

PKG and PKA Signaling in LTP at GABAergic Synapses

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Drugs of abuse usurp the mechanisms underlying synaptic plasticity in areas of the brain, a process that may contribute to the development of addiction. We previously reported that GABAergic synapses onto dopaminergic neurons in the ventral tegmental area (VTA) exhibit long-term potentiation (LTP_{GABA}) blocked by *in vivo* exposure to morphine. The presynaptically maintained LTP requires the retrogradely released nitric oxide (NO) to activate a presynaptic cGMP signaling cascade. Previous work reported that inhibitory GABA_A receptor synapses in the VTA are also potentiated by cAMP. Here, we explored the interactions between cGMP-dependent (PKG) and cAMP-dependent (PKA) protein kinases in the regulation of these GABAergic synapses and LTP_{GABA}. Activation of PKG was required for NO–cGMP signaling and was also essential for the induction of synaptically elicited LTP_{GABA}, but not for its maintenance. Synapses containing GABA_A receptors were potentiated by NO–cGMP signaling, whereas synapses containing GABA_B receptors on the same cells were not potentiated. Moreover, although the cAMP–PKA system potentiated GABA_A synapses, synaptically induced LTP_{GABA} was independent of PKA activation. Surprisingly, however, raising cGMP levels saturated potentiation of these synapses, precluding further potentiation by cAMP and suggesting a convergent end point for both signaling pathways in the regulation of GABAergic release. We further found that persistent GABAergic synaptic modifications observed with *in vivo* morphine did not involve the presynaptic cAMP–PKA cascade. Taken together, our data suggest a synapse-specific role for NO–cGMP–PKG signaling pathway in opioid-induced plasticity of VTA GABA_A synapses.

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INTRODUCTION

Long-term potentiation (LTP) and long-term depression (LTD) are long-lasting synaptic modifications proposed to underlie many examples of experience-dependent plasticity (Malenka and Bear, 2004). Over the last decade, rapid, drug-induced synaptic plasticity has been reported at excitatory glutamatergic synapses in addiction-related brain circuits, suggesting that LTP- and LTD-like changes may also contribute to the development of addiction (Ungless *et al*, 2001; Hyman *et al*, 2006; Kauer and Malenka, 2007). Recent evidence suggests that drug-induced plasticity of ventral tegmental area (VTA) GABAergic synapses may also contribute to the development of addictive behaviors (Mansvelder *et al*, 2002; Melis *et al*, 2002; Liu *et al*, 2005; Nugent *et al*, 2007; Nugent and Kauer, 2008; Pan *et al*, 2008).

Opioids rapidly increase VTA dopamine (DA) cell firing and output through disinhibition, that is, by reducing the

tonic inhibition provided by local interneurons (Johnson and North, 1992). Recently, we reported that 24 h after *in vivo* morphine exposure nitric oxide (NO)-dependent LTP_{GABA} is blocked, providing a long-lasting mechanism by which opioids can enhance the excitability of DA neurons and may contribute to the reinforcing effects of opioids. LTP_{GABA} is heterosynaptic, initiated by glutamate release onto NMDA receptors on the postsynaptic DA neuron. Activation of NO synthase by intracellular Ca²⁺ generates NO, which then travels retrogradely to activate soluble guanylate cyclase (sGC) in neighboring presynaptic GABAergic nerve terminals. Increased levels of cGMP, presumably acting in presynaptic terminals, promote long-lasting potentiation of GABA release at these synapses (Nugent *et al*, 2007). In the context of opiate addiction, it is important to further investigate the precise cellular mechanisms underlying LTP_{GABA}.

Cyclic GMP-dependent protein kinase (PKG) is present in neurons throughout the brain, and is a major target of NO–cGMP signaling (el-Husseini *et al*, 1995; Wang and Robinson, 1997). PKG has previously been implicated in the induction and maintenance of synaptic plasticity (Zhuo *et al*, 1994; Wu *et al*, 1998; Lu *et al*, 1999; Santschi *et al*, 1999; Lu and Hawkins, 2002; Monfort *et al*, 2002, 2004; Chien *et al*, 2003; Liu *et al*, 2003). The cAMP–PKA signaling pathway also regulates synaptic plasticity in many brain

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regions (Huang and Kandel, 1994, 1998; Weisskopf *et al*, 1994; Salin *et al*, 1996a,b; Castro-Alamancos and Calcagnotto, 1999; Linden and Ahn, 1999; Mellor *et al*, 2002). Several studies have implicated cAMP-PKA signaling in responses to drugs of abuse. Following acute withdrawal from chronic morphine, cyclic AMP-dependent increases in GABA release in different regions, including the VTA, have been reported (Bonci and Williams, 1997; Chieng and Williams, 1998; Ingram *et al*, 1998). Furthermore, Melis *et al* (2002) reported that cAMP-PKA signaling is required for induction of a long-lasting potentiation of VTA GABAergic synapses after a single exposure to ethanol. Given that opioids can modulate the release of GABA through an interaction with the presynaptic cAMP cascade (Williams *et al*, 2001), here we have investigated the roles of PKG and PKA as likely downstream targets for cGMP and cAMP in LTP_{GABA}.

MATERIALS AND METHODS

Preparation of Brain Slices

Preparation of slices was as described previously (Jones *et al*, 2000; Nugent *et al*, 2007). Sprague-Dawley rats (15–21 days old) were deeply anesthetized using isoflurane and quickly decapitated in accordance with the Brown University Institutional Animal Care and Use Committee guidelines. The brain was rapidly removed into ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 21.4 NaHCO₃, 2.5 KCl, 1.2 NaH₂PO₄, 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 using a vibratome, stored for at least 1 h at 35°C, and transferred to a recording chamber where the slice was submerged in warmed ACSF.

Electrophysiology

Midbrain slices were continuously perfused with ACSF (no ascorbic acid) at 28–32°C at 2–4 ml/min. To study GABA_A receptor-mediated synaptic transmission, 6,7-dinitroquinoxaline-2,3-dione (DNQX; 10 μM), strychnine (1 μM), and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; 1 μM) were added to block AMPA-, glycine-, and A1 adenosine receptors, respectively. To isolate GABA_B receptor-mediated IPSCs, the superfusion medium contained 2-amino-5-phosphopentanoic acid (AP-5; 100 μM), DNQX (10 μM), picrotoxin (100 μM), strychnine (1 μM), eticlopride (1 μM) and 7-hydroxyiminocyclopropan [b] chromen-1a-carboxylic acid ethyl ester (CPCCOEt; 50 μM) to block NMDA, AMPA, GABA_A, glycine, D₂-, and mGluR1 receptors, respectively. The GABA_B IPSCs were entirely blocked by the GABA_B receptor antagonist CGP55845 (10 μM). Patch pipettes were filled with (in mM): 125 KCl, 2.8 NaCl, 2 MgCl₂, 2 ATP-Na⁺, 0.3 GTP-Li⁺, 0.6 EGTA, and 10 HEPES. To record GABA_AR-mediated IPSCs, cells were voltage-clamped at –70 mV except during HFS, 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. GABA_BR IPSCs were recorded from cells voltage-clamped at –50 mV (see below).

If the steady-state h-current was greater than 60 pA during a step from –50 to –100 mV, the neuron was considered a DA neuron. A recent study showed that expression of I_h alone is not sufficient to identify DA cells unequivocally (Margolis *et al*, 2006, but see the review by Chen *et al*, 2008). Therefore in each set of our experiments, a subset of the neurons recorded from and reported here are possibly non-dopaminergic neurons.

GABA_AR-mediated IPSCs were stimulated at 0.1 Hz (100 μs) using a bipolar stainless steel stimulating electrode placed 200–500 μm rostral to the recording site in the VTA. GABA_BR-mediated IPSCs were stimulated using a train of stimuli; 10 pulses of 250 μs at 66 Hz, repeated once every 60 s (Bonci and Williams, 1996; Fiorillo and Williams, 2000). LTP_{GABA} was induced by stimulating afferents at 100 Hz for 1 s, the train was repeated twice 20 s apart (high-frequency stimulation; HFS). Just before HFS, the recorded neuron was taken from voltage-clamp and into bridge mode, so that the HFS trains were delivered with the membrane potential free to vary.

Statistics

Data are presented as means ± SEM. Significance was determined using a Student's unpaired *t*-test with significance level of *p* < 0.05. Levels of LTP are reported as averaged IPSC amplitudes for 5 min just before LTP induction compared with averaged IPSC amplitudes during the 5 min period from 15 to 20 min after HFS using a Student's paired *t*-test. Paired-pulse ratios (50 ms inter-stimulus interval) were measured over 5 min epochs of 30 IPSCs each as previously described (Nugent *et al*, 2007).

Drug Application

Drugs were bath-applied at known concentrations for at least 15 min before HFS. Control experiments were interleaved with experiments in which drugs were bath-applied. To assess drug effects, IPSC amplitudes were averaged for 5 min during the peak response and were compared with 5 min of averaged data before drug application. Salts and all other drugs were obtained from Sigma-Research Biochemicals International or Tocris Bioscience, except for KT5823, obtained from Calbiochem.

Treatment with Morphine

Rats (15–21 days old) were maintained on a 12-h light/dark cycle and provided food and water *ad libitum*. They were injected intraperitoneally with either 10 mg/kg morphine or a comparable volume of saline, placed in a new cage for 2 h, and then returned to the home cage. They were killed for brain slice preparation 24 h after injection.

RESULTS

As we reported previously (Nugent *et al*, 2007, 2008), GABAergic synapses on VTA DA neurons undergo LTP in response to patterned local electrical stimulation (LTP_{GABA}, Figure 1a). LTP_{GABA} appears to be expressed by an increase in presynaptically released GABA, as the paired-pulse ratio

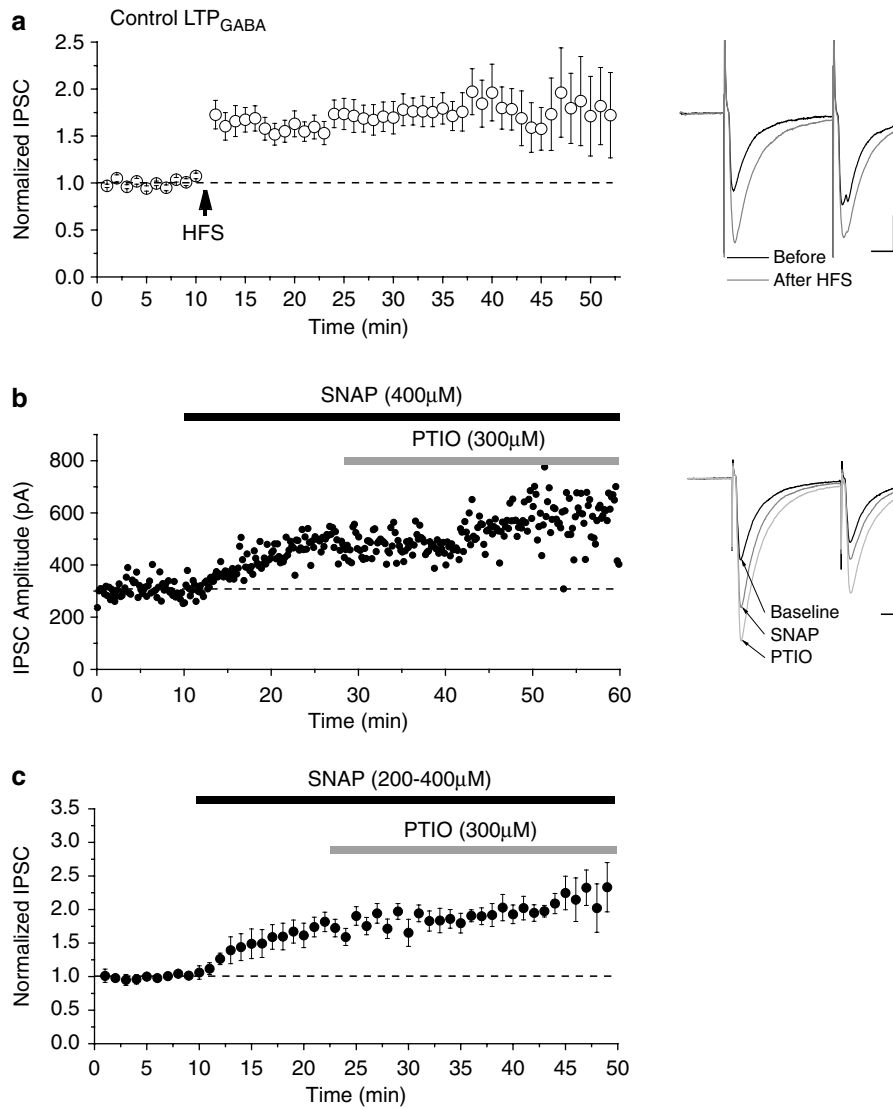


Figure 1 Nitric oxide (NO) is not necessary for the maintenance of LTP_{GABA}. (a) Average of 26 experiments showing LTP_{GABA} recorded from dopamine cells. HFS was delivered at the arrow. LTP_{GABA}: $181 \pm 1\%$ of pre-HFS values, $n = 26$. Inset: averaged IPSCs before and 25 min after HFS from single experiment. In this and all figures, 10 consecutive IPSCs from each condition were averaged for illustration. Calibration for insets: 10 ms, 100 pA. (b) Single experiment illustrating the lack of effect of the NO scavenger, PTIO, on SNAP-induced potentiation of GABAergic IPSCs. SNAP ($400 \mu\text{M}$), an NO donor, potentiated IPSCs. After a new stable level was reached, PTIO ($300 \mu\text{M}$) was bath-applied, but did not reverse the potentiation induced by SNAP. Inset: averaged IPSCs recorded before, after 10 min in SNAP and after 10 min in PTIO. (c) Average of experiments using the protocol outlined in (b) showing that after SNAP potentiated the IPSCs ($150 \pm 4\%$ of pre-SNAP values, $n = 11$), PTIO had no effect on SNAP-induced potentiation ($121 \pm 8\%$ of pre-PTIO values, $n = 11$).

and coefficient of variation change after induction (Nugent *et al*, 2007).

NO is not Needed to Sustain LTP_{GABA}

Sustained activity of protein kinases, such as protein kinase C (PKC) and calcium calmodulin kinase type II (CaMKII), have been proposed to be involved in the maintenance and expression of LTP (Lisman, 1985; Lisman and Goldring, 1988; Malinow *et al*, 1988; Chen *et al*, 2001; Yang *et al*, 2004). In VTA DA cells, the production of presynaptic cGMP in response to NO release triggers LTP_{GABA}. We first asked whether constitutive release of NO is necessary to sustain LTP_{GABA}, or whether instead, a brief exposure is sufficient to persistently enhance GABA release. Consistent with our previous results, the NO donor, SNAP (*S*-nitroso-

N-acetylpenicillamine; $200\text{--}400 \mu\text{M}$), potentiated GABA_AR IPSCs, resembling LTP_{GABA} (Figure 1b and c). Yet we observed that when the NO scavenger, PTIO (2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide, $300 \mu\text{M}$), was added after the NO donor elicited synaptic potentiation, the SNAP-induced potentiation did not decay back to control values (Figure 1b and c). The inability of PTIO to reverse the potentiation strongly suggests that the maintenance of LTP_{GABA} does not require the persistent presence of NO.

The NO-cGMP Signaling Cascade Activates PKG to Potentiate GABAergic Synapses

Consistent with our previous findings, the cGMP analog, pCPT-cGMP (8-(*p*-chlorophenylthio)-cGMP; $100 \mu\text{M}$), potentiated GABAergic IPSCs (Figure 2a) suggesting that

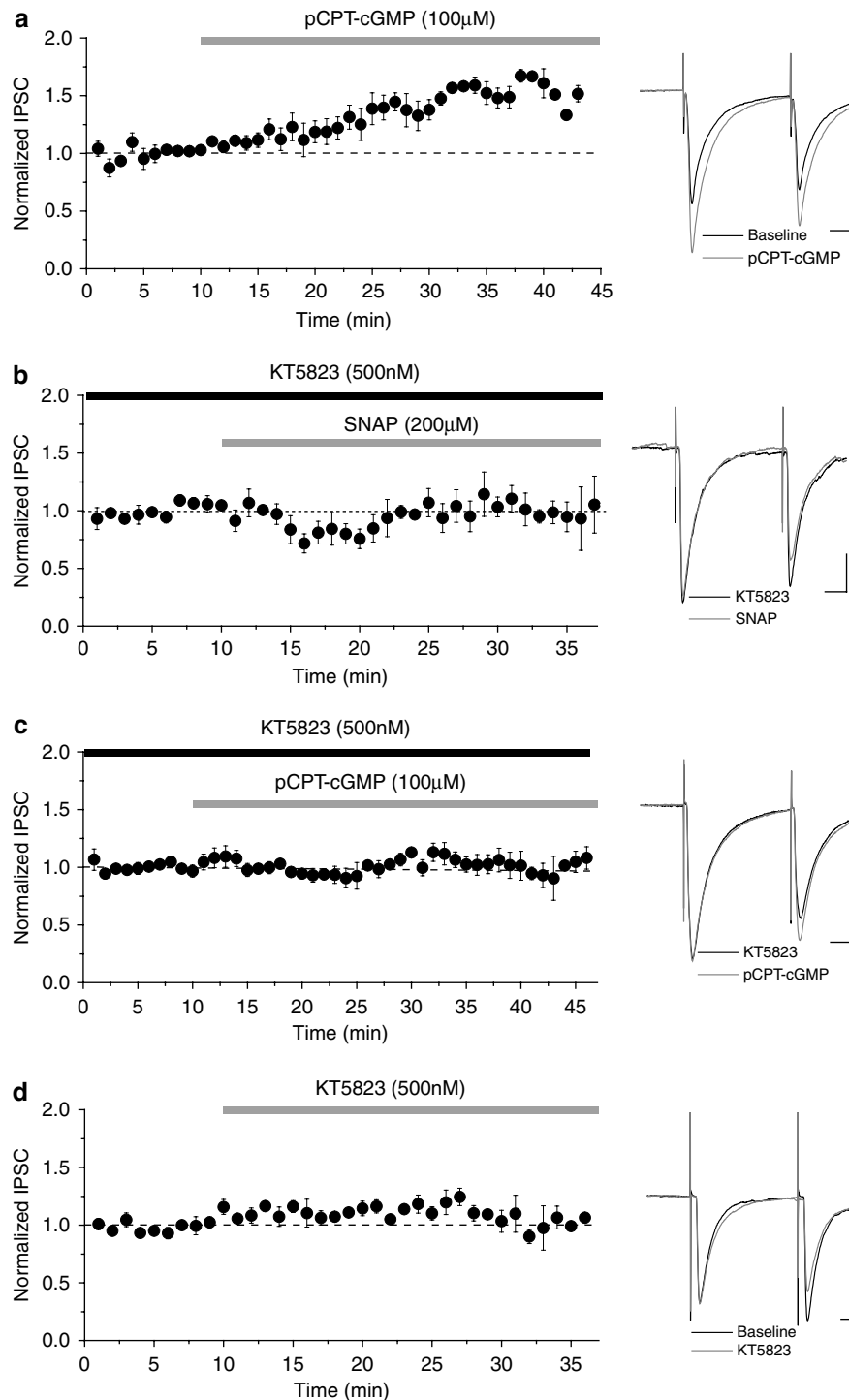


Figure 2 Nitric oxide (NO)-cGMP signaling requires PKG to enhance GABAergic IPSCs. (a) A cGMP analog, pCPT-cGMP (100 μM), potentiates IPSCs ($146 \pm 9\%$ of pre-drug values, $n = 4$). Inset: averaged IPSCs recorded during a single such experiment before and after 15 min in pCPT-cGMP. Calibration for all insets: 10 ms, 100 pA. (b) The PKG inhibitor, KT5823 (500 nM), blocks the enhancement of IPSCs by 200 μM SNAP ($101 \pm 6\%$ of pre-SNAP values, $n = 4$). KT5823 was applied at least 15 min before the addition of SNAP. Inset: averaged IPSCs recorded during single experiment in KT5823 and after 15 min in SNAP. (c) KT5823 (500 nM) also prevents the potentiation of IPSCs by 100 μM pCPT-cGMP ($104 \pm 6\%$ of pre-SNAP values, $n = 6$). KT5823 was applied at least 15 min before the addition of pCPT-cGMP. Inset: averaged IPSCs recorded during single experiment in KT5823 and after 15 min in pCPT-cGMP. (d) KT5823 (500 nM) has no effect on basal GABAergic transmission ($113 \pm 6\%$ of pre-drug values, $n = 5$). Inset: averaged IPSCs recorded during single experiment before and after 10 min in KT5823.

NO-mediated activation of guanylate cyclase is required for NO to enhance GABA release. Furthermore, pCPT-cGMP is a selective activator of PKG, with little effect on cyclic nucleotide-gated ion channels or phosphodiesterases (Wang

and Robinson, 1997). If NO-cGMP signaling must activate PKG to potentiate GABAergic synapses, then a PKG inhibitor should prevent the potentiation induced either by SNAP or pCPT-cGMP. As predicted, KT5823, a selective

PKG inhibitor which interferes at the level of the ATP-binding site of the PKG catalytic domain (Hidaka and Kobayashi, 1992), prevented the potentiation induced by either the NO donor (Figure 2b) or the cGMP analog (Figure 2c). These data support the idea that the sequential activation of the presynaptic sGC and PKG downstream from NO promotes GABA release in these synapses. In addition, KT5823 did not reduce basal synaptic transmission (Figure 2d), implying that PKG activity is not required to maintain basal GABA release from these terminals.

Sequential Activation of GC, and then PKG is Necessary for the Induction of LTP_{GABA} but not for its Maintenance

We further explored the role of PKG in the induction and maintenance of LTP_{GABA} in response to HFS. Application of KT5823 entirely blocked the induction of LTP_{GABA} (Figure 3a). If persistent PKG activity is also necessary for the maintenance of LTP_{GABA}, synaptic potentiation should be reversed if PKG activity is inhibited after induction. To test this hypothesis, we bath applied KT5823 10 min after induction of LTP_{GABA} using synaptic stimulation (HFS). KT5823 had no significant effect on the maintenance of LTP_{GABA} (Figure 3b). Furthermore, after pCPT-cGMP washout and addition of KT5823, the IPSC amplitude remained potentiated, confirming that the maintenance of the potentiation did not require persistent activity of PKG

once the LTP was induced ($178 \pm 4\%$ of pre-drug values, $n = 3$). Taken together, these findings suggest that the induction of LTP_{GABA} requires transient activation of PKG, but the expression of LTP_{GABA} does not require persistent activity of this kinase.

GABA_B Synapses are not Potentiated in Response to cGMP

Anatomically and functionally distinct sets of GABAergic afferents innervate VTA DA neurons at inhibitory synapses containing either GABA_B or GABA_A receptors. For example, GABAergic axons from outside the VTA, such as the nucleus accumbens or ventral pallidum, target GABA_BR-containing synapses, whereas GABA_AR-containing synapses most likely receive their main input from the axons of local GABAergic interneurons in the VTA (Johnson *et al*, 1992; Sugita *et al*, 1992; Cameron and Williams, 1993). As GABA_AR LTP is altered after morphine exposure *in vivo*, and drugs of abuse can also influence GABA_B receptor synapses, we next asked whether the NO-cGMP signaling cascade could modulate GABA_B synapses. We evaluated the effects of SNAP and pCPT-cGMP on GABA_B IPSCs recorded from VTA DA neurons. Unlike GABA_A synapses, after bath application of either the NO donor or the cGMP analog, GABA_B synapses were not potentiated (Figure 4). These findings show that the molecular machinery for NO-cGMP signaling does not potentiate all GABA-releasing axons in the VTA, but is selective for GABA_AR-associated synapses, most likely arising

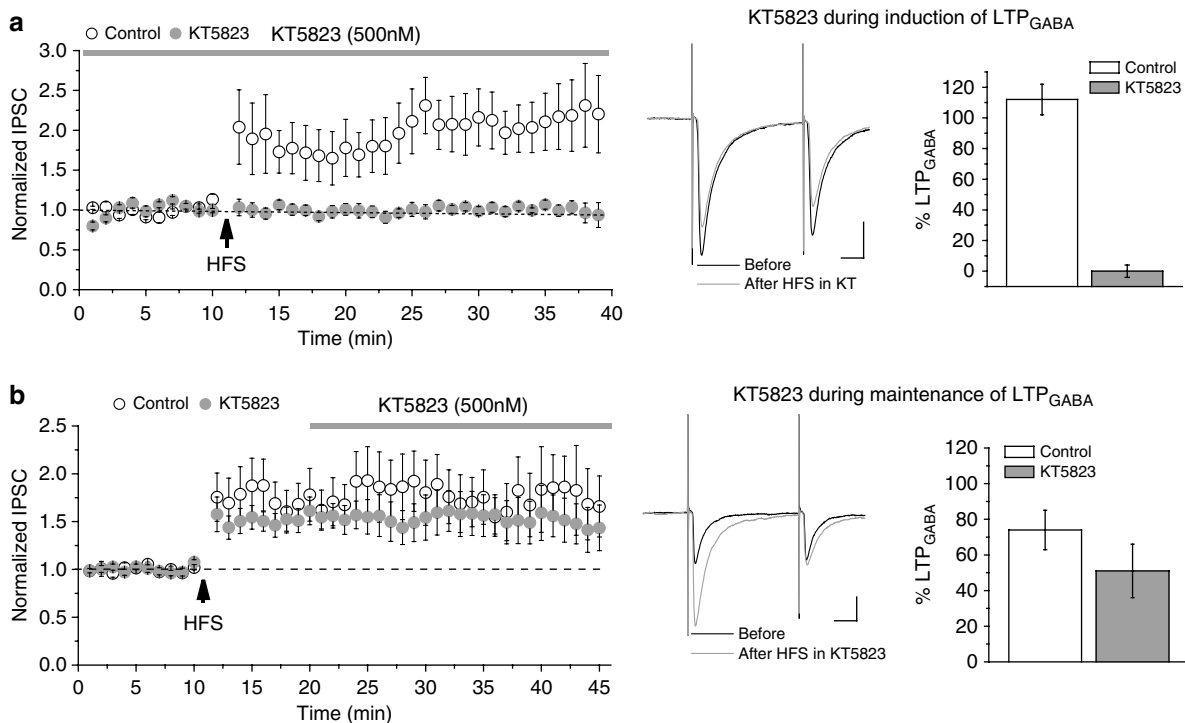


Figure 3 PKG is required for the induction, but not the maintenance of LTP_{GABA}. (a) Bath application of KT5823 (gray bar, 500 nM) prevents LTP_{GABA} (control LTP, open circles: $212 \pm 10\%$ of pre-HFS values, $n = 6$; cells treated for at least 15 min before HFS with KT5823, closed circles: $100 \pm 4\%$ of pre-HFS values, $n = 6$). Insets: averaged IPSCs recorded during a single experiment in KT5823, 20 min after HFS in KT5823. Bar chart illustrating the magnitude of LTP 20 min after HFS in control vs cells treated with KT5823 for at least 15 min before HFS, $p < 0.05$. (b) Bath application of KT5823 (500 nM) 10 min after HFS (gray bar) had no effect on the expression of LTP_{GABA} (control LTP, open circles: $174 \pm 11\%$ of pre-HFS values, $n = 10$; KT5823 cells, filled circles: $151 \pm 5\%$ of pre-HFS values, $n = 10$). Insets: averaged IPSCs recorded during a single experiment before KT5823, and 25 min after HFS in KT5823. Bar chart illustrating the magnitude of LTP 25 min after HFS in control vs cells treated with KT5823 after LTP_{GABA} induction, $p > 0.05$. Calibration for insets: 10 ms, 100 pA.

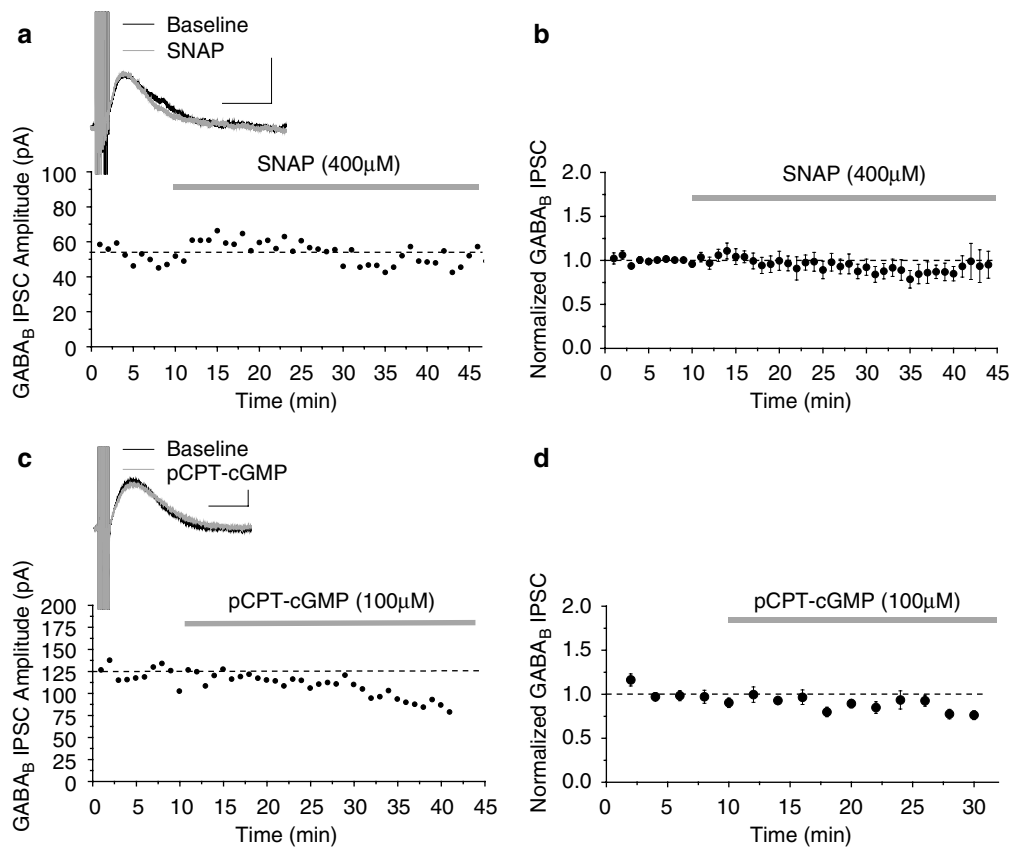


Figure 4 Neither nitric oxide nor cGMP potentiate GABA_B-mediated IPSCs on dopamine neurons. (a) Single example experiment illustrating that SNAP (400 μM) did not potentiate GABA_B IPSCs. Inset: averaged GABA_B IPSCs elicited by a train of synaptic stimuli (see Materials and Methods) recorded before and after 20 min in SNAP. Calibration: 500 ms, 40 pA. (b) Average of six similar experiments using SNAP (400 μM) ($87 \pm 3\%$ of pre-drug values, $n = 6$). (c) Single example experiment illustrating that pCPT-cGMP (100 μM) did not potentiate GABA_B IPSCs. Inset: averaged GABA_B IPSCs recorded before and after 20 min in pCPT-cGMP. Calibration: 500 ms, 40 pA. (d) Average of six similar experiments using pCPT-cGMP (100 μM) ($86 \pm 7\%$ of pre-drug values, $n = 6$).

from local VTA GABAergic neurons. The NO-cGMP potentiating mechanism is either absent or non-functional in the VTA GABA_B nerve terminals originating from GABAergic neurons outside the VTA.

Activation of Adenylyl Cyclase Potentiates GABA_A Synapses and Occludes Further Potentiation by HFS

A rise in presynaptic cAMP following activation of adenylyl cyclase facilitates neurotransmitter release at many synapses, and is involved in the induction and expression of LTP at many excitatory and inhibitory synapses (Briggs *et al*, 1988; Greengard *et al*, 1991; Cameron and Williams, 1993; Chavez-Noriega and Stevens, 1994; Huang and Kandel, 1994, 1998; Weisskopf *et al*, 1994; Bonci and Williams, 1996; Salin *et al*, 1996a; Bonci and Williams, 1997; Castro-Alamancos and Calcagnotto, 1999; Linden and Ahn, 1999; Mellor *et al*, 2002). Furthermore, PKA activation has previously been shown to potentiate GABA_AR synapses on VTA DA neurons (Melis *et al*, 2002). Therefore, we explored the interactions of the cAMP cascade with LTP_{GABA} using forskolin (10 μM) to activate adenylyl cyclase. We confirmed that forskolin enhanced GABAergic responses (Figure 5a and b) and this enhancement was associated with a decrease in the paired-pulse ratio, suggesting that it is likely due to enhanced GABA release, also seen during

LTP_{GABA} (Melis *et al*, 2002; Nugent *et al*, 2007). Dideoxyforskolin, a biologically inactive analog that does not stimulate adenylyl cyclase, had no effect on GABA_A-mediated responses (Figure 5b). Furthermore, once the potentiation by forskolin had plateaued, HFS failed to produce further synaptic potentiation (Figure 5a and c). Thus, forskolin mimicked and occluded LTP_{GABA} through the activation of adenylyl cyclase and the subsequent rise in cAMP. PKA is the major downstream target for cAMP, and if the activation of PKA mediates synaptic enhancement, Sp-cAMPS (a cAMP mimic and specific activator of PKA) should also enhance GABA release. Consistent with this hypothesis, we found that Sp-cAMPS (20 μM) also potentiated GABA_A IPSCs and occluded further potentiation induced by HFS (Figure 6a-c).

Activation of PKA Through the cAMP Signaling Pathway is not Necessary for the Induction or Expression of LTP_{GABA}

Together, these findings indicate that elevation of cAMP or PKA activation enhances synaptic strength through a presynaptic mechanism shared by LTP_{GABA}. However, these experiments do not address whether cAMP/PKA signaling are required for LTP_{GABA}. To test this idea, a specific PKA inhibitor, Rp-cAMPS (20 μM) was bath applied before HFS

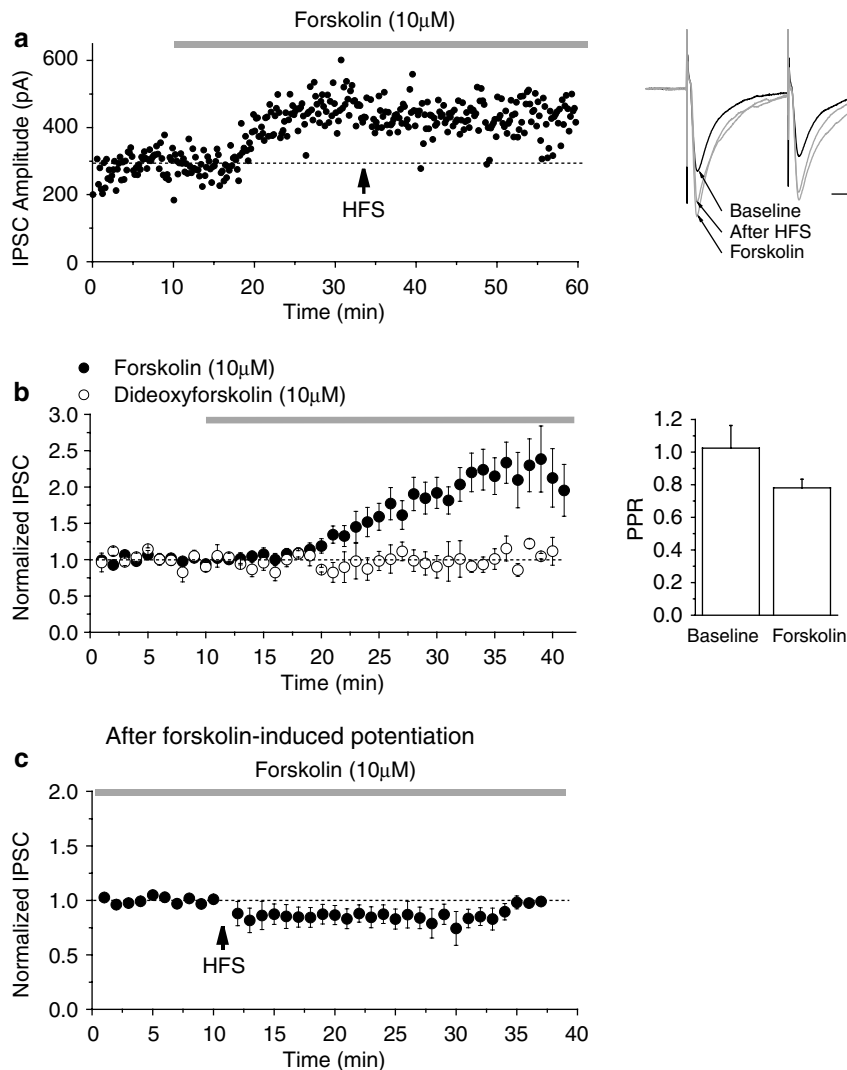


Figure 5 Activation of adenylyl cyclase potentiates GABA_A IPSCs and occludes further potentiation by HFS. (a) Single experiment showing occlusion of the HFS-induced potentiation with forskolin (10 μ M)-induced potentiation. Inset: averaged GABA_A IPSCs recorded during a single experiment before, after 20 min in forskolin and 20 min after HFS). Calibration: 10 ms, 100 pA. (b) Forskolin activated adenylyl cyclase to increase GABAergic transmission, whereas dideoxyforskolin had no effect on GABA_A-mediated responses (forskolin cells, filled circles: $191 \pm 22\%$ of pre-drug values, $n = 10$; dideoxyforskolin cells, open circles: $98 \pm 6\%$ of pre-drug values, $n = 3$). Inset: forskolin-induced potentiation was accompanied by a decrease in the paired-pulse ratio (PPR), suggesting an increase in presynaptic GABA release after forskolin. Five-minute blocks of data are shown (PPR before forskolin: 1.02 ± 0.13 ; in forskolin: 0.78 ± 0.05 ; $p < 0.05$). (c) After the IPSCs in forskolin reached a stable potentiated level, HFS was delivered. Forskolin-induced potentiation occluded further potentiation of IPSCs by HFS ($85 \pm 0.6\%$ of pre-HFS values, $n = 7$). Only the portion of the experiment after forskolin potentiation has plateaued is shown.

and remained throughout the experiment. An even lower concentration of Rp-cAMPS was sufficient to block PKA in an earlier study (Gutlerner *et al*, 2002). The induction and expression of LTP_{GABA} was entirely unaffected by bath application of Rp-cAMPS (Figure 6d). These data suggest that the cAMP-PKA signaling pathway is not required for LTP_{GABA} but apparently shares downstream mechanisms with LTP_{GABA} that underlie the long-lasting enhancement of GABA release from these terminals.

PKG and PKA Signaling Pathways Converge Onto Common Downstream Mechanisms to Sustain the Potentiation of GABAergic Synapses

Our data thus far indicate that elevation of either cGMP or cAMP levels enhances GABA release through the activation of

PKG and PKA, respectively, as shown schematically in Figure 7a. PKA and PKG share common substrates that could serve as a mechanism for convergence. If these two pathways share a common target that promotes persistently enhanced GABA release, saturation of potentiation induced by one signaling pathway should preclude further potentiation through the other. To test this idea, we first bath-applied SNAP to potentiate GABAergic synapses through the NO-cGMP-PKG pathway. Once the potentiation by SNAP had plateaued, application of forskolin did not cause further synaptic potentiation (Figure 7b, c). This finding points to a convergence point for PKG and PKA in expressing and sustaining the increased GABA release. However, a trivial explanation might be that when intracellular levels of cGMP or cAMP are sufficiently high, there is cross-activation of kinases by the cyclic nucleotides (Wang and Robinson, 1997). To rule out this possibility, we examined the effects of

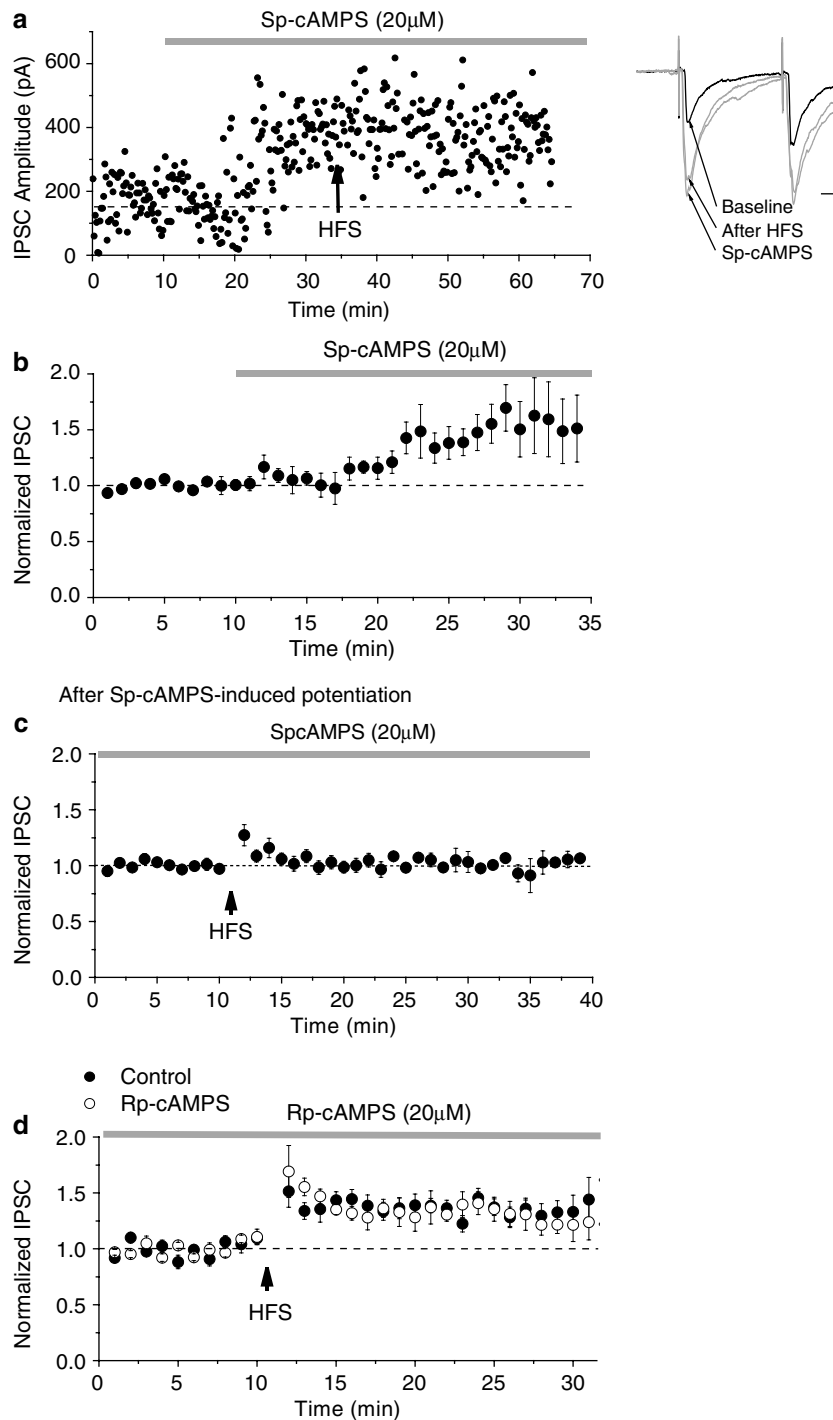
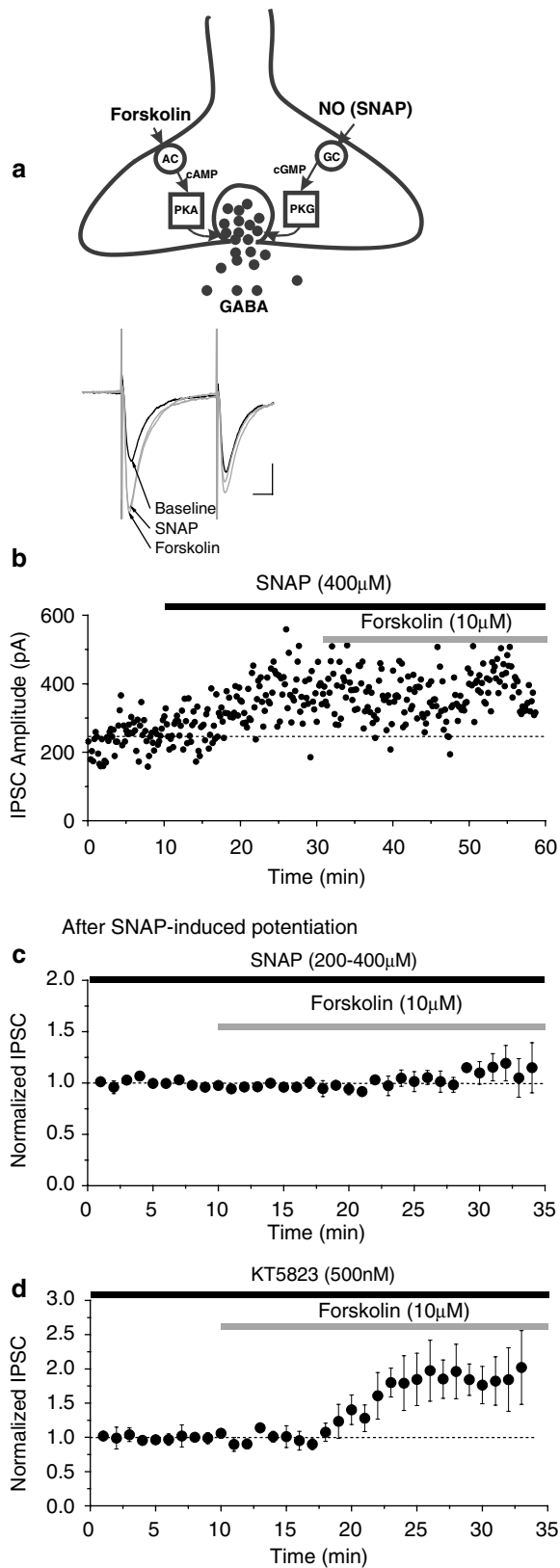


Figure 6 PKA is not involved in the induction or expression of LTP_{GABA}. (a) Single experiment showing occlusion of LTP_{GABA} with Sp-cAMPS (10 μM)-induced potentiation. Sp-cAMPS, a cAMP analog, potentiated GABA_A IPSCs and occluded the potentiation induced by HFS. Inset: averaged GABA_A IPSCs recorded before and after 25 min in Sp-cAMPS and 20 min after HFS. Calibration: 10 ms, 100 pA. (b) Averaged experiments showing that Sp-cAMPS mimics LTP_{GABA} ($152 \pm 10\%$ of pre-drug values, $n = 5$). (c) After the IPSCs in Sp-cAMPS reached a stable potentiated level, HFS was delivered. Only the portion of the experiment after Sp-cAMPS-induced potentiation is shown. Sp-cAMPS-induced potentiation occluded further potentiation of IPSCs by HFS ($100 \pm 0.5\%$ of pre-HFS values, $n = 6$). (d) Bath application of Rp-cAMPS (20 μM), a PKA inhibitor, had no effect on the induction or expression of LTP_{GABA} (control LTP, filled circles: $144 \pm 14\%$ of pre-HFS values, $n = 10$; Rp-cAMPS cells, open circles: $128 \pm 10\%$ of pre-HFS values, $n = 6$). Rp-cAMPS was present in the bath for at least 15 min before HFS.

forskolin on GABAergic synapses in the persistent presence of the PKG inhibitor, KT5823. If the increased levels of cAMP cross-activate PKG (which would subsequently potentiate these synapses), inhibition of PKG should reduce this

potentiation. In contrast, in the presence of the PKG inhibitor, forskolin was still able to induce potentiation comparable with that seen with forskolin alone suggesting that cross talk between these two pathways cannot explain our results.



Instead, the simplest explanation of our data is that the two signaling cascades act on a common target to promote a sustained enhancement of GABA release. Further confirmation of this interpretation comes from bath-application of forskolin for only 10 min. The potentiation induced by brief application of forskolin did not require the persistent activation of PKA and still occluded the further potentiation by HFS, suggesting that both kinases converge on a downstream mechanism that is necessary for LTP_{GABA} ($99.5 \pm 1\%$ of pre-HFS values, $n = 4$).

A Single *In Vivo* Morphine Exposure has no Effect on the Presynaptic cAMP-PKA Signaling Pathway

Our recent work has shown that *in vivo* treatment with morphine persistently modulates GABAergic synaptic plasticity as a result of interference with presynaptic NO-cGMP signaling (Nugent *et al*, 2007). The cAMP-PKA-dependent potentiation of the same GABAergic synapses is also reportedly altered 24 h following ethanol exposure (Melis *et al*, 2002). μ -opioid receptors are negatively coupled to adenylyl cyclase through G_o, and in the VTA, μ -opioid drugs acutely depress GABAergic synaptic transmission (Johnson and North, 1992; Williams *et al*, 2001; Nugent *et al*, 2007). In fact, one of the effectors of opioid receptor activation to decrease GABA release is also the inhibition of adenylyl cyclase. On the basis of our present results, which suggest that cGMP and cAMP signaling cascades coexist in VTA GABAergic synapses, we tested whether the interaction of *in vivo* morphine with cAMP signaling in presynaptic terminals has the potential to interfere with synaptic potentiation by the cAMP-PKA pathway. To address this question, rats were treated either with morphine (10 mg/kg i.p.) or with saline, and 24 h after treatment, the effects of forskolin (10 μ M) were tested on GABAergic synapses. Synapses from both saline- and morphine-treated animals were potentiated after exposure to forskolin (Figure 8a-c), suggesting that the presynaptic cAMP-PKA pathway is unaltered after morphine exposure, in contrast to morphine's effect on the NO-PKG signaling cascade involved in LTP_{GABA}. This result also confirms that the site of disruption of the NO signaling by morphine is upstream to the unidentified converging mechanism for both PKG and PKA.

Figure 7 Convergence of the presynaptic NO-cGMP-PKG and cAMP-PKA signaling pathways on GABA release. (a) Proposed schematic of signaling molecules mediating the effects of SNAP and forskolin in the presynaptic GABAergic terminal. (b) Single experiment illustrating the effect of forskolin added after SNAP potentiated GABAergic IPSCs. After the IPSCs in SNAP (400 μ M) reached a stable potentiated level, forskolin (10 μ M) was bath-applied. SNAP occluded the potentiation induced by forskolin. Inset: averaged IPSCs recorded during a single experiment before, after 10 min in SNAP and after 10 min in forskolin. Calibration: 10 ms, 100 pA. (c) Average of six experiments using the protocol outlined in (b). Only the portion of the experiment showing the effect of forskolin on SNAP-induced potentiation is shown ($108 \pm 7\%$ of pre-forskolin values, $n = 6$). (d) The PKG inhibitor, KT5823 (500 nM), does not prevent the enhancement of IPSCs by 10 μ M forskolin ($187 \pm 8\%$ of pre-forskolin values, $n = 6$), supporting the idea that the effect of forskolin on IPSCs is not mediated by cross-activation of PKG. KT5823 was applied at least 15 min before the addition of forskolin.

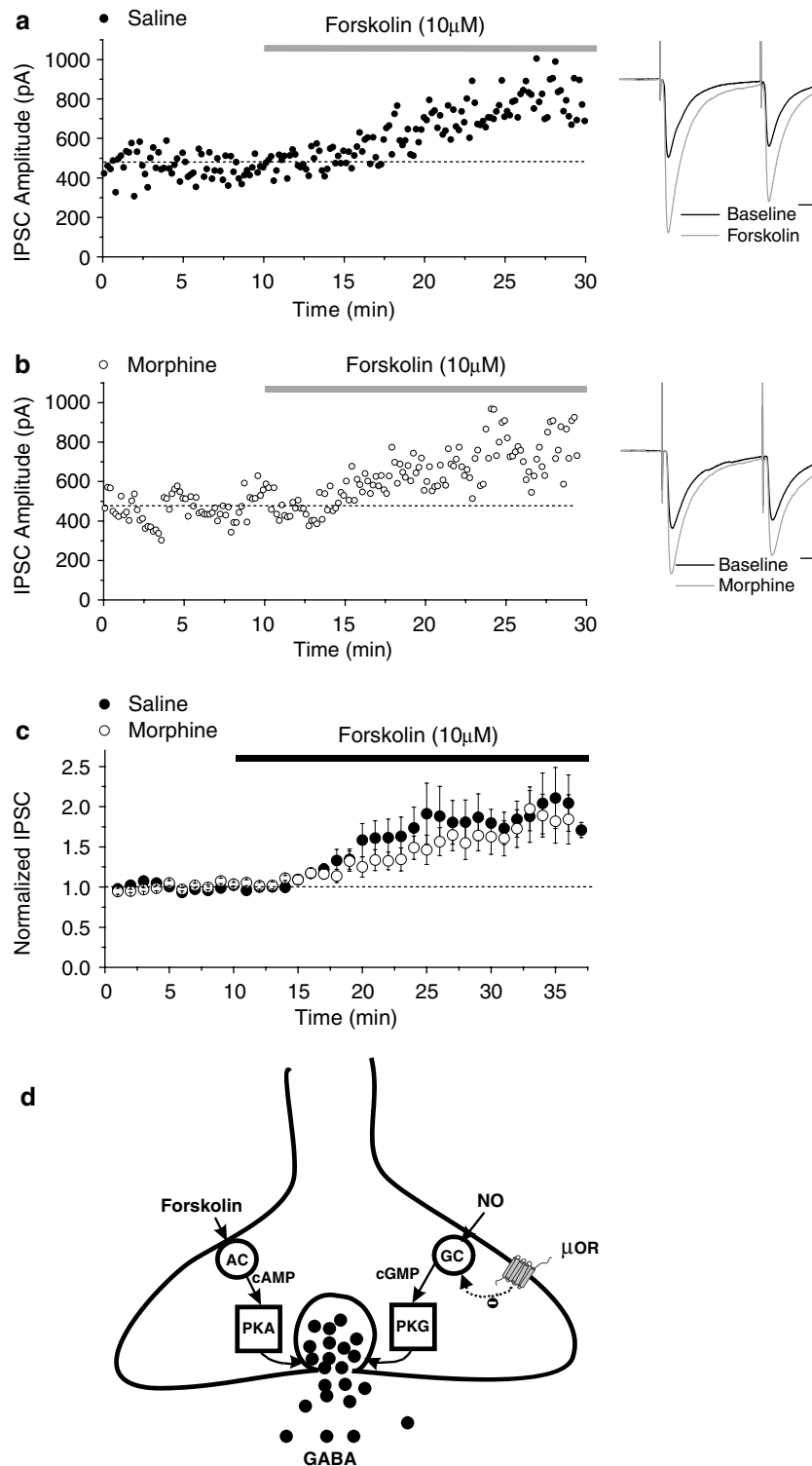


Figure 8 *In vivo* morphine does not modulate the presynaptic cAMP signaling cascade. Rats were treated with either 10 mg/kg morphine or saline. After 24 h, slices were prepared, and IPSCs were recorded from dopamine cells, while 10 μ M forskolin was bath-applied. (a) Single experiment illustrating the potentiating effect of forskolin (10 μ M) on IPSCs from a saline-treated animal. Inset: Averaged GABA_A IPSCs recorded before and after 20 min in forskolin. Calibration for insets: 10 ms, 100 pA. (b) Single experiment illustrating the effect of forskolin (10 μ M) on IPSCs from a morphine-treated animal. Forskolin still potentiates GABA_A-mediated IPSCs. Inset: averaged GABA_A IPSCs recorded before and after 20 min in forskolin. (c) Averaged experiments showing the enhancing effect of forskolin on IPSCs in slices from both saline- and morphine-treated animals, showing that morphine *in vivo* does not alter the effect of forskolin (saline, filled circles, $188 \pm 11\%$ of pre-drug values, $n = 8$; morphine, open circles, $167 \pm 15\%$ of pre-drug values, $n = 10$). (d) Proposed model of signaling molecules involved in opioid-induced plasticity at VTA GABAergic synapses. An *in vivo* injection of morphine alters GABAergic plasticity through modulation of the NO signaling pathway, probably at the level of sGC, without affecting the cAMP signaling cascade.

DISCUSSION

Here we have investigated the involvement of PKG and PKA in the induction and expression of LTP_{GABA}. Furthermore, we provide evidence for the synapse-specificity of NO signaling at VTA GABA_A synapses and confirm that *in vivo* morphine persistently and specifically modulates the plasticity of these synapses through an interaction with the NO signaling pathway without an associated change in the coexistent cAMP signaling cascade.

The NO–cGMP–PKG and cAMP–PKA Signaling Cascades Both Potentiate GABAergic Synapses

Increasing levels of NO exogenously using SNAP, or application of a cGMP analog, pCPT-cGMP, potentiates GABAergic synapses onto VTA DA neurons. Inhibition of PKG prevented the potentiation induced by NO or cGMP, supporting the role of PKG as the downstream effector from NO–cGMP. However, inhibition of PKG had no effect on basal GABAergic tone, suggesting that constitutive PKG activity is not necessary to maintain basal levels of GABA release.

Cyclic GMP-dependent protein kinase is a serine-threonine kinase that mediates most of the effects of cGMP. Two different classes of PKG have been reported, PKG I and PKG II. Although PKG I is highly localized in cerebellar Purkinje cells and a few other sites in brain, the ubiquitous distribution of PKG II and its major localization in neuronal processes make it a major target in mediating cGMP effects in the brain (Wang and Robinson, 1997; de Vente *et al*, 2001; Williams *et al*, 2001; Jouvert *et al*, 2004). Given that pCPT-cGMP is also a specific PKG II activator, PKG II rather than PKG I is the most likely kinase mediating the potentiation of VTA GABA release.

Several studies have shown that stimulation of AC by forskolin increases the release of GABA at VTA GABAergic synapses (Cameron and Williams, 1993; Bonci and Williams, 1996, 1997). We also found that either forskolin treatment or application of a cAMP analog/PKA activator potentiates the GABAergic synapses. We next asked whether the PKG and PKA signaling pathways interact with one another to increase GABA release from these GABAergic terminals. Potentiation of the synapses by using an NO donor prevented subsequent potentiation by forskolin, most likely because these synapses possess the molecular machinery for both NO–cGMP–PKG and cAMP–PKA signaling pathways, with both pathways converging on common downstream effectors to potentiate the GABAergic synapses. PKA and PKG share common phosphorylation substrates, and identification of this unknown converging mechanism in GABAergic release machinery deserves further study. Alternatively, it is formally possible that PKG might phosphorylate an unknown cellular target that could in turn inhibit activation of either AC or PKA.

Synapse Specificity of the NO–cGMP–PKG Signaling to GABA_A Synapses

The NO–cGMP signaling pathway can control GABAergic synaptic transmission and plasticity at GABA_AR synapses (Stern and Ludwig, 2001; Li *et al*, 2002; Yu and Eldred,

2005), but many studies also suggest the involvement of GABA_BRs in drug addiction-related behaviors (Humeniuk *et al*, 1993; Cameron and Williams, 1994; Bonci and Williams, 1996; Shoji *et al*, 1997, 1999; Boehm *et al*, 2002; Leite-Morris *et al*, 2004; Ong and Kerr, 2005). Chronic exposure to either morphine or cocaine modulates GABA_B receptor function (Bonci and Williams, 1996). Moreover, intra-VTA application of baclofen, a GABA_B receptor agonist, interferes with the rewarding properties of intracranial self-stimulation (Willick and Kokkinidis, 1995), with self-administration of several addictive drugs including heroin (Xi and Stein, 1999), with morphine-induced place preference (Tsuji *et al*, 1996), and with opioid-induced motor sensitization (Leite-Morris *et al*, 2002, 2004). We therefore next explored the potential presynaptic effects of NO on synaptic transmission mediated by GABA_B receptors in the VTA. We found, however, that the NO donor or cGMP analog had no effect on GABA_B IPSCs, indicating that the NO signaling pathway selectively potentiates GABA_A synapses in the VTA. Although the NO–cGMP signaling pathway did not potentiate GABA_B synapses, forskolin activation of the cAMP–PKA pathway has previously been shown to increase GABA_B IPSPs (Shoji *et al*, 1999). This functional selectivity is not entirely surprising given that distinct sets of GABAergic inputs with distinct characteristics appear to innervate GABA_A and GABA_B synapses in the VTA. Extrinsic GABAergic afferents arising from forebrain selectively provide synaptic inputs to GABA_B receptors, whereas GABA_A responses are thought to arise from GABA release from local VTA interneurons (Johnson *et al*, 1992; Sugita *et al*, 1992; Shoji *et al*, 1999). In addition to the anatomical differences, D1 and 5-HT1A receptors acting through cAMP–PKA machinery are only expressed on presynaptic GABAergic terminals synapsing on GABA_BRs on DA neurons (Sugita *et al*, 1992; Cameron and Williams, 1993, 1994). The synapse specificity of the NO signaling for GABA_A synapses we have observed here emphasizes the fact that the two GABAergic inputs to these important DA neurons are quite independent, and modulation or alteration in one will likely spare the other. The distinct machinery available to modulate GABA release in distinct cell populations also potentially provides selective targets for drugs of abuse to exert their modulatory effects on GABAergic neurotransmission. These differences may also be exploited by therapeutic agents targeting only a single type of GABAergic synapse.

PKG but not PKA is Involved in LTP_{GABA}

Raising the levels of either cGMP or cAMP increases GABAergic transmission at GABA_A synapses, which mimics LTP_{GABA}. Our earlier work showed the role of cGMP in LTP_{GABA} by ‘occlusion’ experiments in which prior potentiation induced by a cGMP analog prevented further HFS-induced LTP_{GABA}, presumably by maximally activating the release potentiating machinery. Comparable sets of occlusion experiments were performed here with forskolin and Sp-cAMPS, and we found no further LTP_{GABA} after synaptic HFS, again suggesting an interaction of the cAMP cascade with mechanisms used in LTP_{GABA}. To further clarify the involvement of PKG and PKA in GABAergic plasticity, we used compounds that specifically inhibit

protein kinase activity. Although inhibition of PKG completely blocked the induction of LTP_{GABA}, the maintenance of LTP_{GABA} was unaffected. These results show that the induction of NO-dependent LTP_{GABA} is dependent on a rapid activation of PKG; however, the expression and maintenance of LTP_{GABA} does not require persistent PKG activity. On the other hand, inhibition of PKA activity had no effect on the induction or the expression of LTP_{GABA}. The occlusion between SNAP-induced potentiation and forskolin-induced potentiation indicates that LTP_{GABA} requires the NO-cGMP-PKG pathway, and that cAMP-PKA can potentiate GABA_A release by a shared cellular mechanism. Phosphorylation of presynaptic proteins provides a potential molecular mechanism to control transmitter release in a nerve terminal, especially in long-term processes such as presynaptic plasticity (Ghijsen and Leenders, 2005). It is also possible that the cAMP-PKA signaling pathway acts in parallel with PKG to increase phosphorylation of an unknown downstream target whose activation is necessary for the expression of LTP_{GABA}. One possible converging downstream mechanism for both kinases is RIM1 α , an active zone protein and PKA substrate that is involved in long-term changes in neurotransmitter release (Castillo *et al*, 2002; Schoch *et al*, 2002; Chevaleyre *et al*, 2006; Chevaleyre *et al*, 2007). However, it is not yet known whether RIM1 α is a PKG substrate.

The Presynaptic cAMP-PKA Cascade is not Modulated by a Single *In Vivo* Morphine Exposure

We showed previously that a single *in vivo* exposure to morphine acts on the NO-cGMP signaling to block LTP_{GABA} at VTA synapses (Nugent *et al*, 2007). The μ -opioid receptors are coupled through G_o to adenylyl cyclase, which in theory could represent an additional morphine target modulated in parallel with the NO-cGMP-PKG signaling cascade. Potentiation of GABA release after withdrawal from chronic morphine resulted from an upregulation of the cAMP-PKA cascade that is sensitive to inhibition by opioids (Chieng and Williams, 1998; Ingram *et al*, 1998; Shoji *et al*, 1999; Williams *et al*, 2001). Moreover, GABA_A-mediated synaptic transmission is altered in the VTA by the cAMP-PKA cascade after a single *in vivo* exposure to ethanol, and this alteration is proposed to provide an initial maladaptive change at the synaptic level (Melis *et al*, 2002). However, we found here that increasing cAMP levels in morphine-treated animals still potentiated the GABA_A synapses. Although *in vivo* morphine is able to block an increase in GABA release through the NO-cGMP pathway, GABA transmission by the cAMP-PKA pathway is still able to be potentiated 24 h after morphine. These data also indicate a significant difference between the effects of these two addictive drugs. After 24 h ethanol exposure, GABA_A synapses are potentiated and cAMP-PKA cascades elicit no further potentiation (Melis *et al*, 2002), whereas 24 h after morphine exposure, the synapses are responsive to cAMP-PKA. It is possible that in response to the two drugs, synaptic changes occur on different time scales, so that an examination of GABA_A synapses at different time points following ethanol or morphine may show convergence over time.

One day after morphine exposure LTP_{GABA} is inhibited, presumably removing a normal mechanism limiting DA cell firing rate. This inhibition can be bypassed either by cGMP analogs or activation of PKG, or alternatively by activation of the unaffected cAMP-PKA signaling pathway. Our data therefore suggest that raising cAMP or cGMP levels in GABAergic terminals may represent a useful therapeutic strategy to counteract opioid-induced maladaptive changes at GABAergic synapses. Taken together, these data indicate the synapse-specific role of NO-cGMP-PKG signaling in opioid-induced plasticity of GABAergic synapses. Understanding the effects of chronic exposure to morphine on the NO-cGMP-PKG signaling pathway would also provide insight into how drugs of abuse reshape the reward circuitry. It is possible that repeated exposure to morphine would upregulate the cAMP-PKA pathway, while impairing the NO-cGMP-PKG pathway. It will be of interest to ask whether this modulation by chronic morphine provides a form of homeostatic regulation of inhibitory plasticity in the VTA circuitry during establishment of opiate addiction.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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