowed us both to confirm the observations derived from video recording inspection and to isolate and filter out those events before further analysis of the signal. Defecation, wet dog shake and teeth grinding were quantified as the number of episodes per 20 minute session and were analyzed in placebo and morphine animals in three different

conditions: saline injection, naloxone injections and 24 h after naloxone injection.

Tissue processing

Electrode positions were marked at the end of the experiments by performing electrolytic lesions (10 mA DC for 10 sec) under ketamine-xylazine anesthesia. The animals were then perfused with 1% paraformaldehyde and the brain removed, sliced, colored and mounted on slides for histological analysis. Only those tetrodes with tips confirmed within the PFC, the NAC or the BLA were included in the analysis (Fig. 1A).

Electrophysiological data analysis

LFP power and coherence analyses

Session wide power and coherence spectra of the LFP were calculated using 4096 point FFTs in Neuroexplorer (Nex Technologies, MA, USA) and smoothed with a 3-point Gaussian sliding window. For display purposes power spectral density histograms are plotted using a logarithmic scale for power and only values ranging from 0 to 120 Hz are displayed in the present paper. Based on data from the literature we studied power in different frequency bands (Delta 1–4 Hz; Theta 5–10 Hz; Low Gamma 55–65 Hz; High Gamma 70–90 Hz). To compare morphine and placebo conditions power was averaged across the frequency range of interest and further normalized as the percentage of power in baseline condition. Time–frequency power spectra (spectrograms) were calculated using 4096 point discrete FFT in Neuroexplorer yielding a 1 second time resolution and a total of 1200 time steps for each 20 minute session. For display purposes time–frequency plots use a logarithmic scale for power. Spectrograms were calculated for the sake of network state analysis which is detailed below.

Spike-LFP synchronization

The temporal relationship between spikes and LFP was investigated using spike triggered averaging, whereas for their phase relationship we used phase histograms. In both cases we aimed at analyzing spike LFP relationship within the four specific frequency bands of interest with regard to spectral analysis results. For each frequency range of interest, LFP were bandpass filtered in Matlab (Mathworks, MA, USA) using a 3rd order Butterworth filter with cutoff frequencies corresponding to the bands mentioned in the previous paragraph. Spike triggered averages and spike-phase histograms were constructed for single units using the LFP recorded on the same tetrode. Only neurons with spike trains containing more than 50 spikes were included in the analyses.

Spike triggered averaging were computed for each single unit and each frequency band by averaging the filtered LFP in a time window centered on the spike occurrence. For spike phase histograms the phase of each filtered signal was extracted by calculating the Hilbert transform of the signal in Matlab. The phase histogram for each unit was then calculated and tested for homogeneity (see Statistics section).

Network state analysis

We hypothesized that homeostatic dysregulation under morphine leads to modifications of the PFC–NAC–BLA network dynamics and subsequently alters network states present under normal conditions. To gain insight into the dynamics of baseline and morphine brain states we constructed a two-dimensional (2D) state space defined by the first and second component analysis scores of LFP global oscillatory profiles. Those two components generally account for 80% of the variability

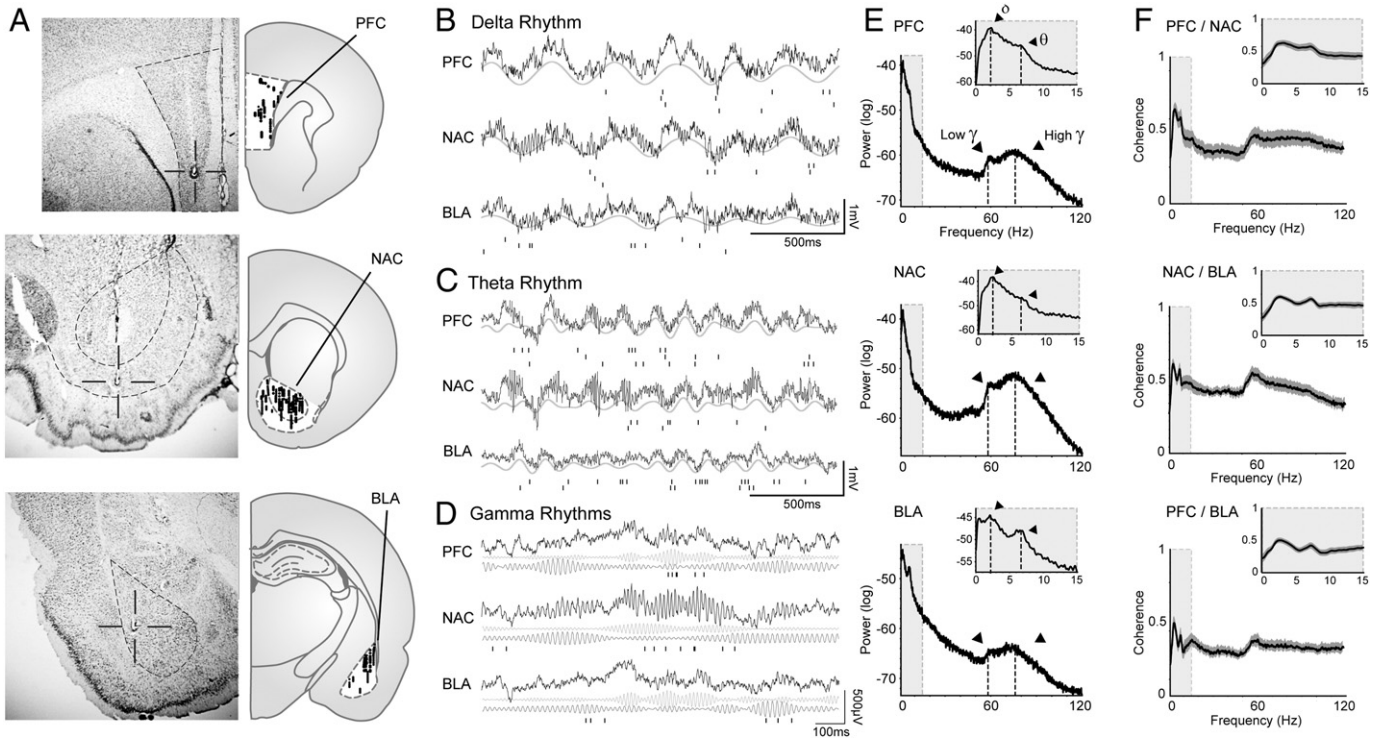

Fig. 1. Prefrontal cortex, nucleus accumbens and basolateral amygdala LFP and single units oscillate in Delta, Theta and Gamma frequency ranges in baseline conditions. (A) Diagram showing example of histological control of electrode tips (left) and overall recording sites (right, black dots) in PFC, NAC and BLA. (B) Example traces showing an episode with prominent Delta oscillations. The grey line represents Delta filtered LFP (1–4 Hz). Example spikes trains are displayed below LFP traces. Each vertical bar denotes the occurrence of an action potential. (C) Example traces showing an episode with prominent Theta oscillations. The grey line represents Theta filtered LFP (5–10 Hz). (D) Example traces showing an episode with prominent Gamma oscillations. Note that Gamma oscillations occur under the form of spindles. Dark and Light grey lines represent the bandpassed filtered LFP in Low (55–65 Hz) and High Gamma (70–85 Hz) range respectively. (E) Power spectral density of LFP in PFC, NAC and BLA calculated for the entire session corresponding to panels B, C and D. Grey boxes: blow up of power spectra between 0 and 15 Hz. Arrows indicate power peaks in the Delta, Theta and Gamma ranges. (F) Coherence spectra of pairs of LFP in baseline condition averaged across all animals. *From top to bottom:* PFC/NAC, NAC/BLA and PFC/BLA coherence.

in the oscillatory activity and therefore capture most of the network possible states. Adapting the method used by Gervasoni et al. (2004), we first concatenated PFC, NAC and BLA spectrograms in the frequency dimension yielding the global oscillatory profile for each 1 s time step. Second we calculated the first (PC1) and second (PC2) principal component scores of each profile. For each rat this calculation was performed on the whole dataset so that the same principal components were used to compare the different conditions (from baseline to day 4). Finally we constructed the 2D state space by plotting the realization of PC1 for each time step against that of corresponding PC2.

Baseline and morphine network states were compared using a Gaussian mixture model followed by unsupervised clustering of the data set. Following the assumption that the network presents 2 distinct states in baseline and under morphine, we modeled the data with a mixture of 2 Gaussians in Matlab and performed an unsupervised clustering algorithm based on the expectation–maximization method. This resulted in the calculation of two clusters for the entire data set and the assignment of network states to cluster 1 or cluster 2 independently of whether they occurred in baseline or morphine conditions. We then compared network state distributions in unsupervised clusters 1 and 2 with that in the different experimental conditions (baseline and morphine day 1 to day 4). For that purpose we calculated the Mahalanobis distance in the 2D space between each point and the centroid of cluster 1 and compared distance distributions between cluster 1, cluster 2, baseline and morphine days 1, 2, 3 and 4.

Statistics

Behavior

Defecation, wet dog shake and teeth grinding episodes per minute in the different groups and conditions were compared with a two-way ANOVA followed by a Bonferroni *post-hoc* test for pairwise comparison. Stupor intervals length was calculated as a percentage of the total session time. Stupor length was compared to baseline level (0%) using a one sample *t*-test and was compared between day 1 and day 2 with a regular *t*-test with Welsh correction for unequal variances.

Electrophysiology

The spike-phase histogram for each single unit was tested for homogeneity using the Rayleigh test for circular data (Fisher, 1993). A unit with a non-homogeneous histogram is considered to present a significantly preferred LFP phase. Mahalanobis distance distributions between cluster 1, cluster 2, baseline and morphine days 1, 2, 3 and 4 were compared using a non-parametric one way ANOVA followed by a Dunnett *post hoc* test. Power was expressed as the percentage of baseline level and was compared across the different conditions using either a *t*-test with Welsh correction for unequal variances in the case of stupor or a 2 way ANOVA followed by a Dunnett *post-hoc* test for pairwise comparison in all other cases.

Results

Oscillation frequencies in the PFC–NAC–BLA network in baseline conditions

Spectral analysis of the PFC, NAC and BLA LFP during alert behavioral state revealed 3 fundamental frequency bands. As shown in Fig. 1 we have found that LFP preferentially oscillate in Delta (1–4 Hz; Fig. 1B), Theta (5–10 Hz; Fig. 1C) and Gamma bands (50–120 Hz; Fig. 1D). Power spectral density analysis confirmed these findings, with the presence of peaks in the histogram within each frequency band (Fig. 1E). In the gamma band, two different rhythms could be discriminated; a slower (average peak ± SD: 61.9 ± 1.4 Hz) and a faster (76.6 ± 1.5 Hz) component that we will respectively name low and high Gamma hereafter. Moreover coherence analysis showed peaks for each frequency component, therefore revealing that not only these four operating modes occur at each node of the network but that the structures also synchronize with one another at these specific frequencies (Fig. 1F). To ensure that LFP represent neuronal activity in each structure, rather than being passively conducted from another structure to the tip of the electrodes we analyzed single unit activity in relation to the four frequency components observed in PFC, NAC and BLA. We recorded a total of 1703 neurons (putative projection or interneurons) in the three structures. We collected 410 putative projection neurons and 65 putative interneurons in PFC, 613 projection neurons and 120 interneurons in the NAC and 417 projection neurons and 78 interneurons in the BLA (Fig. S1). In the baseline condition, a large proportion of total single units (projection neurons or interneurons) recorded in each structure were both time- and phase-locked on LFP in each frequency band of interest (Fig. 2). This confirms that Delta, Theta and Gamma oscillations are present in the neuronal activity of the three structures both in baseline conditions and under morphine simulation.

Morphine induces a shift in network state

Time–frequency analysis in example rat ASD03 (Fig. 3A) showed that, in baseline conditions, power fluctuates across time within each band. At a given time point the relative weight of the different frequency components for the combined three structures defines a global oscillatory profile for the network. To analyze these profiles and their putative alteration by chronic morphine stimulation we concatenated PFC, NAC and BLA power spectra at each time step (1 s) and then calculated the principal component of those complex spectra (Fig. 3B). This was performed in an attempt to isolate network spectral profiles that preferentially occur in awake animals in baseline conditions and, 1, 2, 3 and 4 days after morphine pellets implantation. For each condition spectral profiles aggregated in clear clusters in the 2D state space formed by PC1 and PC2 scores (Fig. 3C). According to the hypothesis that chronic drug administration induces a change in network state, baseline and morphine states should aggregate in two specific clusters. To test this we performed an unsupervised clustering of the entire data set (baseline + morphine) based on a 2-Gaussian mixture model to fit both conditions. With this method, we were able to isolate two clusters representing two significantly distinct combinations of 1st and 2nd principal component scores and therefore two specific network states (Fig. 3D). Figs. 3D–F show that the profiles allocated to cluster 1 encompassed most of baseline while cluster 2 closely corresponded to morphine treatment at days 1 and 2. Analyzing the Mahalanobis distances in the 2D space between each profile and the centroid of cluster 1 showed that baseline and morphine distances on days 3 and 4 did not significantly differ from that of cluster 1 both in ASV03 example rat (Fig. 3E, $p < 0.001$ non-parametric one way ANOVA, Dunn's *post hoc* $p > 0.05$ for each comparison) and at the global population level (Fig. 3F, $p < 0.001$ non-parametric one way ANOVA, Dunn's *post hoc* $p > 0.05$ for each comparison). On the contrary cluster 2 and morphine days 1 and 2 distances were significantly different from baseline and morphine days 3 and 4 in this example animal (Fig. 3E, $p < 0.001$ non-parametric one way ANOVA, Dunn's *post hoc* $p < 0.001$ for each comparison) and at the global population level (Fig. 3F, $p < 0.001$ non-parametric one way ANOVA, Dunn's *post hoc* $p < 0.001$ for each comparison). When analyzed individually, single structure activity state displayed a similar pattern to the whole network (Fig. S2). In summary, chronic morphine induced a significant shift in each structure activity state and more importantly in PFC–NAC–BLA network state. However, this change was only transient since the oscillatory profiles returned to baseline from day 3 onwards.

Behavioral and frequency correlates of morphine-induced network state shift

In baseline condition, when the rats were awake, their normal behaviors were composed of either quiet wakefulness or active exploration. As expected, chronic morphine produced a noticeable

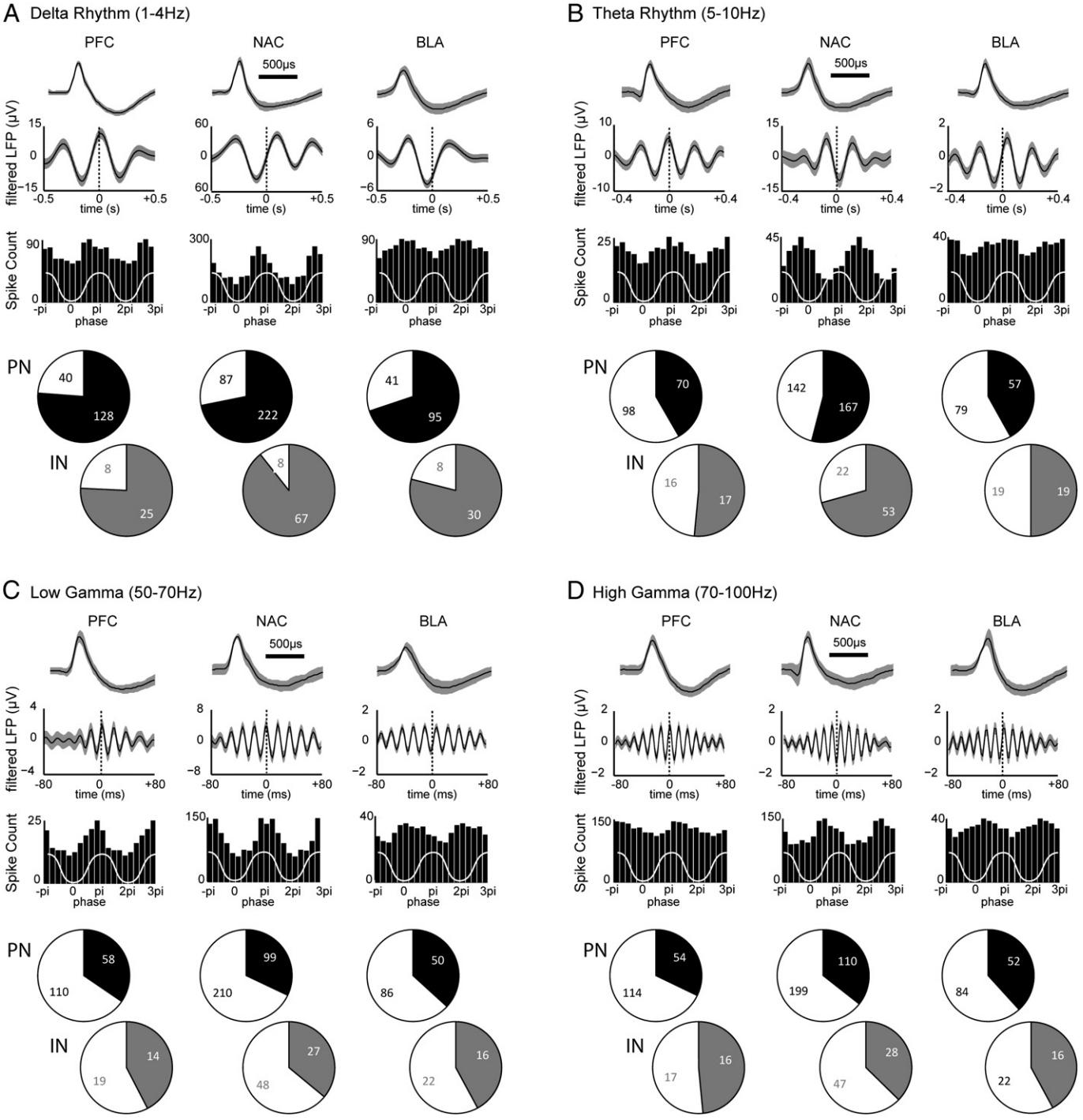

Fig. 2. Entrainment of prefrontal cortex, nucleus accumbens and basolateral amygdala neuron firing by Delta, Theta and Gamma oscillations in baseline conditions. (A) Neuronal oscillations in the Delta range. *From top to bottom*: Spike waveforms for three example putative projection neurons recorded in the PFC, the NAC and the BLA (black line: average waveform; grey area: SD). Spike triggered average of Delta filtered (1–4 Hz) LFP for the same neurons (black line: averaged LFP; grey area: SEM). Phase histograms of the three single neurons showing a preferred phase for LFP Delta oscillations recorded on the same electrode. The significance of phase preference was tested with the Rayleigh test ($p < 0.05$). The white line represents the normalized LFP as a function of phase. Pie charts show the percentage of principal neurons (PN, black) and interneurons (IN, grey) significantly phased-locked to Delta oscillations. This sample merges neurons recorded in baseline conditions in morphine and placebo rats. (B) Neuronal oscillations in the Theta range (5–10 Hz). (C) Neuronal oscillations in the low-Gamma range (55–65 Hz). (D) Neuronal oscillations in the high-Gamma range (70–85 Hz). B, C D panels are organized as described in panel A.

change in the animal's behavior with the appearance of stupor state alongside the quiet and active states. During the 2 days following morphine pellet implantation, most animals displayed characteristic features of stupor with a lack of responsiveness, immobility, prone position and exophthalmos. On day 1 all animals presented at least one of the aforementioned symptoms whereas on day 2 only 5 out of 8 animals displayed such symptoms (Fig. 4A). Moreover for those animals the amount of time spent in stupor significantly dropped from $33.4 \pm 7.1\%$ on day 1 to $11.5 \pm 3.0\%$ on day 2 ($p < 0.05$, *t*-test with Welch correction for unequal variances, Fig. 4A). However, stupor behavior occurrence was transient and completely disappeared from day 3. The animals then displayed again a normal alert behavior with an alternation of quiet wakefulness and active exploration.

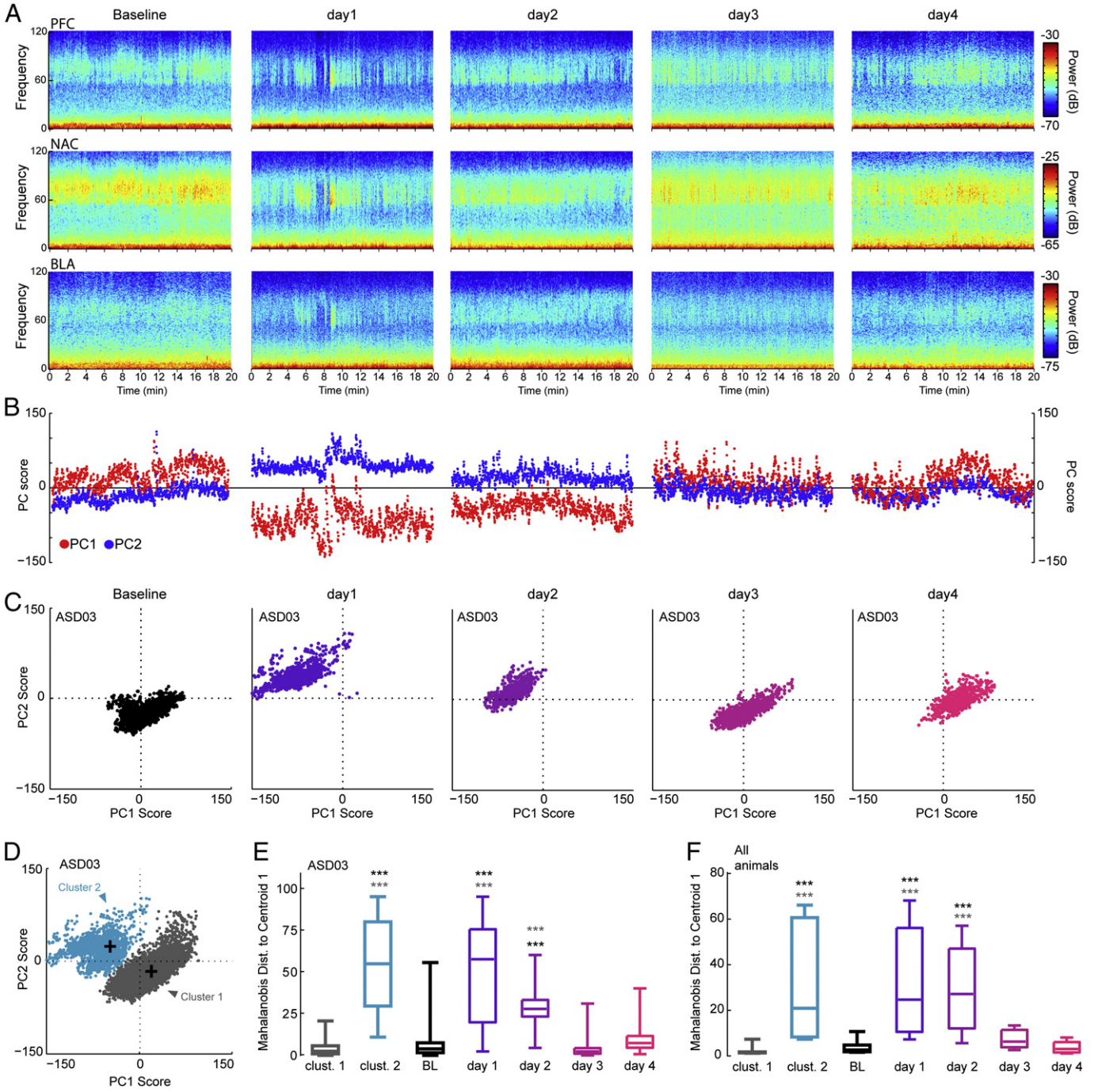

Fig. 3. Chronic morphine transiently disrupts global network dynamics. (A) Example (rat ASD03) of time resolved power spectra of LFP simultaneously recorded in the PFC, the NAC and the BLA before (baseline) and during four consecutive days after morphine pellets implantation. (B) First (red) and second (blue) principal component (PC) score as a function of time. In order to assess morphine induced variability on the whole network we performed principal component analysis of concatenated PFC, NAC and BLA spectra. (C) Raster plot of PC1 score as a function of PC2 score under baseline (black) and chronic morphine (purple to pink) conditions. (D) Unsupervised clustering of PC scores based on mixture model of 2 Gaussians. Black crosses mark the centroids of cluster 1 (grey) and 2 (blue). Note that cluster 1 contains most points belonging to baseline, day 3 and day 4 experiments, whereas the majority of day 1 and day 2 points are grouped in cluster 2. (E) Comparison of PC score Mahalanobis distances in baseline and morphine conditions in ASD03 rat. Baseline, day 3 and day 4 scores do not differ from cluster 1 while day 1 and day 2 scores are significantly different both from cluster 1 and baseline. (F) Comparison of PC scores Mahalanobis distances in baseline and morphine conditions in all animals. Baseline, day 3 and day 4 scores do not differ from cluster 1 while day 1 and day 2 scores are significantly different both from cluster 1 and baseline. Group data indicate that network states are significantly changed on days 1 and 2 before returning to baseline state from day 3 onwards. ***Significant difference with cluster 1 (grey asterisks) or baseline (black) Mahalanobis distance distributions ($p < 0.001$, non-parametric 1 way ANOVA, Dunnett *post-hoc*).

Morphine-induced stupor was paralleled by specific changes in oscillatory activity. Within these epochs we observed large erratic slow waves (Fig. 4C) that were virtually absent in baseline conditions in the awake animal (Fig. 4B). These erratic LFP oscillations ranged from 1 to 15 Hz resulting in a dramatic increase in delta range power compared to baseline both in the PFC, the NAC and the BLA on day 1 ($p < 0.05$, one sample *t*-test) and in the NAC and the BLA ($p < 0.05$, one sample *t*-test) but not in the PFC on day 2 (Fig. 4F, $p > 0.05$, one sample *t*-test). Theta oscillation power did not appear to be modified by morphine. However, the slow wave frequency range covers the theta band considered here (5–10 Hz) (Fig. 4F). Therefore this part of the analysis remains inconclusive as slow wave occurrence is likely to mask any

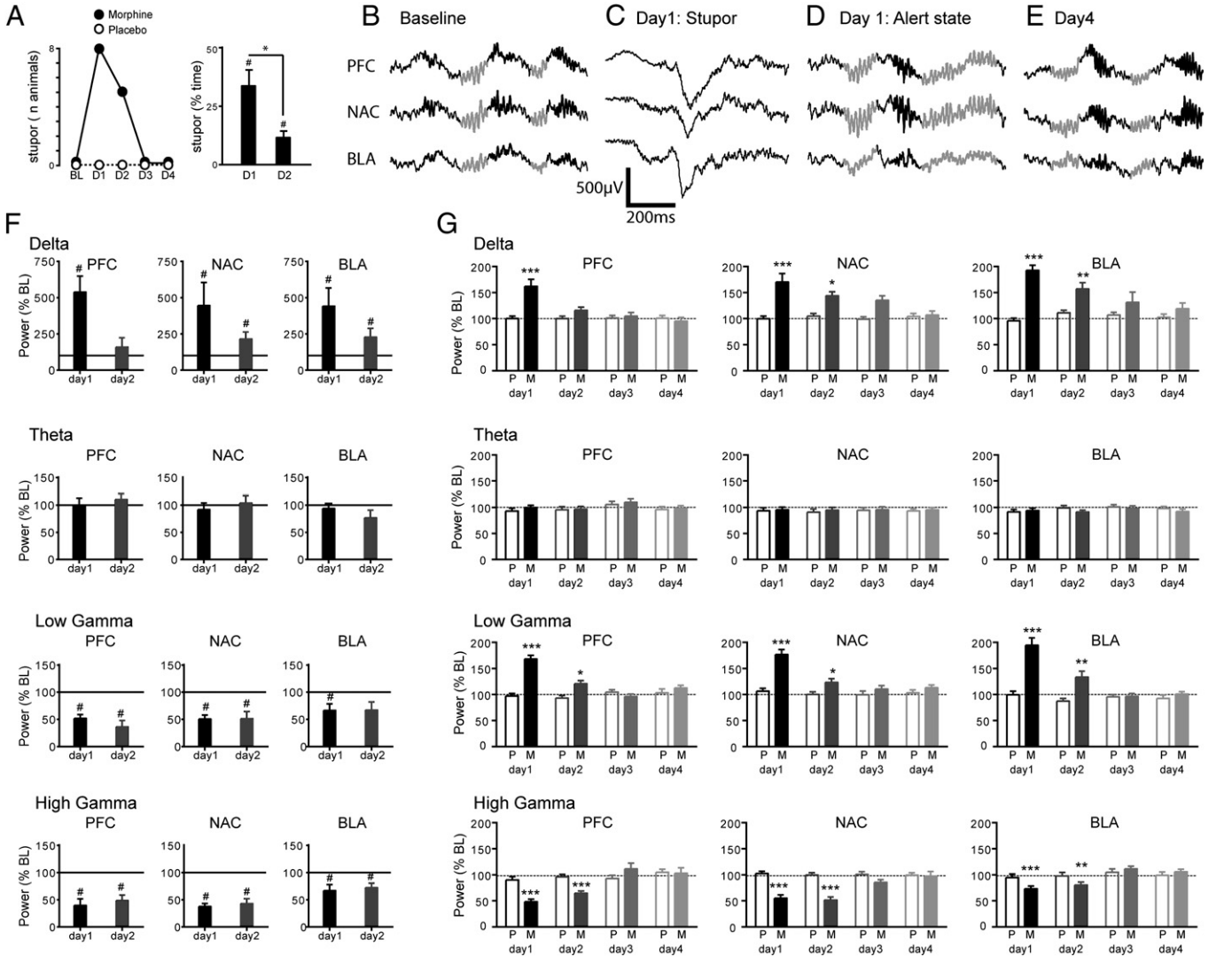

Fig. 4. Morphine induced abnormal behavior and local field potentials normalize after 2 days of chronic treatment. (A) Morphine induced Stupor behavior. *Left:* Number of animals displaying stupor behavior under chronic morphine (M) or placebo (P) treatment. Morphine induced stupor behavior in all 8 animals on day 1 but only in 5 out 8 on day 2. *Right:* Average percentage of time spent in stupor behavior in animals displaying this behavior on days 1 and 2 following morphine pellets implantation. (B, C, D and E) Example traces recorded before (B), 1 day (C, D) and 4 days (E) following morphine pellets implantation. (B) The baseline period before the pellets implantation is characterized by the presence of Delta or Theta oscillations as well as low and high Gamma spindles (thick grey and black portions, respectively). (C) 24 hrs after implantation (day 1) LFP are dominated by epochs with large voltage deflections concurrent with the animal displaying a stupor state. (D) On day 1, stupor epochs alternate with periods of normal behavioral activity (alert state) during which LFP patterns show increased low frequency oscillations in comparison with baseline, together with an increased number and amplitude of low Gamma spindles. (E) On day 4, the same patterns as observed in baseline conditions are present in PFC, NAC and BLA LFP. (F) Average power during stupor state (% of baseline ± SEM) for PFC, NAC and BLA LFP in the Delta, Theta and Gamma ranges. Delta band oscillations were increased while both Low and High Gamma were decreased compared to baseline ($p < 0.05$, one sample t-test). (G) Impact of morphine and placebo chronic treatment on oscillatory activity in the same bands of interest during alert behavioral state. Bars represent the average power (% of baseline ± SEM) for PFC, NAC and BLA LFP in the Delta, Theta and Gamma ranges. Delta and Low Gamma power were increased compared to placebo in all structures during sessions held on days 1 and 2 ($^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$; 2 way ANOVA followed by a Dunnett *post-hoc* test) at the exception of Delta in the PFC on day 2 ($p > 0.05$). High Gamma power was significantly reduced on days 1 and 2 ($^{***}p < 0.001$, $^{**}p < 0.01$; 2 way ANOVA followed by a Dunnett *post-hoc* test).

potential effect of morphine on Theta oscillations. As a result, LFP Theta oscillations and their relationship with single units will not be further commented on in this part of the paper that is concerned with the time course of morphine-related changes. Finally the gamma range was also strongly impacted during stupor as the power of both low and high frequency components was reduced in all structures on days 1 and 2 (Fig. 4F, $p < 0.05$, one sample *t*-test at the exception of BLA on day 2). Analysis of single unit phase-locking within Gamma cycles revealed a very similar pattern. The number of projection neurons phase-locked on Low or High Gamma decreased during stupor epochs compared to baseline (Chi-square test, Fig. S3). Interneurons displayed comparable patterns although the changes observed did not reach significance, probably due to small sample sizes (Fig. S3). Interestingly the percentage of neurons phase-locked on Delta waves did not follow the increase observed in LFP power in that range. However, in the baseline condition, the proportion of phase-locked neurons was between 70% and 89%. One explanation for the absence of change could be the inability of the system to recruit more neurons when Delta oscillations predominate in network activity, hence resulting in a ceiling effect in our sample analysis. An alternative, though not exclusive hypothesis, is that oscillations occurring in the Delta range in the baseline condition and during stupor are of a different nature. This idea is supported from one side by the fact that signal profiles and spectral signatures differ notably during baseline Delta and stupor state erratic slow waves and from the other by the decrease observed in the number of PFC and BLA phase-locked neurons during stupor compared to baseline. Overall, stupor was characterized by a strong prevalence of erratic slow oscillations at the expense of higher frequency range. Interestingly this effect was observed in the entire network and might therefore be correlated with major change in the limbic system function.

Morphine related changes during alert state epochs were substantially different from those observed during stupor. Delta range oscillations were transiently increased compared to placebo animals (Fig. 4G) in all three structures on day 1 (interaction $p < 0.05$, pellets $p < 0.05$, day $p < 0.05$, two way ANOVA, Bonferroni *post-hoc,* $p < 0.001$) and only in the NAC and the BLA on day 2 (Bonferroni *post-hoc,* $p < 0.05$ and $p < 0.01$ respectively). Low gamma oscillations were transiently increased compared to placebo animals (Fig. 4G) in the PFC, the NAC and the BLA on day 1 (interaction $p < 0.05$, pellets $p < 0.05$, day $p < 0.05$, two way ANOVA, Bonferroni *post-hoc,* $p < 0.001$) and on day 2 (Bonferroni *post-hoc,* $p < 0.05$). Conversely, high gamma oscillations were transiently decreased compared to placebo animals (Fig. 4G) in PFC the NAC and the BLA on day 1 (interaction $p < 0.05$, pellets $p < 0.05$, day $p < 0.05$, two way ANOVA, Bonferroni *post-hoc,* $p < 0.001$) and on day 2 (Bonferroni *post-hoc,* $p < 0.01$). Analysis of single unit phase-locking within Gamma cycles also revealed a very similar pattern. The number of projection neurons phase-locked on Low or High Gamma respectively increased and decreased during alert—epochs compared to the same state in baseline (Chi-square test, Fig. S4). Interneurons displayed comparable patterns, although the changes observed did not reach significance, again probably due to sampling (Fig. S4). Interestingly the percentage of neurons phase-locked on Delta waves did not follow the increase observed in LFP power in that range. To explain this discrepancy we favor the presence of a ceiling effect as discussed for the case of Stupor in the previous paragraph. As for stupor the changes observed here occurred at each node of the network and involved a global increase in power and phase-locking in both Delta and Low Gamma combined with a decrease in the High Gamma range. Moreover these modifications were reversed from day 3 as oscillatory profiles returned to baseline level.

Naloxone disrupts morphine-induced network state

We have shown that, in morphine-treated rats, the changes in electrophysiological activity progressively normalized after 2 days resulting in a *de novo* baseline-like network state, even though drug stimulation remained continuous and stable (Gold et al., 1994). We next wanted to investigate whether this new state corresponded to the same physiological network equilibrium as that observed in the drug naive animal or reflected functional adaptation towards a morphine-induced stable state. To test this we interfered with opioid action using peripheral injections of the mu opioid receptor antagonist naloxone (s.c. 15 μg/kg). In morphine treated rats, naloxone induced behavioral signs of withdrawal (Fig. 5A) as the occurrence of dejections, wet dog shakes and teeth grinding episodes were increased in comparison with the placebo group (interaction $p < 0.001$, naloxone $p < 0.001$, pellets $p < 0.05$, 2 way ANOVA, Bonferroni *post-hoc* $p < 0.001$ for each sign). Saline had no overall impact on these signs and naloxone induced changes were no longer observed 24 hrs after the injection.

Naloxone also impacted oscillatory activity in the network specifically in morphine-treated animals with high gamma oscillation power being significantly increased in PFC, NAC and BLA (Figs. 5B–C) as compared to placebo treated rats in which no change was observed (interaction $p < 0.001$, naloxone $p < 0.001$, pellets $p < 0.05$, 2 way ANOVA, Bonferroni *post-hoc* $p < 0.01$ for each structure). Again, saline had no effect on any frequency band and the naloxone effect was not observed 24 hrs after the injection (Bonferroni *post-hoc* $p > 0.05$ for each structure and in each condition). Analysis of single unit phase-locking of projection neurons during Gamma cycles revealed a closely related pattern. The number of projection neurons phase locked on Delta, Theta and Low Gamma were comparable across conditions in morphine and placebo animals. However, High Gamma phase-locking showed an overall tendency towards an increase, though not significant ($0.05 < p < 0.15$, Chi-square test, Fig. S5).Thus, although network oscillatory profiles appeared similar to baseline in placebo and morphine groups, only morphine animals displayed alterations in LFP and related neuronal phase-locking after blockade of opioid receptors.

Discussion

Our group has extensively shown that PFC, NAC and BLA are crucial substrates both for acute opiate withdrawal effects and retrieval of opiate withdrawal memory in dependent and abstinent rats (Frenois et al., 2002, 2005; Lucas et al., 2008, 2012). The present study was designed to investigate the impact of morphine on the dynamics of the PFC–NAC–BLA network using simultaneous electrophysiological recordings in freely-moving rats. Here we show that chronic morphine induced an initial shift in network state. However this shift proved to be transient as oscillatory activity progressively normalized, despite continuous stimulation by the drug. This phenomenon demonstrates significant adaptations to the presence of the drug at the network scale. Blockade of μ opioid receptors in morphine-dependent rats was able to disrupt this apparent stability, revealing that under chronic morphine stimulation, the acquired balance in the PFC–NAC–BLA network depends upon the presence of the drug.

Activity dynamics within the PFC–NAC–BLA network

Recent experiments on single or pair of structures have highlighted that Delta (Fujisawa and Buzsaki, 2011), Theta (Popa et al., 2010; van der Meer and Redish, 2011) and low and high Gamma oscillations (Berke, 2009; Popescu et al., 2009; van der Meer and Redish, 2009) corresponded to functional frequency components and preferred synchronization modes for PFC, NAC and BLA. Here, using simultaneous recordings in the three structures, we have been able to unravel that neuronal activities in these structures oscillate and synchronize with regard to each single rhythm. These findings give strong additional evidence that neuronal coding relies on processing multiple intricate rhythms and occurs not only inside each structure but also between those interconnected elements.

Morphine induces transient changes in PFC, NAC and BLA dynamics

Our results show that morphine induced profound changes in PFC, NAC and BLA network dynamics, but that these changes are transient. Indeed, for the first 2 days, drug stimulation induced a profound reorganization of frequency components and their specific weight within the global network oscillatory profile. More importantly, these changes depend on the different animal behavioral states displayed in association with the drug. During stupor epochs we observed a marked increase in slow oscillations covering both Delta and Theta ranges in combination with a marked decrease across the entire Gamma band, low and high (40–120 Hz). In contrast, during alert epochs, we showed an increase in Delta oscillations and a fine modulation within the Gamma oscillations with an opposite regulation of low and high Gamma bands. Despite the well-known role of this network in reward, drug-associated memories and craving, the changes observed here may reflect a global effect of morphine rather than a specific positive reinforcement effect of morphine. These questions need to be addressed using specific behavioral paradigms to isolate the rewarding and motivational effect of opiates.

Very few studies have investigated the dynamic nature of cortical activity modifications induced by morphine. In the rat frontal cortex, Gamma power is decreased after acute morphine administration (Sun et al., 2006). In parallel, in human subjects, opiate agonist injections induce an increase in slow oscillations, especially in the Delta range (Greenwald and Roehrs, 2005). Our data for the cortical LFP are in line with those two studies, and NAC and BLA Delta and Gamma changes are also consistent with that of the PFC. Thus the morphine-induced modifications observed on days 1 and 2 are likely to reflect the initial acute effects of the drug.

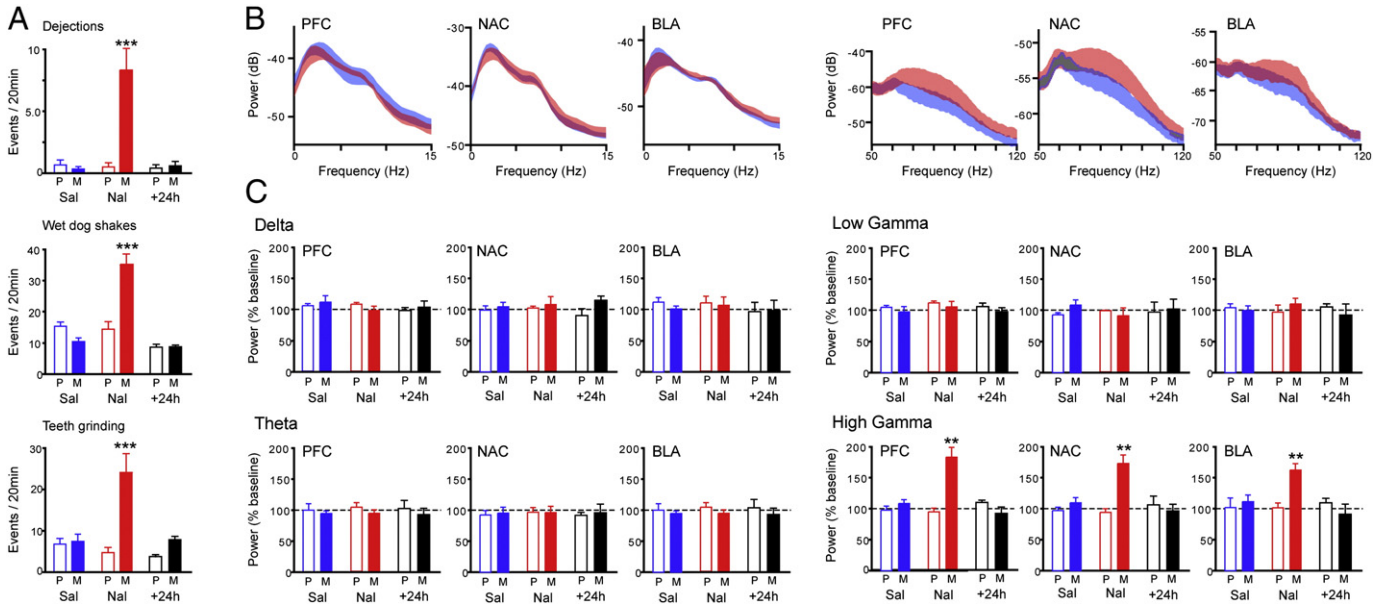

Fig. 5. Naloxone challenge reveals functional imbalance in the PFC, the NAC and the BLA under chronic morphine. (A) Naloxone induces withdrawal signs only in morphine animals. *Top:* number of dejections per 20 minute session in animals implanted with morphine (M) and placebo (P) pellets and treated with saline (Sal), naloxone (Nal. 15 μg/kg) or 24 h after Naloxone treatment. *Middle:* Number of wet dog shakes per 20-minute session. *Bottom:* Number of teeth grinding episodes per 20-minute session. ***Significant difference compared to saline and +24 h conditions ($p < 0.001$, 2 way ANOVA, Bonferroni *post-hoc*). (B) Power spectra (± SEM) of PFC, NAC and BLA LFP after naloxone (red) or saline (blue) injections. (Cn *Left:* Delta and Theta frequency range. *Right*: High and low Gamma frequency ranges. Average power (% of Baseline ± SEM) of PFC, NAC and BLA LFP in the Delta, Theta and Gamma ranges. **Significant difference compared to saline and +24 conditions ($p < 0.01$, 2 way ANOVA, Bonferroni *post-hoc*).

In both stupor and alert states, LFP present abnormal erratic slow Delta oscillations. Interestingly, in human subjects, the positive effects of morphine are associated with an increase in slow waves in the cortex but have also been negatively correlated with morphine induced sedation (Phillips et al., 1994). With rats displaying a significant lack of responsiveness, stupor state reflects an extreme case of sedation. Hence the positive effects of the drug are likely to emerge from neuronal events restricted to alert state epochs rather than that of stupor state. The main difference in LFP oscillatory profiles between these two behavioral states lies in the increase in low Gamma during alert epochs, as compared to the decrease observed during stupor. Increased low Gamma in the NAC has been associated with reward retrieval (van der Meer and Redish, 2009). Considering the present finding that PFC and BLA synchronize with NAC in Delta and Low Gamma ranges, the combination of these two frequencies could act in the network as a drug-related reward signal. The withdrawal signs elicited by naloxone in morphine-dependent rats emerged coincidently with abnormally elevated high Gamma power. This same rhythm was strongly down-regulated throughout the first 2 days following morphine pellet implantation. Low and high gamma therefore present opposite regulation by the drug and exhibit a specific relationship with drug putative rewarding effects and withdrawal aversive effects respectively. Midbrain dopaminergic neuron activity is increased under morphine (Jalabert et al., 2011) and has been shown to lock to cortical LFP Delta rhythm during goal-directed behavior (Fujisawa and Buzsaki, 2011). This suggests that, in opiate dependence, midbrain dopaminergic neurons could interact with the PFC–NAC–BLA network through Delta and Gamma rhythms. Future experiments addressing this issue would be of great benefit for the understanding of the neuronal code behind the rewarding effect of morphine and drugs in general.

Neuronal and behavioral adaptation to chronic morphine

The present study demonstrates that the effect of chronic morphine stimulation on both behavior and network activity developed over time. Indeed the changes attributed to the initial effect of morphine were followed by the late disappearance of stupor epochs together with a reorganization of the oscillatory profiles. This led to a complete reversal of abnormal oscillatory activities in Delta and Gamma bands and an apparent normalization of the activity throughout the network. However, while without effect in placebo animals, naloxone injection was able to promote abnormally elevated high Gamma power and withdrawal signs in morphine-dependent rats. These results extend previous findings obtained in the PFC (Sun et al., 2006) to NAC and BLA activities and show that, although they eventually appear similar, these network activities are intrinsically different in morphine- and placebo-treated animals. More specifically, after 3 days of chronic drug treatment, the network relies on morphine to produce the oscillatory profiles characteristic of a functional system. This indicates that between pellet implantation and day 3, neurons in PFC, NAC and BLA have been the stage of significant neuroadaptations, which could parallel the development of dependence as initially characterized by Gold et al. (1994).

Altered network state and homeostatic dysregulation in addiction

It has been widely documented that chronic morphine administration leads to a large range of neuroadaptations at various levels, including receptors and intracellular signaling as well as synaptic morphology and plasticity (for review, Christie, 2008). Most particularly, complex changes at the level of the mu opioid receptor, the main target of morphine, have been described. But one crucial point is that the morphine potency in inducing tolerance and dependence relies on its low ability to promote receptor internalization and desensitization (for review see Whistler, 2012). However, the identification of the cellular adaptations which are directly relevant to opiate dependence remains a challenge.

Neuronal adaptations are believed to be at the origin of the imbalance in brain function observed in drug addiction and described as a homeostatic dysregulation (Kalivas, 2009; Koob and Le Moal, 1997). Interestingly, the functional activity of the cAMP pathway is initially inhibited by morphine but recovers in spite of a continued opiate exposure. However blocking the opiate effects (e.g. by administration of naloxone) induces an increased rebound of cAMP pathway activity (for review see Nestler, 2004) which is in line with our data and the time course of morphine related changes. Thus beyond the cellular homeostatic adaptations, network activity adaptations are also likely

to contribute to the homeostatic dysregulation processes. Here we analyzed the stability of the PFC–NAC–BLA network as a marker of physiological balance. We showed that morphine is able to challenge this equilibrium as it produced a marked shift in network state which progressively returned to an apparent normal functional state despite the continuous presence of the drug. However when injecting naloxone we also showed that the presence of morphine is a necessary condition for this new equilibrium to endure and for the network to perform normal information processing. In accordance with the allostatic model based on opponent processes (Koob and Le Moal, 1997; Koob et al., 1989), this apparent normalization might actually reflect a new allostatic state corresponding to the net result of adaptive processes engaged within the network to counterbalance drug effects. Our work is therefore the first direct evidence for such inter-systemic allostasis at the level of the network function.

In the pellet model used here, chronic morphine impregnation leads to the induction of a global tolerance to drug effects (Gold et al., 1994). In opiate addicts such tolerance to the substance of abuse is believed to sustain the escalating use with the need to increase doses in order to fully experience the rewarding effect of the drug. We can hypothesize that, as opiate effects progressively disappear between drug taking episodes, the PFC–NAC–BLA network previously exposed to the drug would be in an imbalanced and dysfunctional mode, such as the one shown here under naloxone. In such conditions, the usual opiate consumption will allow the system to reach a functional state that would mimic the normal state; however this dose would not provoke a state shift comparable to that observed with the initial opiate experience. The subject might therefore need to take a higher dose in order to induce the network shift required to produce the acute rewarding effects of the drug. Testing this hypothesis would require simultaneous recordings in a rat model of addiction allowing intermittent episodes of both drug intake and withdrawal (e.g. heroin self-administration or repeated opiate injections). Such studies would improve understanding of how network adaptations following chronic opiate exposure contribute to tolerance, dependence and withdrawal.

Conclusion

Combining original electrophysiological and analytic approaches with a well-established model of opiate dependence, our study provides basic knowledge of oscillation and synchronization frequencies (Delta, Theta, Gamma) underpinning the interaction within the PFC–NAC–BLA network and the complex effect of chronic morphine stimulation on those interactions. This paper also demonstrates new evidence that brainstate stability is affected by chronic drug use, further supporting the allostatic model of drug addiction. Finally our work opens new experimental perspectives to test the mechanisms underpinning both the coding of the rewarding effects of opiates and the neuronal basis of opiate addiction.

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