Orexin Mediates the Expression of Precipitated Morphine Withdrawal and Concurrent Activation of the Nucleus Accumbens Shell

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Background: The lateral hypothalamic neuropeptide orexin (or hypocretin) is implicated in drug addiction. Although a role for orexin has been shown in reward and dependence, the molecular and neural mechanisms are unclear. We investigated the mechanism and neuroanatomic basis of orexin's role in morphine withdrawal.

Methods: C57BL/6J mice received chronic morphine followed by naloxone (0 or 1 mg/kg, subcutaneous) to precipitate withdrawal. Before naloxone, mice received SB-334867 (0 or 20 mg/kg, intraperitoneal), an orexin 1 receptor (Ox1r) antagonist. Using immunohistochemistry, c-Fos, a marker of cell activation, was quantified in the nucleus accumbens (Acb), lateral hypothalamus (LH), ventral tegmental area (VTA), and locus coeruleus (LC). Retrograde tracing with fluorogold (FG) was performed to determine whether orexin neurons project directly to the Acb.

Results: SB-334867 before naloxone significantly attenuated withdrawal symptoms. Withdrawal was accompanied by an increase in c-Fos expression in the Acb shell (AcbSh), which was reduced by SB-334867 but had no effect on the VTA or the LC. Morphine withdrawal increased c-Fos expression in the dorsomedial (DMH) and perifornical (PFA) regions but not in the lateral region of the LH (LLH). Orexin neurons do not appear to form direct connections with Acb neurons.

Conclusions: Altogether, these data demonstrate that orexin, acting via Ox1r, is critical for the expression of morphine withdrawal. AcbSh activation during withdrawal is dependent on Ox1r function and is likely mediated by indirect action of LH orexin neurons.

Key Words: Addiction, drug dependence, heroin, hypocretin, neuropeptide, opiate, orexin, orexin receptor, retrograde tracing

Many models of drug addiction include both positive and negative reinforcement as key components. Continued drug use is partly a result of positive reinforcement from rewarding effects of drug taking and negative reinforcement from withdrawal that accompany cessation of drug taking. The mesocorticolimbic dopamine (DA) system, originating in the ventral tegmental area (VTA) and projecting to terminal regions such as the nucleus accumbens (Acb), amygdala, and prefrontal cortex, has been identified as an essential neural network in which drug-induced neuroadaptations occur that lead to both types of reinforcement. The reinforcing effects of drugs of abuse are associated with increased dopaminergic neurotransmission (1,2) in the Acb (3–6). During cocaine self-administration, animals will lever-press to maintain elevated DA levels (7). In contrast, decreased accumbal DA levels have been associated with morphine withdrawal (8–10).

Drug abstinence results in somatic ("physical") withdrawal as well as motivational ("psychological") withdrawal. Some evidence suggests that these components of withdrawal are mediated by distinct neural systems. In morphine-dependent animals, opiate antagonists in the locus coeruleus (LC) (11–14) and the periaqueductal gray (13,15) precipitate robust somatic withdrawal syndromes, whereas infusions into the Acb generates only a few somatic symptoms (13). Administration of opiate antagonists directly into the Acb and amygdala in morphine-dependent animals results in motivational withdrawal as indicated by the attenuation of lever pressing for food (16) and conditioned place aversion (17). However, other experimental data suggest that some overlap among these neural systems exists. For instance, direct administration of opioid antagonists in the amygdala of morphine-dependent animals is associated with moderate somatic withdrawal (13). Furthermore, systemic DA agonist administration attenuates both conditioned place aversions and somatic withdrawal symptoms in morphine-dependent animals treated with naloxone, while increasing phosphorylation of GluR1 in the Acb (18), indirectly implicating the Acb in both withdrawal components. Altogether, these findings suggest a role for limbic structures in both somatic and motivational withdrawal.

The lateral hypothalamus (LH) has also been shown to be involved in reinforcement and neuroadaptations in response to drugs of abuse. Evidence for the role of the LH in positive reinforcement comes from the work of Olds and Milner (19), which showed that animals will robustly lever-press for LH electrical self-stimulation. Furthermore, animals will self-administer opiates, such as morphine, directly into the LH (20,21), and administration of an opioid antagonist directly into the LH blocks systemic heroin self-administration (22). Similarly, opioid administration directly into the LH results in a conditioned place preference (CPP) effect (23). We have previously presented evidence demonstrating a role for the LH in negative reinforcement by implicating orexin (also called hypocretin), a hypothalamic neuropeptide, in morphine dependence and withdrawal (24).

Orexin-containing neurons are restricted to a few regions of the LH—the lateral region (LLH), perifornical area (PFA), and dorsomedial hypothalamus (DMH) (25–27)—and have been shown to project broadly throughout the brain (26,28,29). The orexin ligands orexin A and orexin B arise from the precursor peptide prepro-orexin by proteolytic processing (27,29) and

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Received August 20, 2007; revised March 4, 2008; accepted March 5, 2008.

0006-3223/08/$34.00
doi:10.1016/j.biopsych.2008.03.006

activate the two G-protein coupled receptors, orexin 1 receptor (Ox1r) and orexin 2 receptor (Ox2r) (27). Interestingly, the orexin receptors and orexinergic projections from the hypothalamus are localized in regions previously shown to play a role in drug addiction, such as the VTA (26,30–32), Acb, substantia nigra (26), and LC (33,34). Orexin neurons located in the LH innervate several regions along the trajectory of the mesocorticolimbic pathway (26,32). Ox1r mRNA is colocalized with the selective DA neuronal marker tyrosine hydroxylase (TH) in the VTA and in vitro activation of orexin receptors activates dopaminergic VTA neurons, leading to excitation (34), whereas activation of orexin receptors in the Acb leads to inhibition (35). In the LC, orexin has been shown to excite neurons primarily via Ox1r activation (36).

Early studies suggest a role of orexin in sleep regulation as orexin knockout mice are narcoleptic and loss of orexin neurons leads to narcolepsy (25,37–39). Further behavioral experiments have expanded beyond sleep regulation to orexin's role in general arousal, feeding, and metabolism (40,41) and suggest a possible role of orexin in drug and natural reinforcement. For instance, animals that express conditioned place preference (CPP) in response to morphine, cocaine, or food express increased c-Fos activation in orexin neurons of the LH, and blockade of orexin receptors attenuates the CPP effect in response to such stimuli (42). Systemic administration of orexin A leads to increased reinstatement of extinguished lever-pressing for cocaine (43) and blockade of Ox1r at the level of the VTA significantly blocked the development, but not the expression, of cocaine sensitization (44). By using orexin knockout mice, our laboratory has previously shown that orexin neurons respond to morphine withdrawal and that orexin mutant mice demonstrate attenuated somatic morphine withdrawal symptoms (24).

Although it is clear that orexin is important in components of drug addiction, little is known about the neural circuits and mechanisms by which orexin mediates drug dependence. In this study, pharmacological Ox1r blockade, c-Fos analysis, and retrograde tracing techniques were used to establish a role for Ox1r in naloxone-precipitated somatic morphine withdrawal.

Methods and Materials

Subjects

Subjects were 72 male C57BL/6J mice, obtained from Jackson Laboratories (Bar Harbor, Maine), between 8 and 12 weeks of age, before the onset of the experiments. Mice were housed in groups of five and maintained on a 12:12-hour light:dark cycle with ad libitum access to food and water except during the morphine withdrawal observation session.

Morphine Treatment and Withdrawal

For chronic morphine treatments, mice ($n = 36$) were injected with escalating morphine doses (20, 40, 60, 80, 100, and 100 mg/kg, intraperitoneal [IP]) every 8 hours for 2.5 days. To assess morphine withdrawal, each mouse was injected with naloxone (0 or 1 mg/kg, subcutaneous) 2 hours after the last morphine injection. Mice were removed from their home cage and placed in a standard clear plastic mouse home cage without the bedding, which served as the observation chamber. Withdrawal sessions took place in the early phase of the light, or inactive, cycle; naloxone injections were administrated 4 hours into the light cycle. Before naloxone treatments, the presence of Straub tail was noted to confirm morphine dependence (45). The occurrence of each withdrawal symptom (jumping, paw tremors, gnawing, head swoops, tremors, wet-dog shakes, ptosis, and backward walking) was counted by an investigator blind to treatment conditions for 20 min following naloxone treatment. To evaluate the severity of withdrawal, a global withdrawal score was computed by multiplying the sum total obtained for each sign by a constant and adding the scores for each sign.

The constant assigned to each symptom, designed to reflect the severity and occurrence of a particular symptom, was adapted from previously published global score calculations for both rats (46) and mice (47,48). Jumps and paw tremors were multiplied by .1; gnawing, ptosis, and head swoops were multiplied by .5; tremors, wet-dog shakes, and backward walking were multiplied by 1.0. To assess the effects of orexin antagonism in the expression of morphine withdrawal, mice received an injection of SB-334867 (0 or 20 mg/kg, IP), an Ox1r antagonist, 20 min before naloxone injections and global withdrawal scores were calculated.

Immunohistochemical Studies

Please see Supplement 1.

Retrograde Tracing

All surgeries were performed under aseptic conditions, using sodium pentobarbital for anesthesia. Fluorogold (FG) injections (.05 μL of .5% FG in .9% saline) were made bilaterally using a 5 μL Hamilton microsyringe (Hamilton, Reno, Nevada). The coordinates for Acb injections were as follows: 1.2 mm rostral to bregma, ± 0.5 mm from midline, and 3.5 mm below the surface of the dura matter. Mice were perfused 7 days postinjection, and the brains were removed and immunostained for orexin or TH following the same immunohistochemical procedures described earlier.

Drugs

Morphine sulfate (provided by the National Institute on Drug Abuse) and naloxone (Sigma, St. Louis, Missouri) were dissolved in saline. SB-334867 (1-(2-methylbenzoxazol-6-yl)-3-[1,5]naphthyridin-4-yl urea hydrochloride) was synthesized by GlaxoSmithKline (Harlow, United Kingdom) and dissolved in 10% (w/v) (2-hyroxypropyl)-β-cyclodextrin in sterile water. The dose of SB-334867 is consistent with other recent studies (42,50).

Data Analysis

To assess the role of orexin antagonism on the expression of morphine withdrawal, the results were expressed as mean ± SEM of global withdrawal scores. To quantify colocalization of orexin with c-Fos in the LH and TH with c-Fos in the VTA and LC, results were expressed as mean ± SEM of percentage of c-Fos positive orexin or TH cells. Multiple two-way analysis of variance (ANOVA) were conducted with dose of SB-334867 and dose of naloxone as between-subject factors.

Results

Ox1r Blockade Attenuates the Expression of Morphine Withdrawal

To investigate the role of Ox1r in the expression of morphine withdrawal, mice chronically injected with morphine received naloxone to precipitate withdrawal. Precipitation of withdrawal by naloxone was accompanied by somatic behavioral signs of withdrawal. Animals pretreated with SB-334867 (20 mg/kg, IP) before naloxone-precipitated withdrawal demonstrated a striking reduction in several withdrawal symptoms, including tremors, wet-dog shakes, backward walking, and ptosis, and global withdrawal scores were significantly lower than animals pre-