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DYNAMIC CHANGES IN ORBITOFRONTAL NEURONAL ACTIVITY IN RATS DURING OPIATE ADMINISTRATION AND WITHDRAWAL

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Abstract—The orbitofrontal cortex is involved in the reinforcing effects of drugs of abuse. However, how the dynamic activity in OFC changes during opiate administration and withdrawal period has not been investigated. We first tested the effects of opiates and drug craving with the conditioned place preference paradigm, using manganese-enhanced magnetic resonance imaging and traditional electroencephalograph recording techniques in rats. T1-weighted 2D MRI (4.7 T) was used after unilateral injection of $MnCl_2$ (200 nL, 80 mM) into the right orbitofrontal cortex. The manganese-enhanced magnetic resonance imaging data suggested that the OFC activity decreased during the opiate administration period but recovered increasingly during the withdrawal period. Also, we found decreases and increases in gamma-band (20–100 Hz) activity during the opiate administration and withdrawal period, respectively. Our results showed that orbitofrontal cortex activity decreased during morphine administration and then went up progressively over several days during withdrawal. The time course of the recovery of orbitofrontal activity from inhibition during the withdrawal period may be related to the experience of drug craving. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: conditioned place preference, manganese-enhanced MRI, gamma-band EEG, drug craving.

The orbitofrontal cortex (OFC) is anatomically connected with brain areas known to be involved in the reinforcing effects of drugs of abuse. For example, the OFC receives the projection of the nucleus accumbens (Koob and Bloom, 1988; Ray and Price, 1993) and dopamine (DA) cells in the ventral tegmental area (Oades and Halliday, 1987), which are considered to be the targets of the reinforcing effects of drugs of abuse (Koob and Bloom, 1988). Nucleus accumbens and ventral tegmental area are both recipients of the midbrain dopamine pathway, which is important for mediating the reinforcing and dependence-creating properties of drugs that enhance dopamine function.

Several sources of information suggest that the OFC plays a role in behavioral disorders such as drug abuse and compulsive repetitive behavior. With the use of brain imaging techniques, previous studies have provided evidence of the involvement of the OFC in drug abuse. For example, a [^{15}O]-H_2O positron emission tomography individualized cue exposure paradigm reported that drug craving was associated with activity in the left OFC (Daglish et al., 2001). Similar techniques were used to show that the 'urge to use' correlated strongly with increased regional cerebral blood flow in the inferior frontal and orbitofrontal cortices (Sell et al., 2000). However, it is still unknown how the dynamic activity in OFC changes during opiate administration and withdrawal period. Therefore, in the current studies, we used the manganese-enhanced magnetic resonance imaging (MEMRI) and electroencephalograph (EEG) techniques to investigate the OFC activity in rats during opiate administration and opiate withdrawal period.

Calcium is an essential second messenger required for proper brain function. Depolarization of neurons leads to a rise in intracellular Ca^{2+} transport across the plasma membrane through ligand-gated or voltage-gated Ca^{2+} channels. Thus the activity of Ca^{2+} is an index of neuron activity. However, it is very difficult to observe directly the Ca^{2+} activity *in vivo*. Previous work has shown that Mn^{2+} can be taken up through voltage-gated Ca^{2+} channels (Hu et al., 2001; Narita et al., 1990; Pautler and Koretsky, 2002; Pautler et al., 1998; Takeda et al., 1998), which are ubiquitous in neurons (Silva et al., 2004). Therefore, Mn^{2+} accumulates in neurons through voltage-gated calcium channels in an activity-dependent manner (Silva et al., 2004). Some experiments have suggested that Mn^{2+} has shown potential as an MRI contrast agent that is directly sensitive to brain activity (Lin and Koretsky, 1997).

In addition, EEG gamma rhythms (20–100 Hz) are seen as an emergent property of networks of principal cells and fast-spiking interneurons (Traub et al., 2004). A number of elegant studies have shown that subpopulations of

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Abbreviations: ANOVA, analysis of variance; AP, anteroposterior; CPP, conditioned place preference; EEG, electroencephalograph; MEMRI, manganese-enhanced magnetic resonance imaging; ML, mediolateral; MR, magnetic resonance; OFC, orbitofrontal cortex; ROI, region of interest.

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GABA neurons form networks which oscillate at different frequencies including gamma, and these networks then train the principal cells of the cortex, pyramidal neurons, to oscillate at the same frequency that are capable of generating powerful gamma frequency outputs (Traub et al., 2004). Thus, gamma rhythms, like Mn^{2+} accumulation, could be an index of principal neuron activity. Therefore, in this study, the gamma-band EEG activity was also used as an index to probe the OFC activity during opiate administration and withdrawal period.

As an experimental measure of the mechanisms in which opiate drugs change behaviors in rats, we used the conditioned place preference paradigm (CPP). CPP is a useful method for screening of morphine-induced psychological dependence (Lee et al., 2003) and has been suggested as one of the animal models for drug craving.

EXPERIMENTAL PROCEDURES

Animals

Experiments were performed on male Sprague–Dawley rats (Animal House Center, Kunming General Hospital, Kunming, PR China), 6–8 weeks old, weighing 200–250 g. Animals were group-housed, with a 12-h light/dark cycle and a thermoregulated environment. All experiments conformed to local guidelines on the ethical use of animals. The number of animals used was minimized, and the experiments were performed using anesthetic, without any suffering. All procedures were conducted in strict adherence to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Morphine injection

Rats were injected twice per day at 12 h intervals with morphine hydrochloride (10 mg/mL; First Medicine Factory of Shenyang, Shenyang, PR China) at a dose of 10 mg/kg body weight (Pu et al., 2002; Trujillo and Akil, 1991; Yang et al., 2004). The administration of morphine lasted for 12 days continuously and the withdrawal of morphine lasted for 7 days. The control rats were injected with saline at the same volume.

CPP

The withdrawal behavior and drug craving of one week after morphine withdrawal were tested using the CPP paradigm. The CPP apparatus consisted of three compartments: two equal size large compartments (45×45×30 cm) and the center choice compartment (45×22.5×30 cm) with a smooth gray PVC floor that allowed entry to the main compartments via two guillotine doors. One large compartment was white with a smooth floor and the other was black with a textured floor. An unbiased, counterbalanced and randomized CPP procedure was utilized. There were three phases to the conditioning of place preference: the preconditioning (1–3d), conditioning (4–15d) and postconditioning tests (CPP test 16–22d). Pre-conditioning phase: rats were placed in the center choice chamber with the guillotine doors removed to allow access to the entire apparatus for 15 min. The amount of time spent in each compartment was recorded. In this apparatus, rats show no consistent preference for either big compartment prior to conditioning (Carr and White, 1983). Conditioning phase: rats were immediately confined to one compartment for 50 min after morphine injections and to the other compartment for 50 min after saline injections. All to-be-conditioned rats were injected with either saline (10 mg/kg, i.p.) or morphine hydrochloride (10 mg/kg, i.p.) twice one day. The injection interval was at least 6 h. The order of the injection (morphine or vehicle, i.p.) and compartment (white or black) was counterbalanced across subjects. Postconditioning phase: rats treated with either morphine or vehicle were placed in the choice compartment of the apparatus with doors open and were allowed free access for 15 min. The time spent in each compartment during a 900 s session was recorded. The difference in time spent between the morphine-paired and saline-paired compartments was measured. Comparisons of time spent in the morphine-associated compartment from the different treatment groups were statistically analyzed using two-way analysis of variance procedures (ANOVA).

Manganese stereotaxic injections and perfusion

Stereotaxic injections of $MnCl_2$ were performed respectively 40 min after morphine injection at the 1st, 6th and 12th day of the morphine administration and at the 1st, 3rd, 5th and 7th day after the withdrawal of morphine. Rats (n=5 per group) were anesthetized with pentobarbital (40 mg/kg; Shanghai Chemical Factory, Shanghai, PR China). Stereotaxic microinjection (Shanghai Anting Factory, Shanghai, PR China) of 200 nL of 80 mM $MnCl_2$ (Fourth Factory of Chemistry, Shanghai, PR China) into the right OFC was performed on anesthetized rats. The injection was completed in two minutes (100 nL/min) and the 500 nL syringe was left at the injection site for 5 min before being withdrawn. Stereotaxic coordinates for the OFC were determined from stereotaxic atlas: 3.7 mm anterior from bregma, −2.4 mm mesolateral from the midline, and −4.6 mm ventral.

A time course analysis has revealed optimal contrast-to-noise ratios at about 5–6 h after Mn^{2+} injection (Van der Linden et al., 2002; Watanabe et al., 2004). Therefore, 5 h after Mn^{2+} injection, rats were anesthetized with ketamine (40 mg/kg, i.p.) and were perfused with 10% formalin solution containing 1% potassium ferrocyanide in order to wash out extracellular Mn^{2+}. Subsequently, rats were decapitated and the heads were stored in 10% formalin solution for 6–7 days. Finally, the heads of the rats were scanned by magnetic resonance (MR) imaging.

MR imaging

All MR experiments were performed on a Bruker Biospec 4.7 T/30 cm spectrometer equipped with a 20-cm diameter gradient insert. The volume coil was used for excitation and a surface coil for reception. Contiguous, coronal T_1-weighted images were obtained using an inversion recovery spin echo technique with a slice thickness of 0.8 mm, TR of 3 s, TE of 14 ms, TI of 600 ms, FOV of 3.0×3.0 cm^2 and matrix size of 128×128. The in-plane spatial resolution of the images was 230 μm×230 μm.

Signal intensity

MRI signal intensity of a specific anatomical region of interest (ROI) was analyzed by measuring the average pixel intensity value. A representative ROI in the OFC ipsilateral to the injection site was used for quantitative measurement in the coronal slice. A corresponding ROI of the similar size and location contralateral to the injection site served as control. The correlate ratio of the ipsilateral ROI and the contralateral ROI was calculated. The results are presented as mean±standard error of mean (S.E.M.). The data were analyzed by one-way ANOVA (SPSS 10.0). P value less than 0.05 was considered statistically significant. All data were treated with PARAVISION software (Bruker, Inc).

EEG recording surgery

Surgery was performed under pentobarbitone sodium (40 mg/kg, i.p.) anesthesia. After a midline scalp incision, a burr hole was drilled in the skull over the OFC. A single tungsten wire (100 μm in diameter) insulated and supported by a matched quartz-tube was implanted in the left hemisphere in OFC anteroposterior (AP),

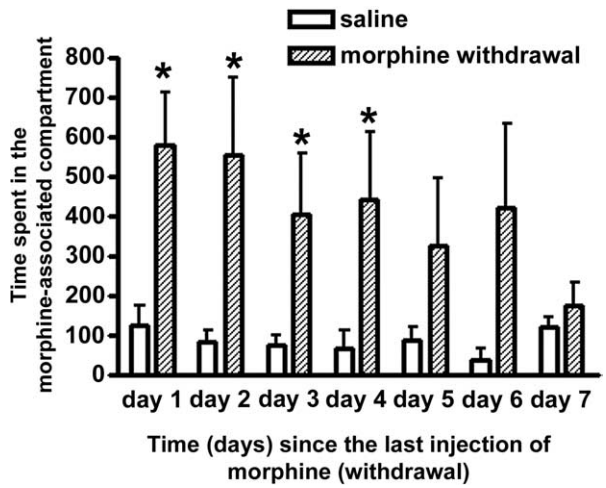

Fig. 1. The difference in time spent between the morphine-paired and saline-paired compartments. The time spent in the morphine-paired compartment was significantly longer than in the saline-paired group for 4 days after withdrawal. The time spent in the morphine-paired compartment was not significantly different from the saline-paired group at the 5th, 6th, and 7th days after withdrawal (two-way ANOVA, * $P<0.05$).

3.7 mm to bregma; mediolateral (ML), 2.4 mm; dorsoventral (DV), 4.6 mm, according to Paxinos and Watson (1986). Two stainless steel watch screws in the bone above the right cerebral regions served as reference (AP, 3; ML, 2) and ground (AP, −5; ML, 2) electrodes. Two or more additional support screws were positioned, and the entire ensemble was secured to the skull with dental acrylic. All electrodes were attached to male pins that were secured in a rectangular three by one pin array and secured with dental acrylic. The rats were allowed 10 days to recover.

EEG recording

Morphine doses were increased from 8 to 30 mg/kg over 3 days and subsequently maintained on 30 mg/kg (day 4 to day 6). Only the morning injection was performed on day 6. This morphine administration schedule has been shown to reliably result in physical dependence (Hutcheson et al., 2001). During the withdrawal period, behavioral procedures identical to that during conditioning period but without the injections were performed on day 7 to day 11 continuously. The EEG signal was recorded at 25 min after the morphine injection and lasted for 2 min. The same procedures were followed during the morphine withdrawal period. Bioelectrical activity was recorded monopolarly against the reference electrode in freely behaving rats. The amplifiers served to eliminate cable movement artifacts. The EEG signal was amplified, filtered (bandpass, 0.5–100 Hz) and digitized (1000 Hz), and data were saved in Microsoft Excel format.

EEG analysis

EEG data recorded during one session were continuously separated into n segments the exact number depended upon the length of the recordings without artifacts by 1.024 s epoch, and every segment was filtered (bandpass: 20–100 Hz, notch: 48–52 Hz). Secondly, the power of every segment was calculated as follows: $P=\Sigma x^2/1024$; and averaged power over N segments served as fast EEG activity power during one session. Normalized fast EEG activity power indicating morphine acute effects was also calculated: $Pnmy=(Pmy-Py)/Py\times100\%$, (Pmy, power of OFC at 25 min after morphine injection; Py, power of OFC before morphine injection at same day; $y=1$–11). All analysis was accomplished using Matlab 6.0 software. Statistical comparisons were made between groups by two-way repeated measures ANOVA, and $P<0.05$ as significant level.

RESULTS

Time differences in the morphine- and saline-paired compartments by CPP apparatus

There were substantial differences in the time spent in the morphine-paired and saline-paired compartments of the CPP apparatus. The time spent in morphine-paired compartment was significantly longer than in saline-paired group (two-way ANOVA, $F(1,10)=9.197$, $P=0.013$, Fig. 1). These results suggested that during the conditioning phase the rats had learned that certain environmental cues were associated with the hedonic experience of the morphine and that during withdrawal the learned relationship resulted in place preferences (Fig. 1, two-way ANOVA, * $P<0.05$) and thus the CPPs were subject to extinction when the pairings between morphine and the sensory cues of the environment were discontinued.

The MEMRI data in OFC during the morphine administration and morphine withdrawal period

Fig. 2 shows MEMRI images in OFC during morphine administration (days 1, 6 and 12) and withdrawal period (days 1, 3 and 5) (only one picture was selected in each group). The data suggested that the Mn^{2+} intensity in OFC

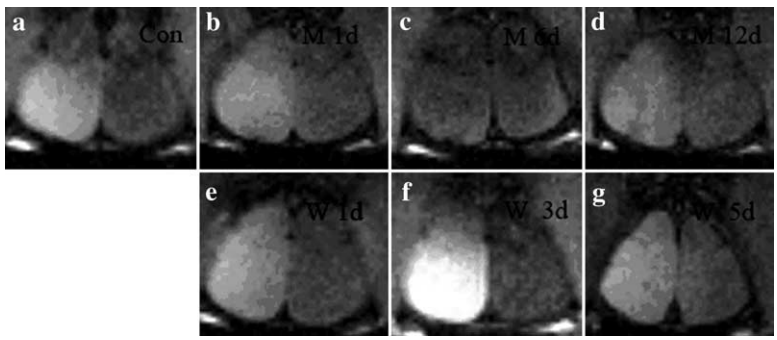

Fig. 2. The differences of the MEMRI signal intensity in OFC between the control group, the morphine administration group and the withdrawal group in rats. (a) The control group. (b–d) The MEMRI signal intensity in OFC during morphine administration. (b) Morphine administration 1st day. (c) Morphine administration 6th day. (d) The morphine administration 12th day group. (e–g) The MEMRI signal integrity in OFC during withdrawal period. (e) Withdrawal 1st day. (f) Withdrawal 3rd day. (g) Withdrawal 5th day.

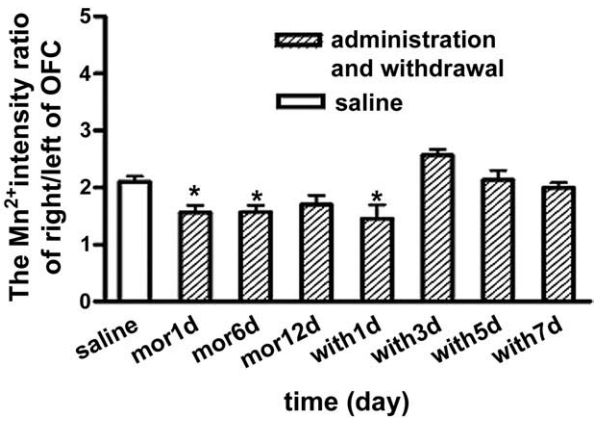

Fig. 3. The results of one-way ANOVA indicate that the Mn^{2+} intensity ratio of the right/left at the morphine administration the 1st–6th days and at the morphine withdrawal the 1st day exhibited respectively a significant decrease than in the saline control group (one-way ANOVA, post hoc Dunnett's C tests, * $P<0.05$). The other days did not show any significant difference compared with the saline group.

exhibited significant decrease compared with the saline control group at the 1st and 6th day of morphine administration (Fig. 3; one-way ANOVA, post hoc Dunnett's C tests, $F(6,28)=7.242$, $P<0.001$). During withdrawal, the Mn^{2+} intensity in OFC decreased significantly at the 1st day after withdrawal but recovered the previous level at the 3rd day after withdrawal. Moreover, the Mn^{2+} intensity on the 5th and 7th day after morphine withdrawal was not significantly different compared with the control group (one-way ANOVA, * $P<0.05$).

Normalized orbitofrontal gamma-band EEG power in OFC during morphine administration and morphine withdrawal period

EEG activity in the OFC was recorded in order to examine possible dynamic changes in brain activity during the morphine administration and withdrawal period. Results showed that acute morphine could induce significant decreases of gamma-band EEG activity, compared with saline injection during all administration period (Fig. 4; * $P<0.05$, ** $P<0.01$, *** $P<0.01$, two-way ANOVA, $F(1,10)=13.626$, $P=0.006$). These data seemed to suggest that the OFC activity is decreased by acute morphine administration. However, the OFC gamma-band EEG power began to rise on the 1st day after withdrawal. The increase in gamma-band EEG power achieved significance compared with the control group on the 3rd and 5th day after withdrawal (Fig. 5; * $P<0.05$, ** $P<0.01$, *** $P<0.01$; two-way ANOVA).

DISCUSSION

The aim of the current study was to investigate dynamic activity changes in OFC in rats during the morphine administration and withdrawal period. Our results showed that OFC activity decreased during morphine administration and then went up progressively over several days during the withdrawal period.

Before discussing these issues in detail, we will address two technical issues that relate to the use of the MEMRI technique. Firstly, in the MEMRI protocol, rats were perfused with 10% formalin solution containing 1% potassium ferrocyanide to wash out extracellular manganese in order to minimize the background noise of the extracellular manganese. In fact, this resulted in MEMRI data indicating the accumulated OFC activity during the time course from the injection of Mn^{2+} to the perfusion in our experiments.

Secondly, a major drawback to the use of Mn^{2+} as an MR detectable contrast agent is its cellular toxicity. Chronic manganese poisoning primarily involves the CNS (Silva et al., 2004). In our experiments, the effects of Mn^{2+} influenced activity for only five hours, in contrast to other studies in which rats were exposed to Mn^{2+} ions for several

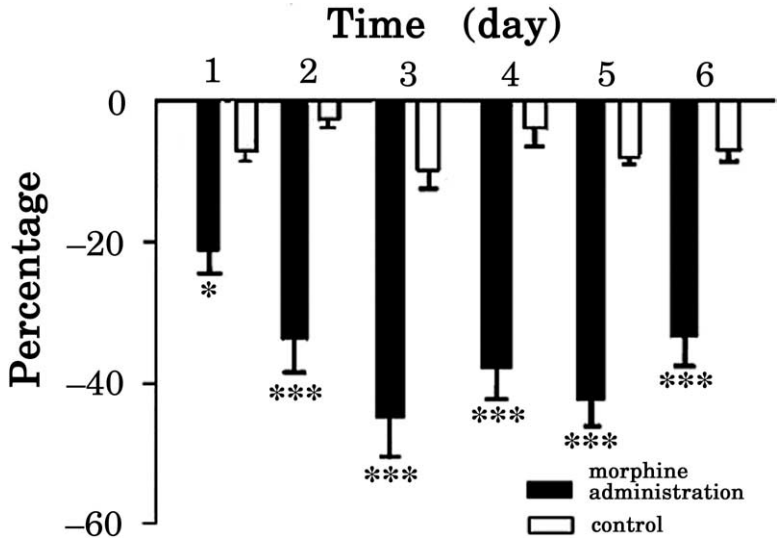

Fig. 4. Normalized orbitofrontal gamma-band EEG powers (mean±S.E.M.) recorded at the 25th minute after acute morphine ($n=8$, filled squares) versus saline ($n=6$, open squares) injection for 2 min during a continuous 6 day period. Morphine doses were increased from 8 to 30 mg/kg over 3 days and subsequently maintained on 30 mg/kg (day 4 to day 6). Two-way ANOVA, * $P<0.05$, ** $P<0.01$, *** $P<0.01$.

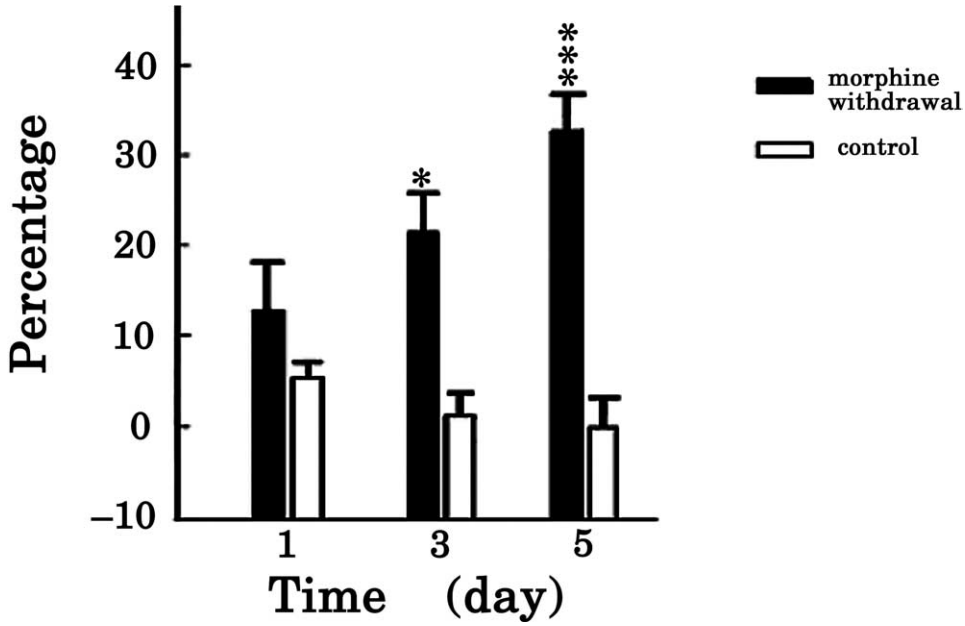

Fig. 5. Normalized absolute orbitofrontal gamma-band EEG powers (mean±S.E.M.) recorded. Recordings were performed during morphine administration and withdrawal (day 1 to day 11). Two-way ANOVA, * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

days, and even weeks (Pautler et al., 2003; Takeda et al., 1998). Thus we believe that the toxicity effect of Mn^{2+} is quite limited in our study.

The CPP data showed that the experience of morphine in the presence of specific environmental cues plays a very significant role in determining the rats' places preferences. Nominally, we term these preferences drug craving though we note that the expression of CPPs reflects Pavlovian conditioning rather than instrumental learning (Lei et al., 2005) and thus we cannot directly show that the rats' behavior was goal-directed.

The MEMRI data showed that the Mn^{2+} intensity in OFC decreased compared with the control group (Fig. 2 and Fig. 3) during morphine administration, which suggests the OFC activity decreased. Few studies have measured regional brain activity during drug intoxication, and most of these studies have employed a single drug exposure. Such studies have shown lower glucose metabolism throughout the brain, including the frontal cortex during morphine intoxication (London et al., 1990). In contrast, the Mn^{2+} intensity at the 1st day after withdrawal showed a significant decrease and recovered to the previous level at the 3rd and 5th day after withdrawal, which suggests that OFC activity went up progressively compared with control group during the withdrawal period.

A comparison of the MEMRI and EEG techniques leads to similar conclusions about the activity of the OFC cortex during morphine administration and withdrawal. The power of OFC gamma EEG decreased during the acute morphine administration period but increased during the morphine withdrawal period (Figs. 3 and 4). Thus, these techniques resulted in similar changes in OFC activity though we note that the methods of morphine injection were different.

Based on the results of MEMRI and gamma-band EEG, it appears that there is an increase in OFC activity during morphine withdrawal. Previous studies have showed lower glucose metabolism in the frontal cortex during morphine intoxication (London et al., 1990). Some studies also showed that, early in the withdrawal period, metabolism in the OFC in cocaine abusers was significantly higher than in a control group. Moreover, metabolism in the OFC was significantly correlated with the intensity of the craving. It has also been shown that increases in brain metabolism are correlated with craving intensity (Volkow et al., 1991).

Considering the results of CPP, the increase in OFC activity during withdrawal may reflect drug craving (defined as a preference for a place in which the rats had the hedonic experience of morphine). We postulate that the increase of OFC excitatory activity during withdrawal may play a role of inhibition in drug craving.

Response inhibition has been previously studied in a neuroimaging paradigm (Vaidya et al., 1998). The OFC was activated in a go/no-go task in fMRI studies. Strong measures of response inhibition were associated with greater volume of activation in the OFC, possibly implicating the OFC in the effort exerted when inhibiting a response (Casey and Rapoport, 1997). More direct evidence was provided for the role of the prefrontal circuit in response inhibition in drug addiction (Goldstein et al., 2001). For cocaine and alcohol abusers, higher orbitofrontal gyrus activation was associated with lower conflict. For the controls, higher OFC activation was associated with higher conflict. Thus, at baseline, increased relative activation of the OFC is associated with worse performance in controls and better performance in substance abusers on the Stroop task, suggesting reversal of the role of the OFC as a function of addiction (Goldstein et al., 2001).

CONCLUSION

In conclusion, our study shows that dynamic changes in brain activity are amenable to study across the addiction and withdrawal process, and we also believe that the MEMRI paradigm has particular promise for exploring brain function underlying behavioral disorders such as addiction.

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