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Extinction of morphine-dependent conditioned behavior is associated with increased phosphorylation of the GluR1 subunit of AMPA receptors at hippocampal synapses

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Abstract

In abstinent opiate addicts, relapse can be triggered by exposure to environmental cues associated with drug use; thus, the disruption of these learned associations may be an effective approach for reducing relapse. Interestingly, glutamatergic systems are thought to be involved in opiate-induced behavioral plasticity. In this study, changes in expression and phosphorylation levels of AMPA glutamate receptor subunits (GluR1, GluR2) in the hippocampus were investigated in rats showing a conditioned response to an opiate-paired environment as well as in animals in which this conditioned behavior was extinguished. Additionally, another set of animals went through a drug-unpaired paradigm (without conditioning) in order to examine the effects of the pharmacology of the drug itself. Subcellular fractionation techniques were used to analyze the local distribution of AMPA glutamate subunits within the synapse, especially at the postsynaptic density (PSD). Results showed that morphine-dependent conditioned responses did not alter expression or redistribution of GluR1 or GluR2; however the unpaired administration of morphine resulted in an increase in the phosphorylation of the GluR1 subunit at extrasynaptic sites. Interestingly, the extinction of the conditioned response significantly increased phosphorylation of the GluR1 subunit at the PSD. Therefore we propose that, within the synapse, the phosphorylation of the GluR1 subunit at the PSD may be a key mechanism in the extinction of opiate-associated conditioned responses.

Keywords

glutamate; CPP; extinction; PSD; rat

Introduction

Relapse in abstinent human opiate addicts can be triggered by the exposure to environmental cues previously associated with drug use (O'Brien *et al.*, 1977). Learned associations that develop between the abused opiate and the environment in which it is consumed are engendered through Pavlovian conditioning processes (Sideroff & Jarvik, 1980). This conditioned response (CR) is long-lasting and can occur despite years of abstinence after drug use (O'Brien *et al.*, 1992). However, little is known regarding the mechanisms by which the conditioned stimulus (CS) may lose its ability to elicit this CR; this process is called extinction. It has been suggested that the previously learned drug-environment association and its extinction are two distinct

learning processes involving different molecular mechanisms (Bouton, 1988; Bouton, 2000; Bouton, 2002; Quirk & Mueller, 2008).

Learning and memory are primary components in the development of context-associated cues (Everitt & Wolf, 2002; Koob *et al.*, 1998; Parker *et al.*, 2006), likewise, they are key to extinction. The hippocampus plays a crucial role in associative memory networks and in the learning of relational information between environmental stimuli (Morris, 2003). Indeed, studies have implicated a role for the hippocampus in morphine-dependent conditioned behavior (Corrigall & Linseman, 1988; Ferbinteanu & McDonald, 2001).

Addictive processes involve a key element of neuronal plasticity (Nestler & Aghajanian, 1997; Robbins & Everitt, 1999). Neuronal plasticity is an enduring change in synaptic efficacy that is thought to underlie the capacity for learning and memory. Indeed, the neuroadaptations in hippocampal function that develop with repeated exposure to opiates may play a role in the modulation of opiate-associated cues (Frohardt *et al.*, 2000; Maren, 2001; Wilson *et al.*, 1995). Though not yet well characterized, glutamatergic systems are thought to be involved in this opiate-induced neuronal plasticity (Trujillo, 2000). In this regard, it has been recently suggested that the AMPA glutamate receptor may represent a key player in the regulation of the molecular mechanisms underlying reactivity to opiate-associated cues (Harris *et al.*, 2004; Moron *et al.*, 2007; Trujillo, 2000; Zhong *et al.*, 2006). Indeed, it has been described that morphine administration alters levels of the GluR1 and GluR2 subunits of AMPA receptors in the hippocampus (Zhong *et al.*, 2006). Therefore, we hypothesize that modulation of AMPA receptors may underlie synaptic plasticity in response to opiate-dependent behaviors (see rev. by (Malenka, 2003).

To test this hypothesis, the expression and synaptic localization of GluR1 and GluR2 subunits of AMPA receptors in the hippocampus were analyzed in rats showing a conditioned response to an opiate-paired environment as well as in animals in which this conditioned behavior was extinguished. To this end we employed the conditioned place preference (CPP) paradigm in combination with an extinction training protocol. Subcellular fractionation was performed to examine the expression and synaptic localization of AMPA receptor subunits in response to these morphine-associated behaviors. We found that extinction of morphine-CPP increases phosphorylation of the GluR1 subunit at the synapse and that this effect may be independent of the molecular pharmacology of the drug itself or drug withdrawal. Thus, these findings provide new evidence for the key role of the GluR1 subunit in the extinction of opiate-induced conditioned responses.

Materials and Methods

Animals and drug

A total of 50 male Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Houston, TX, USA) were used. Animals weighted 175–199 g at the beginning of the experiments and were housed 2 per cage with food and water *ad libitum*. Rats were allowed to acclimate for 10 days in a colony room at a constant temperature (21–23 °C) and humidity (45–50%) on a 12 h light–dark cycle (light 07:00–19:00 h). They were handled daily during the first week after arrival. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch. Morphine sulfate (Sigma-Aldrich, St. Louis, MO) was diluted in sterile saline (0.9% NaCl) and administered subcutaneously (s.c.). All vehicle injections consisted of sterile saline (0.9% NaCl). All rats in the same cage received the same treatment.

Apparatus

A three-compartment place preference apparatus made of Plexiglas (Med Associates, Inc., Georgia, VT), consisting of two main compartments (14.5 cm × 21.5 cm × 21 cm W × L × H), separated by a grey center compartment (30 cm × 21.5 cm × 21 cm W × L × H) was used. One of the main compartments was black with flooring consisting of 0.4 cm stainless steel rods spaced 1.5 cm apart. The other main compartment was white with steel mesh floor spaced 1.2 cm apart (0.1 cm diameter). The center zone was gray with a sheet-metal floor. The system was automated (Med Associates, Inc., Georgia, VT) and used 12 infrared photocell beams in the two main compartments and 6 in the grey one (spaced 5 cm apart and 3 cm above the chamber floor) to allow assessment of side preference. The signal was then processed by the software to extract the “time spent” in seconds in each compartment of the apparatus. Chambers were illuminated with overhead fluorescent lights and lighting was adjusted to 7 lux in each conditioning environment and 30 lux in the grey chamber.

Place preference paradigm

Figure 1A shows the experimental design for the CPP paradigm. Briefly, animals (N=32) received a single preconditioning test (day 0) in which each rat was placed in the center box, and allowed to freely roam the entire CPP apparatus for 15 min in order to assess the unconditioned chamber preference. Absence of a strong preference or aversion for one of the compartments was verified. The next day, rats were assigned to receive saline or morphine paired with one of the two conditioning environments in a counterbalanced manner (unbiased procedure, (Bardo *et al.*, 1995; Cunningham *et al.*, 2003; Cunningham *et al.*, 2006), such that half of the rats were conditioned in their preferred-chamber, and the other half were conditioned in their non-preferred chamber. During conditioning (days 1, 2, 3 and 4), twice-daily sessions were conducted, with animals receiving a 30 min conditioning session by confining them in one side of the CPP chamber in the morning and a second 30 min conditioning session by confining them in the opposite side of the CPP chamber 6 h later. Rats were divided into 2 treatment groups (n=16/group). The morphine-CPP group received morphine (10 mg/kg, s.c.) in one side of the CPP chamber (morphine-paired) and saline in the other side (saline-paired) of the chamber. The saline-CPP group received saline in both sides of the CPP chamber. On the expression test day (day 5), each animal was placed into the central compartment with free access to all three compartments; their activities were monitored over a period of 15 min. A CPP is defined as an increase in the time spent in the chamber formerly paired with morphine (calculated as time spent in the morphine-paired compartment minus time spent in the saline-paired compartment) as compared to saline-conditioned animals (see (Mueller *et al.*, 2002; Mueller *et al.*, 2008)). For subsequent biochemical analyses (see section below), half of the animals were sacrificed immediately following the expression test and their hippocampi were dissected and stored at −80°C. These rats (n=8/subgroup) were designated as morphine-CPP, for morphine-conditioned and saline-CPP for saline-conditioned animals (see Fig. 1A).

Extinction training

Figure 1B shows the experimental design for the extinction training. The extinction of morphine-CPP was performed with the remaining animals (N=16) using an injection-free paradigm (Biala & Budzynska, 2006; Shoblock *et al.*, 2005). Following the initial CPP test, rats were given 15 min tests once a day until the extinction criteria was reached (no injections were given during this extinction period). Extinction criteria was reached when rats spent less than 55% of the total time in the morphine-paired chamber during 2 consecutive days. When a rat reached the criteria it was immediately sacrificed along with a randomly chosen saline control rat. Upon sacrifice, rats hippocampi were dissected and stored at −80°C for subsequent biochemical analyses (see section below). These rats (n=8/subgroup) were designated as

morphine-CPP extinction for morphine-CPP extinguished and saline-CPP extinction for saline-CPP extinguished animals, respectively (see Fig. 1B).

Drug-unpaired treatment

Figure 1C shows the experimental design for the drug-unpaired treatment. This paradigm was performed to distinguish the effects of drug-induced learning from the effects based on the molecular pharmacology of the drug itself. To this end, rats (N=16) received 10 mg/kg s.c morphine or saline following the same protocol than that described above for the place preference paradigm. However, in this case rats were not subjected to any conditioning, i.e. they were not exposed to the CPP apparatus, and treatment conditions (e.g., room for the injection, boxes where rats were placed after the injection, and the experimenter who injected the drug) were randomly changed to avoid any conditioning to those stimuli. Animals were sacrificed 12 h after the last injection and hippocampi were dissected and stored at -80°C for further biochemical studies (see section below). These rats (n=8/group) were designated as morphine-unpaired, for morphine treated and saline-unpaired for saline treated animals, respectively (see Fig. 1C).

Subcellular fractionation and PSD isolation

Subcellular fractionation was performed essentially as described previously (Moron *et al.*, 2007). Briefly, hippocampi (with approximately same wet weight) from individual animals were homogenized in 1.5 ml of 0.32 M sucrose, 0.1 mM CaCl_2 containing protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO). The homogenate was brought to a final concentration of 1.25 M sucrose by adding 2 M sucrose and 0.1 mM CaCl_2 . The homogenate was then placed in an ultracentrifuge tube and overlaid with 1 M sucrose and subjected to centrifugation at 100,000g for 3 h at 4°C . The synaptosomal fraction was collected at the 1.25 M/1 M sucrose interface. To obtain the synaptic junctions, the synaptosomal fraction was diluted with 20 mM Tris-Cl, pH 6, 0.1 mM CaCl_2 , containing 1% Triton X-100 (TX-100) and mixed for 20 min at 4°C , and centrifuged at 40,000g for 20 min at 4°C . The pellet containing the isolated synaptic junctions was collected. To separate presynaptic proteins from the PSD, the pellet was resuspended in 20 mM Tris-Cl, pH 8, 1% TX-100, 0.1 mM CaCl_2 . The mixture was again mixed for 20 min at 4°C , and centrifuged at 40,000g for 20 min at 4°C . The insoluble pellet containing the PSD fraction was collected and stored at -80°C until use. The supernatant containing the presynaptic fraction (PAZ) was collected to exclude presence of AMPA receptors at the presynapse.

Immunoblotting

For immunoblotting, 10 μg of protein from each synaptic fraction obtained from individual animals was resolved in 7.5% SDS-PAGE gels and transferred to nitrocellulose membranes (Scheicher & Schuell, Bioscience, Keene, NH) by electroblotting. Membranes were incubated with selective antibodies to AMPA receptors subunits: anti-GluR1 C-terminal (1:1000; Chemicon, Temecula, CA), anti-GluR2 N-terminal (1:2000; Chemicon, Temecula, CA), and anti-pGluR1 at Serine 845 (1:1000; Chemicon, Temecula, CA). It has been reported that these antibodies do not show cross-reactivity with other AMPA receptor subunits (Gerges *et al.*, 2004; King *et al.*, 2006; Osten *et al.*, 1998; Vissavajhala *et al.*, 1996). After incubating with appropriate fluorescent secondary antibody membranes were scanned directly by the Odyssey Infrared Fluorescent Imaging System (Li-Cor Biosciences, Lincoln, NE). Blots were reprobed with an anti-actin antibody (1:10,000, Sigma-Aldrich, St. Louis, MO) to ensure equal loading and transfer. Blots were also blotted with antibody to tubulin. Band densities were analyzed using Odyssey Software (Li-Cor Biosciences, Lincoln, NE). Quantification was performed by measuring the intensity of the band with protein specific antibodies and comparing it to that of actin since quantitative analysis did not show any changes in actin levels across conditions.

In contrast, tubulin levels were altered upon morphine administration and therefore it was excluded as a housekeeper marker. Additionally, validation of the subcellular fractionation protocol was performed using antibodies to PSD95 (Upstate, Lake Placid, CA), CaMKII alpha subunit (Upstate, Lake Placid, CA), and syntaxin 1 (Chemicon, Temecula, CA).

Data analysis

Morphine-CPP values are reported as mean time $\pm$ SEM difference in time (sec) spent in the morphine-paired chamber versus saline-paired chamber for the morphine treated animals and time spent in the black chamber versus the white chamber for the saline group. During preconditioning, the individual baseline preference for each of the compartments was analyzed using a two-tailed paired Student's t-test. The CPP level was evaluated during the expression test using a two way ANOVA, followed by a two-tailed paired Student's t-test comparing the drug-paired chamber versus the saline-paired chambers for the morphine-CPP, and the black versus the white chambers for the saline-CPP. For the immunoblotting studies, measurement of pGluR1, GluR1 and GluR2 levels were normalized to actin levels in the lane, to control for variation in loading and transfer. Values were normalized to saline control values. Differences between groups were determined using a two-tailed Student's t-test. Significance was set at $p < 0.05$.

Results

Morphine-induced CPP

During preconditioning it was found that animals generally spent more time in the black chamber compared to the white one (data not shown). Therefore, the apparatus and subject assignment procedure were randomly selected to generate an unbiased conditioning procedure (Bardo *et al.*, 1995; Cunningham *et al.*, 2003), such that animals were assigned to the drug-paired chamber in a counterbalanced manner (see Methods section). In CPP level test, a two way ANOVA showed a significant drug effect ($F(1,60)=19.00$; $p<0.0001$) and a significant drug $\times$ chamber interaction ($F(1,60)=4.56$; $p<0.05$). Animals which received saline during each of the conditioning sessions exhibited no significant preference for either of the place cues. The mean time (sec) spent in the black and white compartments respectively, were: 208.7 ± 9.3 sec and 185.6 ± 13.56 sec ($n=16$ /group). Figure 2A shows that animals trained with morphine (10mg/kg s.c., 4 conditioning sessions with the drug) presented a significant place preference for the morphine-paired compartment ($t(15)=6.977$; $p < 0.001$, $n=16$ /group). The mean time (sec) spent in the drug-paired and saline-paired compartments respectively, were: 241.4 ± 7.52 sec and 161.1 ± 8.55 sec ($n=16$ /group). This is in agreement with previous studies showing that morphine (10 mg/kg) is effective as a conditioning stimulus when a total of four drug conditioning sessions are employed (Milekic *et al.*, 2006; Ribeiro Do *et al.*, 2005).

Extinction of morphine-induced place preference

Extinction training was performed using an injection-free paradigm (Biala & Budzynska, 2006; Shoblock *et al.*, 2005), in contrast to other published studies in which animals are injected with saline in the previously drug-associated chamber to accelerate extinction (Mueller *et al.*, 2002; Romieu *et al.*, 2004). Since the present study was designed to elucidate synaptic mechanisms underlying learning processes related to the expression and the extinction of a previously acquired CPP, we chose to perform this injection-free extinction training in order to avoid any confounds that the saline injection might have on the expression of synaptic proteins.

Conditioning was performed as described above. Following the initial CPP test, rats ($n=8$ /group) were given 15 min tests once a day until the extinction criteria was reached (such as rats spent less than 55% of the total time in the morphine-paired chamber during 2 consecutive

days). Place preference for the morphine-paired chambers gradually diminished over time until it did not differ from that of the saline-paired groups. However, an individual temporal variation between subjects in reaching extinction was observed (see Fig. 2B). Indeed, time to extinction ranged from 3 to 13 days depending on the animal (Fig. 2B). Therefore, given this variability, a molecular examination of individual animals will be crucial to understanding the mechanisms underlying extinction of opiate-associated cues.

Isolation of PSD fraction from hippocampal synapses

To study the contribution of hippocampal synaptic proteins (i.e. AMPA glutamate receptors) to morphine-dependent conditioned behaviors, subcellular fractionation was performed in order to purify a fraction enriched in PSD proteins. By examining the expression of AMPA receptors in different synaptic fractions, we were able to elucidate whether morphine-induced behavioral responses led to changes in the synaptic localization of AMPA receptors. For the isolation of hippocampal PSD we used our recently published protocol (Moron *et al.*, 2007). Other studies have used fractionation protocols that yield PSD fractions containing presynaptic proteins (Cotman *et al.*, 1974). The protocol used in this study allows the selective enrichment of PSD-associated proteins, and in addition requires smaller amounts of starting material, making this method more suitable for studies of specific brain regions from individual animals. Therefore, PSD fractionation was conducted individually with hippocampal tissue derived from each animal. Aliquots from different steps of the fractionation process were kept in order to examine expression of AMPA receptors in different synaptic fractions. Using western blotting with PSD markers (PSD-95 and CAMKII) enrichment of PSD proteins during fractionation and absence of those proteins in the presynaptic fraction was confirmed (Fig. 3A). Absence of presynaptic contaminants was confirmed by using antibody to the presynaptic marker syntaxin I, which was enriched at the presynaptic fraction (Fig. 3A).

Phosphorylation of the GluR1 subunit and expression of GluR1 and GluR2 subunits of AMPA receptors was examined in the different synaptic fractions. Figure 3B shows that phosphorylation of GluR1 at serine 845 (pGluR1) is increased at the PSD. In addition, levels of GluR1 and GluR2 were also increased at the PSD when compared to the synaptosomal fraction or the total homogenate. Furthermore, we did not observe the presence of AMPA receptor subunits at the presynaptic fraction (PAZ) (see Fig. 3B).

Morphine-CPP does not alter levels of synaptic AMPA receptor subunits

Next, the synaptic localization and expression of GluR1 and GluR2 AMPA receptor subunits in the hippocampus was examined in morphine-conditioned animals. In addition, the phosphorylation state of GluR1 at Ser845 (pGluR1) was also analyzed. Phosphorylation at this site is mediated by protein kinase A (PKA) (Lee *et al.*, 2000) and it has been correlated with insertion of this subunit at the synapse (Ehlers, 2000). Upon initial CPP testing, animals were sacrificed, hippocampi dissected and subcellular fractionation performed. Although all morphine-conditioned animals showed a strong preference for the drug-paired chamber (see Fig. 2A), biochemical analysis did not show any changes in the expression or synaptic localization of GluR1 and GluR2 AMPA receptor subunits (Fig. 4B, C, respectively) when compared to saline-conditioned controls. In addition, phosphorylation of GluR1 in any synaptic fraction was not affected by this conditioned response to morphine (Fig. 4A). Taken together, these biochemical results show that morphine-CPP does not induce any visible alterations in the expression, phosphorylation and/or synaptic localization of AMPA receptor subunits in the hippocampus.

The extinction of morphine-CPP is associated with an increase in GluR1 phosphorylation at the PSD

Next, we sought to examine whether the extinction of a previously learned association between morphine and environmental cues was associated with changes in expression and synaptic localization of AMPA subunits in the hippocampus. Although we found that morphine-CPP did not have any effect on the expression of AMPA receptors, the extinction of this conditioned response led to a significant increase in the phosphorylation of the GluR1 subunit (Fig. 5A); and this effect was independent of the time elapsed to reach the extinction criteria. Interestingly, this increase was seen locally at the PSD ($t(6)=2.94$; $p<0.05$, total of 7 samples, Fig. 5A) since no significant changes were observed in other synaptic fractions. These data, therefore, suggest that the extinction of morphine-CPP leads to an increase in the phosphorylation of GluR1 at synaptic sites.

In contrast, when total levels of GluR1 were examined, no significant changes were found in any synaptic fraction (Fig. 5B). These data indicate that the extinction of morphine-CPP is affecting the phosphorylation state of this subunit rather than its total expression levels. Since the function of this subunit is regulated by phosphorylation/dephosphorylation, these findings further confirm our hypothesis that the extinction of morphine-CPP may affect the function of GluR1 without affecting its total expression levels. In addition, levels of GluR2 were not affected in any synaptic fraction studied (Fig. 5C). This is in agreement with the notion that most AMPA receptors in the hippocampus are GluR1/2 complexes (Wenthold *et al.*, 1996), therefore the absence of a change in GluR1 levels seems to be consistent with the observed absence of a change in GluR2 levels.

Furthermore, we also performed control groups to examine whether the effects produced were related to extinction of morphine-CPP as opposed to drug withdrawal (see Supplementary Material). To this end, animals were conditioned as described above. Upon morphine conditioning animals were kept at their home cages and sacrificed at 3 or 13 days in order to analyze the effects of morphine withdrawal in the expression and redistribution of AMPA receptors. We selected these time points since time to extinction ranged from 3 to 13 days depending of the animal (see Fig. 2). Data obtained showed that neither the phosphorylation of GluR1 nor the expression and synaptic localization of both GluR1 and GluR2 subunits were altered at 3 days or 13 days upon initial CPP testing (see Supplementary Figure 1). Therefore, these data suggest that the extinction of morphine-CPP is responsible for the observed increase in the phosphorylation of GluR1 at the PSD and excludes a possible effect due to drug withdrawal.

Repeated morphine administration leads to changes of AMPA receptor subunits in synaptic fractions

Finally, the present study examined whether the observed changes were due to morphine-dependent associated behaviors, or were related to the molecular pharmacology of the drug itself. For this purpose, rats were repeatedly treated with morphine using a protocol comparable to that of the CPP but avoiding any possible drug-environment association (drug-unpaired treatment, see Methods section above). Repeated administration of morphine led to a significant increase in the phosphorylation of the GluR1 subunit at the synaptosomal fraction ($t(7)=2.657$; $p<0.05$, Fig 6A). No changes in GluR1 phosphorylation were observed in the total homogenate, which indicates that morphine treatment alters local phosphorylation of this subunit. In addition, no changes were observed at the PSD fraction, which suggests that repeated morphine administration leads to an increase in the phosphorylation of GluR1 at extrasynaptic sites (since AMPA receptor subunits were absent at the presynaptic fraction).

Interestingly, analysis of the total levels of GluR1 and GluR2, after repeated morphine administration showed a significant decrease in the expression of these two subunits at the PSD ($t(7)=4.92$, $p<0.01$) and ($t(7)=4.23$; $p<0.01$); Fig. 6B, C, respectively). In contrast there were no significant changes in the levels of GluR1 and GluR2 in any of the other synaptic fractions examined. Thus, morphine administration seems to decrease total levels of GluR1 and GluR2 locally at the PSD. Therefore, these findings indicate that upon morphine administration there is an increase in the phosphorylation of GluR1 at extrasynaptic sites and a concomitant decrease in total GluR1 and GluR2 levels at the PSD. In addition, these effects seem to be likely due to the pharmacology of the drug itself rather than to a drug-environment learned association.

Discussion

While significant advances have been made in the treatment of opiate addiction through the development of pharmacotherapies, return to drug use after periods of abstinence (relapse) continues to impede the success of opiate addiction treatment programs. Indeed, subsequent exposure to opiate-associated cues is thought to contribute to relapse phenomena (O'Brien, 1997). It has been shown that reactivity to drug-cues and the extinction of this behavior constitute two singular learning processes that are modulated by different neuronal mechanisms (Quirk & Mueller, 2008). In this regard, recent studies have shown evidence implicating glutamatergic neurotransmission, and more specifically the AMPA receptor, in the mechanisms that underlie reactivity to opiate-associated cues (Harris *et al.*, 2004; Kang *et al.*, 2006). This study demonstrates that the extinction of a previously morphine-dependent contextual learned association leads to changes in the phosphorylation of AMPA receptors at hippocampal synapses. In contrast, we do not observe any changes in the expression or phosphorylation of these receptors in morphine-conditioned animals. Therefore, data presented here are specific to drug-related extinction learning versus contextual conditioning alone.

Repeated morphine exposure, but not morphine-CPP, increases GluR1 phosphorylation in hippocampal synaptic fractions

Addictive processes involve a key element of neuronal plasticity (Nestler & Aghajanian, 1997), in that repeated exposure to drugs of abuse produces molecular and neurochemical changes. These changes can be short-lived within the brain and due largely to the molecular pharmacology of the drug itself (Nestler, 2005). In addition, these changes can be persistent and may alter brain circuits that regulate cognitive responses to environmental stimuli (Kalivas & O'Brien, 2008). This study was designed to dissociate neurochemical changes induced by morphine-associated conditioned behavior from those changes based on the molecular pharmacology of the drug itself. To this end, an additional group of animals was repeatedly treated with morphine avoiding any drug-environment association (the morphine-unpaired group). Special attention was paid in order to randomly modify treatment parameters and thus avoid any association between morphine injection and any stimuli. Rats that underwent this treatment showed elevated phosphorylation levels of the GluR1 subunit (at Ser845) at the synaptosomal fraction, with no changes at the PSD. These data suggest, therefore, that administration of the drug itself leads to an increase in pGluR1 levels at extrasynaptic sites (since we did not detect expression of AMPA receptor subunits at the presynaptic fraction). Morphine-induced phosphorylation of GluR1 at Ser845 maybe mediated by PKA, since it is known that repeated morphine administration results in upregulation of adenylyl cyclase and PKA (Duman *et al.*, 1988; Pu *et al.*, 2002). PKA-mediated phosphorylation of GluR1 potentiates excitatory currents mediated by AMPA receptors (Roche *et al.*, 1996) and promotes AMPA insertion at the membrane, ultimately leading to an enhancement of glutamatergic transmission (Ehlers, 2000). In our findings, we do not observe changes in total levels of GluR1 that correlate with the observed increase in pGluR1 levels in the synaptosomal fraction after unpaired administration of morphine. The synaptosomal fraction contains both membrane and

cytosolic compartments within the synaptic terminal (Phillips *et al.*, 2001). The absence of a change in GluR1 levels, thus, may be due to the fact that our synaptosomal preparation contains a pool of both intracellular and cell surface AMPA receptors. Therefore, morphine-induced phosphorylation of GluR1 subunits may result in increased insertion of these subunits at the membrane by shifting the equilibrium between the surface and internal pools of GluR1 and favoring the cell surface pool (pGluR1) without affecting total levels of the receptor at the synaptosomal fraction, although further studies need to be performed to confirm this idea. In this regard, it has been described that synaptic potentiation promotes phosphorylation of GluR1 at S845 in hippocampal neurons and that this effect is correlated with an increase in the insertion of the receptor in the cell surface at extrasynaptic sites (Oh *et al.*, 2006). Interestingly, our data also show that unpaired administration of morphine leads to a decrease in GluR1 levels at the PSD. Therefore, we could hypothesize that by decreasing levels of synaptic GluR1, unpaired morphine administration promotes translocation and phosphorylation of GluR1 at extrasynaptic sites. However, we do not observe an increase in GluR1 levels at the synaptosomal fraction. As mentioned earlier, one possible explanation could be that by decreasing GluR1 levels at the PSD morphine may favor surface delivery of GluR1 from the intracellular to the cell surface pool at extrasynaptic sites. Overall, this effect could have an impact on glutamatergic transmission (Ehlers, 2000). In this regard, it has been recently reported that morphine withdrawal increases glutamate release and, thus, promotes glutamatergic transmission in this brain area (Guo *et al.*, 2005). In addition, it has also been shown that morphine withdrawal increases glutamate uptake and cell surface expression of glutamate transporter at hippocampal synapses (Xu *et al.*, 2003). Therefore, these findings together with our data highlight the role of glutamatergic systems in morphine-induced adaptive changes in the hippocampus.

In contrast, animals that showed a strong morphine-induced place preference did not exhibit any changes in the expression or synaptic localization of AMPA receptors. This is in agreement with a recent study showing that the expression of amphetamine-associated conditioned behavior does not correlate with any changes in the phosphorylation of the GluR1 subunit in the hippocampus (Sakurai *et al.*, 2007). Based on these results, the authors suggested that phosphorylation of the GluR1 subunit did not play a key role in the mechanisms underlying reactivity to drug-cues. Therefore, it is possible that when hippocampal circuits are engaged, entirely different mechanisms are involved in opiate-induced associative learning.

Extinction of morphine-CPP promotes persistent changes at glutamatergic synapses in the hippocampus

It is thought that extinction training may decrease relapse to drug-seeking behavior by replacing previously drug-dependent learned associations with a new learning. Indeed, extinction is now defined as a new and specific learning process (Bouton *et al.*, 1993; Myers & Davis, 2002; Quirk & Mueller, 2008). Additionally, it has been reported that Pavlovian conditioning and extinction diverge according to the particular brain structure involved and the underlying molecular mechanism (see reviews: (Lattal *et al.*, 2006; Myers & Davis, 2002)). The present study shows that rats that underwent extinction training upon the initial CPP test exhibited a significant increase in GluR1 phosphorylation at the PSD, with no changes in total GluR1 levels. These data suggest that by increasing GluR1 phosphorylation the extinction of morphine-CPP may result in facilitation of AMPA-mediated currents at the synapse (Roche *et al.*, 1996) without altering the number of receptors at the PSD. In light of the importance of the PSD in synaptic regulation and plasticity underlying learning and memory (Milner *et al.*, 1998), the observed effects may play a key role in the extinction of morphine-induced environmental associations. Interestingly, a significant increase in pGluR1 levels at the hippocampal PSD was found in all extinguished morphine-CPP rats, regardless of the time they took to reach extinction (3 to 13 days). In addition, this effect seems to be specific to

extinction training as opposed to morphine withdrawal since animals that were initially conditioned and then withdrawn from morphine at their home cages did not show any changes in the phosphorylation or synaptic redistribution of the GluR1 subunit in the hippocampus.

Therefore, these findings suggest that extinction training promotes enduring postsynaptic changes at glutamatergic synapses and that these changes are not dependent on the time elapsed after discontinuation of drug administration. Moreover, our results show that extinction of the previously learned morphine-dependent association promoted an increase in the phosphorylation of GluR1 subunit within the hippocampus and that this effect was absent in animals showing a conditioned response to morphine. It is known that drug-dependent conditioned responses and the extinction of this behavior constitute two different types of learning processes (Myers & Davis, 2002; Quirk & Mueller, 2008), and this study provides further biochemical evidence that both learning processes may be regulated by different mechanisms in the hippocampus. In addition, this study provides new information about the role that the GluR1 subunit of AMPA receptors, within the hippocampus, may play in extinction learning. Indeed, the hippocampus is a brain region known to participate in associative processes and more specifically in the multimodal and contextual representations (Eichenbaum, 2003; Lavenex & Amaral, 2000). Therefore, our data also provides supportive evidence for the role of the hippocampus in the extinction of the previously drug-dependent learned association. This is in agreement with other studies of the role of the hippocampus in extinction processes (Rossato *et al.*, 2006; Szapiro *et al.*, 2003; Vianna *et al.*, 2001; Vianna *et al.*, 2003).

In summary, this study shows that the extinction of morphine-induced conditioned responses results in an increase in phosphorylation levels of the GluR1 subunit of AMPA receptors at hippocampal synapses and that this effect is not associated to drug withdrawal. Therefore, this synaptic adaptation may be one of the key mechanisms that could trigger extinction of opiate-dependent conditioned behavior and, therefore, prevent relapse to drug-seeking behavior.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CPP	conditioned place preference
PSD	post synaptic density
PAZ	presynaptic fraction

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Figure 1.

Schematic representation of the experimental design. (A, B) After a single preconditioning test (day 0), rats received two daily conditioning sessions from days 1 to 4. Morphine-conditioned animals received morphine (10 mg/kg, s.c) in one side of the CPP chamber and saline in the other side. Saline controls received saline in both chambers. Expression test was performed on day 5. (A) Upon expression test, half of the animals (morphine-CPP and saline-CPP, n=8/subgroup) were immediately sacrificed and their hippocampi dissected. (B) Extinction training was performed with the remaining animals. Following expression test, animals received a single daily extinction training. When an animal reached extinction criteria it was immediately sacrificed along with a randomly chosen saline control (morphine-CPP extinction, saline-CPP extinction, n=7–8/subgroup). (C) In the unpaired group, rats received 10 mg/kg s.c, morphine or saline (morphine- and saline-unpaired respectively, n=9 per group) following the same protocol than that described for the place preference paradigm but without any conditioning. Animals were sacrificed 12 h upon last morphine administration.

A Expression of Morphine-CPP B Extinction of Morphine-CPP

Figure 2.

(A) Conditioned place preference to morphine. Animals trained with morphine exhibited a significant place preference for the morphine-paired compartment. Rats were conditioned with morphine (10 mg/kg s.c.) or saline for 4 days (n=16 per group). Data represent the time spent (sec) in the morphine-paired chamber versus the saline-paired chamber for the morphine treated rats and time spent in the black chamber versus the white chamber for the saline group (mean $\pm$ SEM). *** $p < 0.001$, two-tailed paired t-test. (B) Extinction of conditioned place preference to morphine. Following initial CPP testing, rats were given daily tests until the extinction criteria was reached. Place preference for the morphine paired chambers gradually diminished over time. Summary of the variability between subjects in the extinction of morphine-dependent CPP. Time to extinction ranged from 3 to 13 days depending on the animal.

Figure 3.

Biochemical validation of the subcellular fractionation protocol performed to isolate PSD from rat hippocampus. Enrichment of PSD proteins during fractionation was confirmed by western blotting. (A) 10 ug of protein from hippocampus homogenate (Homo), synaptosomes (Syn), synaptic junctions (Syn Junc), PSD, and presynaptic fraction (PAZ) were separated by SDS-PAGE and probed with antibodies to known postsynaptic (PSD95, CaMKII) and presynaptic markers (syntaxin 1). (B) Synaptic localization of AMPA receptor subunits within the hippocampal synapse. Western blot analysis indicated that phosphorylation of the GluR1 subunit and expression of GluR1 and GluR2 subunits of AMPA receptors is increased at the PSD and absent at the presynaptic fraction (PAZ).

Figure 4.

The expression of morphine-CPP does not induce changes in expression of GluR1 and GluR2 receptors in synaptic fractions. Upon initial CPP testing, rats were sacrificed, hippocampi dissected, and subcellular fractionation performed in individual samples. Expression and phosphorylation of AMPA subunits within the synapse were analyzed by western blotting using antibodies to phospho-GluR1 Ser845 (pGluR1) (A), GluR1 (B) or GluR2 (C). Although rats exhibited a strong CPP to morphine, western blotting analysis did not show any changes in the phosphorylation state or the expression levels of GluR1 (A, B) and GluR2 (C) subunits of AMPA receptors in any synaptic fraction studied. A representative western blot is shown for each of the receptor subunits studied. Measurement of pGluR1, GluR1 and GluR2 levels were normalized to actin levels in the lane. Data (mean $\pm$ SEM) are normalized to saline controls and represent the sum of 8 individual animals. *Homog*, homogenate; *Synap*, synaptosomal fraction; *S*, saline; *M*, morphine.

Figure 5.

The extinction of morphine-CPP increases phosphorylation of GluR1 at the PSD. Upon initial CPP testing, animals underwent extinction training. When an animal reached extinction criteria it was sacrificed along with a randomly chosen saline control. Subcellular fractionation was performed in individual samples. Expression and phosphorylation of AMPA subunits within the synapse were analyzed by western blotting using antibodies to phospho-GluR1 Ser845 (pGluR1) (A), GluR1 (B) or GluR2 (C). (A) Extinction of morphine-CPP led to a significant increase in the phosphorylation of GluR1 at the PSD; no significant changes were observed in other synaptic fractions. (B, C) No changes were observed in the levels of GluR1 and GluR2 in any of the synaptic fractions studied. A representative western blot is shown for each of the receptor subunits studied. Measurement of pGluR1, GluR1 and GluR2 levels were normalized to actin levels in the lane. Data (mean $\pm$ SEM) are normalized to saline controls and represent the sum of 7–8 individual animals. * $p < 0.05$, two-tailed paired t-test. *Homog*, homogenate; *Synap*, synaptosomal fraction; *S*, saline; *M*, morphine.

Figure 6.

Repeated unpaired administration of morphine (without any conditioning) leads to changes in the phosphorylation and expression of GluR1 and GluR2 receptors in synaptic fractions. Animals were sacrificed 12 h upon the last morphine administration (10 mg/kg s.c.) and their hippocampi dissected. Subcellular fractionation was performed in individual samples. Expression and phosphorylation of AMPA subunits within the synapse were analyzed by western blotting using antibodies to phospho-GluR1 Ser845 (pGluR1) (A), GluR1 (B) or GluR2 (C). (A) Unpaired morphine administration led to a significant increase in the phosphorylation of GluR1 at the synaptosomal fraction with no changes in other synaptic fractions. (B, C) Levels of GluR1 and GluR2 were decreased locally at the PSD. A representative western blot is shown for each of the receptor subunits studied. Measurement of pGluR1, GluR1 and GluR2 levels were normalized to actin levels in the lane. Data (mean $\pm$ SEM) are normalized to saline controls and represent the sum of 8 individual animals. * $p < 0.05$; ** $p < 0.01$, two-tailed paired t-test. *Homog*, homogenate; *Synap*, synaptosomal fraction; *S*, saline; *M*, morphine.