Morphine-induced modulation of LTD at GABAergic synapses in the ventral tegmental area
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ABSTRACT
Adaptive behaviors often require the learning of appropriate responses to rewarding stimuli, yet aberrant learning processes can lead to serious diseases such as addiction. Dopamine (DA) neurons of the ventral tegmental area (VTA) play an essential role in the treatment of rewarding stimuli, and they exhibit plasticity in response to such stimuli, but also to drugs of abuse. Previously we discovered a form of presynaptic nitric oxide (NO)-mediated long-term potentiation (LTPGABA) at GABAergic synapses onto VTA DA neurons that is prevented with morphine in vivo 24 h after exposure. Here we investigated whether the same GABAergic synapses are capable of exhibiting long-term depression (LTD in addition to LTPGABA) and its possible modulation by morphine in vivo. We found that indeed the efficacy of VTA GABAergic synapses can be down-regulated through induction of a novel form of LTD (i.e., LTDGABA) in response to synaptic stimulation. Paired pulse ratio (PPR) and coefficient of variance (CV) analyses of evoked IPSCs confirmed that this plasticity may be postsynaptic. Consistently, LTDGABA did not involve presynaptic cannabinoid CB1 receptors (CB1Rs). Moreover, NMDAR activation was not necessary for LTDGABA. However, blockade of D2 dopamine receptors (D2R) significantly attenuated LTDGABA proposing a novel synaptic mechanism for the regulation of excitability of DA neurons by endogenous DA and D2R activation. Interestingly, 24 h after a single in vivo exposure to morphine, LTDGABA was absent in slices from morphine-treated rats but unaffected in slices from saline-treated rats, confirming a bidirectional impact of morphine on GABAergic synaptic plasticity in the VTA. The control of bidirectional GABAergic plasticity by morphine in the VTA may represent the neural correlates necessary for the addictive properties of opiates.

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1. Introduction
Drugs of abuse to include opiates, hijack synaptic plasticity mechanisms in reward-related brain areas. This highlights their value and reinforces their abuse (i.e., establishment of compulsive drug-seeking and drug-taking behaviors) (Kauer and Malenka, 2007). More recently, a critical link was found between losses of synaptic plasticity in the nucleus accumbens (NAc) of cocaine-addicted animals to their inability to control their cocaine-intake (Kasanetz et al., 2010). Such convincing evidence in line with previous findings supports the idea that synaptic plasticity is required not only for reinforcement of reward-based learning and motivated behaviors, but also for the addiction process. The well-studied forms of synaptic plasticity implicated in learning and addiction are LTP (strengthening of synapses) and LTD (weakening of synapses).

The VTA consisting of DA and GABA neurons is an area crucial for reward and drug reward. DA release originating from VTA DA neurons in a VTA target (specifically in the NAc) is believed to initiate drug-induced neuroplasticity necessary for the formation of addictive behaviors (Kauer and Malenka, 2007). Both glutamatergic and GABAergic synapses regulating VTA DA cell activity are found to be drug-sensitive and subject to plasticity (Nugent and Kauer, 2008). Glutamatergic synapses onto VTA DA neurons can be potentiated in response to a single in vivo exposure to different classes of addictive drugs including opiates which is believed to be essential for initiation of behavioral sensitization (Saal et al., 2003;
Ungless et al., 2001). On the other hand, GABA enhancers have been found to be effective in impeding the learning of drug-seeking behaviors (Volkow et al., 2004) but many unacceptable side effects cannot be avoided. Interestingly, local facilitation of GABAergic inhibition in the VTA prevents addictive behaviors in animal models of addiction (Backes and Hemby, 2008; Lee et al., 2007; Xi and Stein, 2000) supporting the idea that selective GABAergic intervention in this area will likely offer new and promising treatments for drug addiction. In addition to drugs elevating GABA levels or GABA agonists directly targeting GABA receptors, an increase or a decrease in GABAergic inhibition can be achieved through synaptic plasticity. Our current knowledge about the control of GABAergic synaptic strength in the VTA is sparse but presents an important area of interest. Our previous work provided the first evidence of modulation of LTP at GABAergic synapses by morphine in vivo. We discovered a novel presynaptic form of LTD (LTPGABA) at GABAergic synapses onto VTA DA neurons mediated by the NO/cGMP/cGMP-dependent protein kinase (PKG) signaling cascade. We found that 24 h after a single exposure to opiates, LTPGABA is blocked presumably by opiate interaction at the level of guanylyl cyclase (Nugent et al., 2009, 2007). Opiate-induced blockade of LTPGABA could emerge as early as 2 h after morphine exposure and could last up to 5 days. Intriguingly, LTPGABA is also found to be a major target for other addictive drugs (nicotine, cocaine and ethanol) and even stress suggesting that GABAergic plasticity in the VTA may in fact be a common synaptic target for all addictive drugs (Guan and Ye, 2010; Niehaus et al., 2010). It is worthwhile to be mentioned that the common method for identification of DA neurons in the VTA slices is the measurement of a hyperpolarization-activated current (Ih) including the present study. Interestingly, it has been shown that non-DA neurons can also express Ih currents, therefore Ih alone may not reliably represent DA cells (Margolis et al., 2006). Nevertheless, a recent study also confirmed that the larger Ih is a consistent characteristic of DA neurons (Zhang et al., 2010). Therefore in our experiments, a small subset of the neurons recorded from and reported here are also non-DA neurons. Here we report that GABAergic synapses onto Ih-positive neurons, presumably DA neurons, exhibit a form of postsynaptic LTD (LTDGABA) that can also be modulated by morphine in vivo. The bidirectional regulation of GABAergic plasticity in the VTA by morphine provides additional cellular correlates by which opiates and maybe other drugs of abuse could regulate DA cell excitability and DA release.

2. Materials and methods

2.1. Animals

All experiments were carried out in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Uniformed Services University Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering, and to reduce the number of animals used. Animals were 2–3 weeks old Sprague–Dawley rats provided food and water ad libitum, on a 12 h light/dark cycle.

2.2. Preparation of brain slices

Rats were anesthetized using isoflurane and quickly decapitated. The brain was rapidly removed into ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 2.14 NaHCO3, 2.5 KCl, 1.2 Na2HPO4, 2.4 CaCl2, 1.2 MgSO4, 11.1 glucose, 0.4 ascorbic acid, saturated with 95% O2/5% CO2 (pH 7.4). Horizontal midbrain slices containing the VTA (250 μm thick) were cut and incubated for at least 1 h at 34 °C, and then transferred to a recording chamber where the slice was submerged in warmed ACSF.

2.3. Electrophysiology

Whole cell recordings were performed on midbrain slices using a patch amplifier (Multiclamp 700B) under infrared-differential interference contrast microscopy. Data acquisition and analysis were carried out using DigiData 1440A and pClamp 10 (molecular devices, Union City, CA). Signals were filtered at 3 kHz and digitized at 10 kHz. The recording ACSF was the same as the cutting solution except that it was ascorbic acid-free. Slices were perfused at 2–4 ml/min and recorded at 28–29 °C. To study GABAβ-mediated synaptic transmission, 6,7-dini- troquinoxaline-2,3-dione (DNQX; 10 μM), and strychnine (1 μM) were added to block AMPA- and glycine-receptor-mediated synaptic currents, respectively. Patch pipettes were filled with (in mM): 125 KCl, 2.3 NaCl, 2 MgCl2, 2 ATP–Na+, 0.3 GTP–Na+, 0.6 EGTA, and 10 HEPES (pH adjusted 7.28 with KOH; 275–280 mOsm).

Cells were voltage-clamped at −70 to −80 mV except during LTD protocol, and the cell input resistance and series resistance were monitored throughout the experiment; experiments were discarded if these values changed by more than 10% during the experiment. Istead current measurement was used to identify DA neurons by stepping cells from −50 mV to −100 mV. In our experimental conditions, the average of Istead current was 213 ± 27 pA, n = 62. However, in each set of our experiments there is a small subset of non-DA neurons that express Istead currents. Paired GABAβ, IPSCs were stimulated using a bipolar stainless steel stimulating electrode placed 200–500 μm rostral to the recording site in the VTA at 0.1 Hz (100 μs). The stimulation intensity was adjusted so that the amplitude of IPSCs ranged between −200 and −600 pA. To induce LTDGABA, afferents were stimulated at 1 Hz (low frequency stimulation, LFS) for 6 min while cells were held at −40 mV (voltage-clamp mode).

2.4. Drug application

Drugs were superfused at indicated concentrations for at least 15 min before LTD protocol. Interleaved control experiments were performed with experiments in which drugs were bath applied. Salts and all other drugs were obtained from Sigma–Research Biochemicals International or Tocris Bioscience.

2.5. Morphine treatment

For treatment with morphine, rats were injected once 24 h prior to sacrifice for slice preparation. Morphine sulfate was dissolved in 0.9% saline, and saline was used to treat control animals. Rats were brought up from the animal facility in the morning, weighed, and injected intraperitoneally with either 10 mg/kg morphine or a comparable volume of saline. They were then returned to their home cages after 2 h. Rats were brought back up to the lab for sacrifice and brain slice preparation 24 h post-morphine.

2.6. Statistics

Values are presented as means ± s.e.m. Statistical significance was determined using a two-tailed pair-sample or two-sample Student’s t-test with significance level p < 0.05. Levels of LTD are reported as averaged IPSC amplitudes for 5 min just before LTD induction compared with averaged IPSC amplitudes during the 5 min period from 20 to 25 min after LFS. PPR and CV were measured as previously described (Nugent et al., 2007). Briefly, PPR (50 ms interstimulus interval) and CV were measured over 5 min epochs of 30 IPSCs each. The peak values of the evolved paired IPSCs were measured relative to the same baseline. A stable baseline value was considered in each sweep of paired pulses starting at 20–50 ms right before the emergence of the inward IPSC current using pClamp 10 software. The average value for the amplitude of all 30 IPSCs was divided by the average value for the amplitude of the corresponding 30 IPSCs, and reported as the mean PPR for that epoch. 1/CV was measured by dividing the mean amplitude of IPSCs recorded over 5 min epochs by the variance of these IPSCs (only IPSCs were used to determine this value).

3. Results and discussion

3.1. LTD at GABAergic synapses onto VTA DA neurons (LTDGABA)

In continuation of our major finding of morphine-blockade of LTPGABA (Nugent et al., 2007), we explored the possibility that GABAergic synapses originating from GABA interneurons in the VTA are capable of bidirectional plasticity (expression of LTD in addition to LTP). Using a pairing LTD protocol, low frequency synaptic stimulation with modest depolarization, we were able to reliably induce an enduring and stable LTDGABA in DA cells (Fig. 1a–b, paired t-test: p < 0.000001). The same pairing stimulation paradigm has been shown to induce LTD of excatory synapses onto DA neurons in the VTA (Jones et al., 2000; Thomas et al., 2008). The induction of LTD was not observed in GABAergic neurons in the VTA could provide a natural mechanism to increase DA cell excitability by decreasing GABA inhibition and also promoting an excitatory glutamatergic LTP in the VTA (i.e., metaplasia),
3.2. LTD\(_{\text{GABA}}\) is expressed postsynaptically

To identify the locus of LTD\(_{\text{GABA}}\) expression, PPR and CV analyses were performed in control experiments. PPR and CV are measures that reliably change with changes in probability of release and can be used to predict the nature of synaptic plasticity. The apparent advantage of CV is that it is independent of quantal size; therefore if CV changes similarly to the mean values of IPSCs, the locus of expression of plasticity is presynaptic, whereas if CV is unaffected, synaptic plasticity is expressed postsynaptically.\(^{[30]}\) In our experiments, we did not detect any significant changes associated with LTD\(_{\text{GABA}}\) in PPR or CV (Fig. 1c–d, paired t-test: \(p = 0.121\) for PPR, and \(p = 0.579\) for CV) which strongly points to a postsynaptic nature of LTD\(_{\text{GABA}}\). This is in contrast to the nature of LTP\(_{\text{GABA}}\) which is induced postsynaptically but expressed presynaptically (i.e., through an increase in presynaptic GABA release). Our results suggest that postsynaptic LTD\(_{\text{GABA}}\) may result from a decrease in number and conductance of GABA\(_A\) receptors similar to a postsynaptic form of LTD of GABA inhibition in rat deep cerebellar nuclei.\(^{[93, 96]}\) However, in contrast to deep cerebellar nuclear inhibitory LTD, LTD\(_{\text{GABA}}\) is NMDAR independent (see below).

3.3. LTD\(_{\text{GABA}}\) does not require NMDAR and CB\(_1\)R activation

NMDAR activation is required for induction of some forms of LTD at inhibitory GABAergic synapses in the brain.\(^{[93, 96]}\) Like LTD\(_{\text{GABA}}\) in VTA DA neurons, we explored whether eCBs also play a critical role in LTD\(_{\text{GABA}}\). Unlike eCB-LTD, the CB\(_1\)R antagonist, AM251 (10 \(\mu\)M), did not have any effect on the induction of LTD\(_{\text{GABA}}\), suggesting that LTD\(_{\text{GABA}}\) does not involve presynaptic CB\(_1\)Rs (Fig. 2b; paired t-test: \(p = 0.007\) for control cells and \(p = 0.004\) for AM251 cells). This is consistent with our results derived from PPR release.
and CV analyses which points to the postsynaptic nature of LTD_{GABA}. Given that CB1Rs are exclusively expressed on GABAergic terminals in the VTA (Szabo et al., 2002), it is not surprising that these receptors are not required for expression of LTD_{GABA}.

3.4. D2Rs are involved in induction of LTD_{GABA}

Somatodendritic release of DA in the VTA and its action on D2 autoreceptors provides an endogenous feedback inhibitory mechanism for controlling the excitability of DA neurons. Interestingly, activation of D2Rs (proposed to be located onto GABA terminals which is not anatomically verified) in the VTA could also facilitate the induction of cocaine-induced eCB-LTD through inhibition of the cAMP-PKA signaling cascade (Pan et al., 2008a). Given that drugs of abuse could increase somatodendritic release of DA (Bradberry and Roth, 1989; Campbell et al., 1996; Klitenick et al., 1992) and the critical role of D2Rs in induction of cocaine-induced eCB-LTD and of glutamatergic LTD in the VTA (Jones et al., 2000; Thomas et al., 2000), we next examined whether D2R activation is also required for LTD_{GABA}. In the presence of D2R antagonist, sulpiride (10 µM), we attempted to induce LTD_{GABA}. Sulpiride significantly attenuated LTD_{GABA} suggesting a critical role of D2Rs in induction of this plasticity (Fig. 3a–b; paired t-test: p = 0.002 for control cells and p = 0.333 for sulpiride cells). It still remains to be determined whether post synaptic disruption of G protein signaling and also Ca^{2+} signaling block the induction of LTD_{GABA}. Based on the post-synaptic nature of LTD_{GABA}, we favor the idea that activation of D2 autoreceptors triggers and/or interacts with postsynaptic signaling cascades essential for induction of synaptic plasticity. In fact, compelling evidence suggest a D2R-triggered activation of calcineurin (i.e., protein phosphatase 2B) mediated by the Inositol 1, 4, 5-Trisphosphate (IP3) signaling in the striatum (Hernandez-Lopez et al., 2000) that may also underlie LTD_{GABA} in the VTA. Based on this observation in the striatum, our hypothesis predicts that D2Rs may activate phospholipase Cβ (PLCβ1) through Gβγ signaling, which in turn could trigger IP3 signaling pathway and therefore, could increase intracellular levels of Ca^{2+}. Activation of a Ca^{2+} dependent phosphatase such as calcineurin may eventually lead to dephosphorylation of GABA_{A} receptors or their associated regulatory subunit as shown previously in a form of LTD of GABAergic transmission in neurons of the deep cerebellar nuclei (Morishita and Sastry, 1996). Further work on the characterization of LTD_{GABA} and other forms of synaptic plasticity in the VTA should...
help elucidate the neural substrates of reward-motivated behaviors.

3.5. LFS-induced LTD_{CABA} is modulated by morphine in vivo

We have previously shown that 24 h after morphine in vivo, LTD_{CABA} is blocked providing a mechanism by which opiates could modulate DA cell excitability and DA release (Nugent et al., 2007). Modulation of LTD_{CABA} by opiates seemed to be very likely, therefore we next examined whether 24 h after in vivo exposure to morphine, LTD_{CABA} is disrupted. We found that while slices from saline-treated rats exhibited LTD_{CABA} in response to LFS, LTD_{CABA} was absent in slices from morphine-treated rats (Fig. 4a–c, paired t-test: $p = 0.042$ for saline and $p = 0.507$ for morphine). This remarkable finding suggests that opiates can regulate a bidirectional inhibitory plasticity in the VTA. It is still not clear and yet to be determined whether morphine blocks or occludes LTD_{CABA}. It is tempting to favor the latter hypothesis where morphine induces LTD_{CABA} and therefore occludes LFS-induced LTD_{CABA}. Analyses of basal PPR and CV of GABAergic IPSCs in slices from saline and morphine animals showed no difference in these measures (data not shown, mean basal PPR values from saline-treated rats: 0.907 ± 0.16, morphine-treated rats: 0.95 ± 0.28, t-test: $p = 0.528$) which is consistent with our previous finding (Nugent et al., 2007). Based on this data, in vivo morphine does not seem to induce any form of presynaptic plasticity (LTP or LTD). However, because LTD_{CABA} is postsynaptic, it is likely that in vivo morphine could trigger or block LTD_{CABA}. In a scenario where morphine induces LTD_{CABA}, we assume that an increase in the somatodendritic release of DA by morphine through μ opioid receptors (Chefer et al., 2009) and DA’s action on D2 autoreceptors could trigger the morphine-induced plasticity. As a result, morphine-induced LTD_{CABA} in parallel with morphine-induced blockade of LTD_{CABA} and morphine-induced glutamatergic LTP could work together to over-excite DA neurons, thereby increasing DA release. On the other hand, if morphine prevents LTD_{CABA}, this may occur through a presynaptic inhibition of the somatodendritic release of DA by morphine through κ opioid receptors (Ford et al., 2007). In this situation, morphine-blockade of LTD_{CABA} would serve as a physiological brake to dampen the over-excitability of DA neurons produced by morphine-induced glutamatergic LTP and morphine-blockade of LTD_{CABA}. In sum, our finding of morphine-modulation of LTD_{CABA} is significant and provides an additional novel target for drugs of abuse to mediate their addictive properties.

4. Concluding remarks

Here we report a novel form of postsynaptic LTD (LTD_{CABA}) at GABAergic synapses onto VTA DA neurons that is independent of NMDARs and CB1Rs but requires D2R activation. Remarkably, we found that 24 h after a single in vivo exposure to morphine, LTD_{CABA} is absent from slices of morphine-treated rats. The induction of LTD at inhibitory GABAergic synapses provides an additional mechanism to promote the induction of synaptic plasticity at adjacent excitatory synapses. Moreover, the existence of LTD_{CABA} in addition to other forms of inhibitory plasticity in the VTA provides a physiological mechanism to reinforce the learning of reward-related behaviors. More importantly, drug-induced modulation of this plasticity could provide a means to promote or counteract maladaptive drug-induced changes at GABAergic synapses. The mechanisms by which morphine and other drugs of abuse block/trigger LTD_{CABA} are our next important questions. Our experiments further characterizing LTD_{CABA} will provide us avenues to pursue. Our results here provide new information on the learning mechanisms of the VTA inhibitory plasticity that is associated with reward and drug-induced reward and present possible novel therapeutic approaches to drug craving in addicts.

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