# The Synaptic Regulation of Ventral Midbrain Dopamine Neurons and its Modulation by Repeated Cocaine Treatment

by

Christopher D. Fiorillo

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[redacted]

Gary Westbrook

[redacted]

Craig Jahr

[redacted]

[redacted]

Steve Johnson

[redacted]

John Williams

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APPENDIX 2 / ADDITIONAL MANUSCRIPT

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## Abstract

The dopamine neurons of the ventral midbrain have been intensely studied for many years due to the central role they play in a number of prominent neurological and psychiatric conditions, including Parkinson's disease, schizophrenia, and drug addiction. I have investigated the synaptic regulation of dopamine neurons in an in vitro slice preparation of rat midbrain. The first component of this thesis is a description of a slow inhibitory postsynaptic potential (IPSP) mediated by glutamate. The second part of this work demonstrates that repeated cocaine treatment selectively suppresses this IPSP through an increase in endogenous adenosine.

L-glutamate is the primary excitatory neurotransmitter in the mammalian central nervous system, acting rapidly through ligand-gated ion channels and more slowly through G-protein-coupled receptors. However, we have found that glutamate mediates an inhibitory postsynaptic potential (IPSP) in dopamine neurons. Activation of a metabotropic glutamate receptor (mGluR1) by synaptically released transmitter caused mobilization of intracellular calcium from caffeine/ryanodine sensitive stores and activation of an apamin-sensitive potassium current. This mechanism is distinct from previously characterized slow IPSPs, which are mediated by a membrane-delimited pathway and activation of an inwardly rectifying potassium current. With more sustained activation of mGluR1 the hyperpolarizing response desensitized and a depolarization was produced. In addition to demonstrating a glutamate-mediated IPSP, this indicates that a single type of receptor can mediate inhibition or excitation in the same cell depending on the frequency and duration of synaptic activity.

Dopaminergic neurotransmission is known to be altered long after withdrawal from repeated exposure to psychostimulants such as cocaine and amphetamine. It was previously found that increased adenosine tone results in inhibition of slow IPSPs in

dopamine neurons of guinea pigs 7-10 days withdrawn from cocaine or morphine. We have examined the synapse specificity of the increased adenosine tone in slices from the ventral tegmental area of rats 10-20 days withdrawn from repeated cocaine treatment. It was found that the selective A1 receptor antagonist, DPCPX, selectively enhanced the slow, mGluR-mediated IPSP in slices from cocaine-treated animals. DPCPX was without effect on fast, glutamate receptor-mediated EPSCs. However, in the presence of a low, physiologically relevant concentration of amphetamine, DPCPX did augment fast EPSCs in cocaine-treated rats. Although DPCPX increased slow $GABA_B$-mediated IPSPs, this was not changed by cocaine pretreatment. Even in the presence of amphetamine, cocaine pretreatment was without effect on the modulation by DPCPX of $GABA_B$ IPSPs. This suggests that the elevated adenosine tone acts preferentially or exclusively on glutamate terminals. Furthermore, endogenous adenosine selectively suppresses the mGluR inhibitory component of the glutamate synaptic response.

# INTRODUCTION

The dopamine-containing neurons of the ventral midbrain are thought to provide the common target of many addictive drugs, and to play a critical role in all phases of addiction. Changes at the cellular level that may underlie addiction are not well understood. We have therefore studied the synaptic regulation of dopamine neurons in slices from drug-naive as well as cocaine-experienced rats. In the introduction, an overview of postsynaptic mechanisms in the nervous system in general and in dopamine neurons in particular will be presented. The dopamine system will then be introduced, and the function of the dopamine signal as it relates to drug addiction will be discussed. Finally, the physiological changes in the dopamine system caused by repeated psychostimulant administration will be addressed.

## Synaptic Potentials

Synaptic potentials throughout the nervous system fall into two broad classes: those mediated by fast, ligand-gated ion channels, and those mediated more slowly by G protein-coupled receptors. Ligand-gated ion channels can also be divided into two types. Cation-permeable channels are excitatory, and these include the AMPA and NMDA subtypes of glutamate receptor, as well as nicotinic acetylcholine receptors, 5-hydroxytryptamine type 3 (5-$HT_3$) receptors, and P2X purinergic receptors. Anion-permeable channels are usually inhibitory, and these include the $GABA_A$ and glycine receptors.

Two types of slow synaptic potential have been characterized, and each is mediated by a distinct type of G protein-coupled receptor. The best understood is the slow IPSP mediated by receptors coupled to pertussis toxin-sensitive $G_i$ / $G_o$ proteins and activation of an inwardly rectifying potassium conductance (Hille, 1992). This signalling pathway has been best characterized in cardiac myocytes, and more recently in cells heterologously

expressing recombinant proteins. Acetylcholine binds to a M2 muscarinic receptor, causing it to release G protein, which dissociates into $\alpha$ and $\beta\gamma$ subunits. The $\beta\gamma$ subunits, which are membrane bound, then directly activate GIRK channels to produce a hyperpolarizing current (Reuveny et al., 1994). In the nervous system, slow IPSPs working through a similar or identical mechanism have been shown to be mediated by M2 muscarinic (Calabresi et al., 1998), as well as $\alpha_2$ adrenergic (Surprenant and Williams, 1987), 5-$HT_{1A}$ (Pan et al., 1989), and $GABA_B$ receptors (Otis et al., 1993). The most ubiquitous slow IPSP in the central nervous system is that mediated by $GABA_B$ receptors.

Slow excitatory postsynaptic potentials (EPSPs) have also been observed. These are mediated by receptors known to couple to phosphoinositide (PI) hydrolysis, including muscarinic M1 acetylcholine (Cole and Nicoll., 1983; 1984), $\alpha_1$-adrenergic (Oleskevich and Williams, 1995), 5-$HT_2$ (Bobker, 1994), and group I metabotropic glutamate receptors (mGluR1 and mGluR5; Charpak and Gahwiler, 1991; Batchelor et al., 1994; Pozzo Miller et al., 1995; Congar et al., 1997; Shen and Johnson, 1997). The mechanisms of this signalling pathway are not well understood. Typically, these receptors have been found to couple to pertussis toxin-insensitive, $G_q$ proteins, which can directly or indirectly inhibit potassium channels and activate cation channels (Charpak et al., 1990; Crepel et al., 1994; reviewed by Pin and Duvosin, 1995). It is well established that these receptors couple to activation of phospholipase C, which metabolizes phosphatidylinositol to diacylglycerol and inositol 1,4,5-trisphosphate ($IP_3$, Pin and Duvosin, 1995). Diacylglycerol can then activate protein kinase C, while $IP_3$ binds to the $IP_3$ receptor. The $IP_3$ receptor is a ligand-gated ion channel which opens to release $Ca^{2+}$ into the cytosol from smooth

endoplasmic reticulum (reviewed by Berridge, 1997). Although this signalling pathway is found in most if not all neurons, there is very little evidence to date for its synaptic activation or its involvement in mediating synaptic potentials (Berridge, 1998). Indeed, the depolarizing effect of PI-coupled receptors has generally been found to be independent of $Ca^{2+}$ (Charpak et al., 1990; Batchelor et al., 1996) and perhaps the PI-pathway as well (Pozzo Miller et al., 1995).

Although PI-coupled receptors have generally been found to be excitatory, there is no *a priori* reason to think that this should be the case. Indeed, inhibition by these receptors has occasionally been observed, though it has not been demonstrated synaptically. Inhibition by mGluRs was characterized by Shirasaki and others (1994) in dissociated CA1 pyramidal neurons. Group I mGluRs (presumably mGluR5) mobilized $Ca^{2+}$ and activated voltage-independent, $Ca^{2+}$-dependent $K^+$ channels that were insensitive to the toxins apamin and iberiotoxin. The response appeared to be mediated by $IP_3$, and was also blocked by caffeine and ryanodine, suggesting that ryanodine receptors might amplify $IP_3$-induced $Ca^{2+}$ release through the process of $Ca^{2+}$-induced $Ca^{2+}$ release. This response was observed using perforated-patch recordings, and quickly ran down during whole-cell recordings. Similar phenomena have been observed in the same lab with muscarinic acetylcholine receptors (Wakamori et al., 1993), and with mGluRs in CA3 pyramidal neurons (Harata et all., 1996). Interestingly, this last study (Harata et al., 1996) also found excitatory responses to mGluR agonists, as have most studies in CA3 pyramidal neurons (Charpak et al., 1990; Charpak and Gahwiler, 1991; Pozzo Miller et al., 1995) as well as CA1 neurons (Crepel et al., 1994; Congar et al., 1997). The parameters determining whether excitation or inhibition is exhibited have not been elucidated.

Other groups have also shown inhibitory actions of glutamate in mammalian

neurons. Group I mGluRs activate a large-conductance, voltage- and $Ca^{2+}$-dependent $K^+$ channel (BK) in cerebellar granule cells (Fagni et al., 1991). Again, mGluRs and muscarinic receptors in these same cells release $Ca^{2+}$ from internal stores, apparently through both $IP_3$ and ryanodine receptors (Irving et al., 1992). A BK channel may also be activated in neurons of the basolateral amygdala, though this appears to involve a group II mGluR (Holmes et al., 1996). Very recently, it has been shown that group II mGluRs, which like $GABA_B$ receptors couple to $G_i$ / $G_o$ proteins, can activate GIRK channels in some types of neuron (Knoflach and Kemp, 1998; Dutar et al., 1999), as they can in an expression system (Saugstad et al., 1996). Glutamate can therefore have inhibitory effects through activation of mGluRs, though this appears to be the exception rather than the rule.

Synaptic inhibition by glutamate has been observed in hippocampal CA1 neurons, and is dependent on a $Ca^{2+}$-activated $K^+$ conductance (Nicoll and Alger, 1981). The receptor mediating this response could not be determined at the time of this study due to the lack of selective pharmacological agents. However, the hyperpolarizing response was not observed independent of the preceding EPSP, and may have been dependent on $Ca^{2+}$ influx through NMDA receptors. An effect of mGluRs cannot be ruled out.

In summary, two types of slow synaptic potential have been demonstrated in neurons. Slow IPSPs are mediated by $G_i$ / $G_o$-coupled receptors and GIRK channels, and are most often mediated by $GABA_B$ receptors. Slow EPSPs, such as those mediated by group 1 mGluRs, are produced by synaptic activation of receptors coupled to PI hydrolysis and modulation of a number of ionic conductances.

## Synaptic Potentials in Dopamine Neurons

In dopamine neurons of the ventral midbrain, each of the four basic types of

synaptic potential discussed above have been demonstrated. Fast EPSPs are mediated by both AMPA and NMDA subtypes of glutamate receptor (Mereau et al., 1991; Johnson and North, 1992), while mGluR1 receptors give rise to slow EPSPs (Shen and Johnson, 1997). GABA mediates both fast and slow IPSPs through activation of $GABA_A$ and $GABA_B$ receptors, respectively (Johnson and North, 1992a; Hauser and Yung, 1994). While a single stimulus is sufficient to evoke fast synaptic potentials, a train of stimuli (5-15 at 50-100 Hz) is necessary to reliably elicit slow synaptic potentials in dopamine neurons in vitro (Johnson and North, 1992a; Shen and Johnson, 1997). This is consistent with the G protein-coupled receptors being located perisynaptically (Baude et al., 1993) and requiring transmitter to escape the synaptic cleft.

The synaptic potentials in the VTA, as elsewhere, have been shown to be modulated presynaptically by activation of numerous receptors. The two that are critical to the present work are the dopamine D1 receptor and the adenosine A1 receptor. A1 receptors inhibit both glutamate and GABA-mediated synaptic potentials in VTA dopamine neurons (Wu et al., 1995). Presynaptic D1 receptors activate adenylyl cyclase to augment $GABA_B$ IPSPs, but not $GABA_A$ IPSPs (Cameron and Williams, 1993). This occurs tonically in response to endogenous dopamine, as indicated by a decrease in IPSP amplitude following application of D1 receptor antagonists. More recently, it has been shown that D1 receptor mRNA is present in excitatory afferents to the VTA (Lu et al., 1997), and activation of D1 receptors in the VTA enhances glutamate release in vivo (Kalivas and Duffy, 1995). D1 receptors are not thought to be present on dopamine neurons.

## The Dopamine System

The dopamine neurons of the ventral midbrain are most often divided into two

primary groups based on topography and functional connectivity (Fallon and Loughlin, 1995). Those dopamine neurons with soma in the substantia nigra pars compacta (SNpc) send their projections primarily to the dorsal striatum. The selective destruction of these nigrostriatal dopaminergic neurons underlies Parkinson's disease, which is characterized by difficulties in the initiation and planning of movement. The dopamine neurons in the adjacent ventral tegmental area (VTA) project to the ventral striatum, limbic areas, and frontal cortex. Dopamine in these brain regions is important for goal-directed behavior and cognitive function. The mesocorticolimbic dopamine system is disrupted in schizophrenia, and is thought to play the more critical role in drug addiction relative to the nigrostriatal system. Despite functional differences between the mesolimbic dopamine system of the VTA and the nigrostriatal dopamine system of the SNpc, only very few small, quantitative differences have been found between the neurons in these areas. Even their activity in behaving animals is very similar (reviewed by Schultz, 1998). This suggests that much of the functional difference is likely due to differences in projection areas rather than differences in afferent input or intrinsic membrane properties. However, the dopamine neurons of the VTA will be emphasized here.

It appears that all major classes of addictive drugs increase extracellular dopamine levels in projection areas (Di Chiara and Imperato, 1988). Nicotine, opiates, and ethanol enhance dopamine release by directly or indirectly increasing the rate of discharge of dopamine neurons. By contrast, the target of the psychostimulants is the dopamine transporter, which provides the dominant mechanism for terminating the action of extracellular dopamine. Although extracellular levels of other monoamines such as norepinephrine and 5-hydroxytryptamine are also increased by psychostimulants through a similar mechanism (e.g. Surprenant and Williams, 1987; Bobker, 1994), effects on these transmitters have not been proven necessary for their reinforcing properties.

Cocaine binds to the dopamine transporter and effectively blocks the reuptake of

dopamine (reviewed by Amara and Sonders, 1998). Amphetamine is a substrate of the dopamine transporter, and therefore competes with dopamine for transport. However, it is thought that the more significant action of amphetamine is its ability to release dopamine, apparently by promoting the reverse transport of dopamine out of the terminals (Amara and Sonders, 1998). A key functional difference between cocaine and amphetamine is that the increase in extracellular dopamine caused by cocaine is impulse-dependent, while that by amphetamine is largely impulse-independent.

As stated above, the dopamine neurons of the VTA project in a very divergent manner to the ventral striatum, limbic structures, and frontal cortex. Numerous studies have indicated that dopamine release in the nucleus accumbens (NAcc), which is part of the ventral striatum, is particularly critical to the reinforcing actions of addictive drugs (Koob, 1992). The NAcc sends a reciprocal, GABAergic projection back to both the VTA and SNpc (Nauta et al., 1978; Berendse et al., 1992). The ventral pallidum, another major target of the NAcc, also sends a GABAergic projection to the ventral midbrain (Hattori et al., 1975; Zahm, 1989; Smith and Bolam, 1990). The other GABA input to dopamine neurons in the VTA is from intrinsic interneurons. The interneurons are spontaneously active and produce tonic inhibition of dopamine neurons. The increase in activity of dopamine neurons by μ-opioids is thought to occur primarily through inhibition of these interneurons and subsequent disinhibition of dopamine neurons (Johnson and North, 1992b).

GABA terminals arising from all three of the above inputs appear to release GABA onto $GABA_A$ receptors on dopamine neurons (Hausser and Yung, 1994; Paladini et al., 1999). However, it seems that synaptically released GABA from striatal afferents, but not from interneurons, has access to $GABA_B$ receptors on dopamine neurons (Sugita et al., 1992; Cameron and Williams, 1993; Hausser and Yung, 1994). Conversely,

pharmacological evidence suggests that the $GABA_A$-mediated inhibitory postsynaptic potential (IPSP), evoked by local, low intensity stimulation in brain slices, derives almost entirely from interneurons (Sugita et al., 1992; Cameron and Williams, 1993). My work on GABAergic inhibition of dopamine neurons has focused entirely on the $GABA_B$ IPSP, and presumably is relevant to inhibition by accumbal afferents.

Excitatory amino acid-containing projections to the VTA from the prefrontal cortex have been well documented (Beckstead, 1979; Christie et al., 1985; Sesack and Pickel, 1992). An excitatory projection from the subthalamic nucleus to dopamine neurons of the SNpc is well established, but there may not be a corresponding projection to VTA (Kita and Kitai, 1987). There is also a presumably excitatory projection to the VTA from the central nucleus of the amygdala (Wallace et al., 1989; Gonzales and Chesselet, 1990), and an electrophysiological study in brain slices suggests an excitatory amino acid projection from the habenula to dopamine neurons (Matsuda and Fujimura, 1992). The VTA also receives a cholinergic projection from the laterodorsal tegmental and pedunculopontine nuclei (Oakman et al., 1995). Glutamate may be a co-transmitter in these cholinergic neurons (Lavoie and Parent, 1994), and appears to mediate the excitation of SNpc dopamine neurons after stimulation of the pedunculopontine region (Di Loreto et al., 1992).

The prefrontal cortex has received particular attention, as it has been shown to control the burst firing of dopamine neurons in rats (Svensson and Tung, 1989; Murase et al., 1993). Dopamine neurons in vivo have been found to fire in two modes, single spike firing and burst firing (Grace and Bunney, 1984a, 1984b; Freeman et al., 1985). However, higher-frequency bursts (20 Hz) elicit significantly more dopamine release in projection areas per action potential than does lower frequency single-spike firing (5 Hz; Gonon, 1988; Suaud-Chagny et al., 1992). Burst firing of dopamine neurons is dependent

on NMDA receptor activation in the ventral midbrain (Chergui et al., 1993; reviewed by Overton and Clark, 1997).

## The Function of the Dopamine Signal

It is widely maintained that drugs become addictive by mimicking natural primary reinforcers such as food and sex (Wise and Rompre, 1989). The exceedingly powerful influence of addictive drugs on behavior is thought to result from an artificially large reward signal, many times larger than that of any naturally occurring reward. Furthermore, this reward signal is hypothesized to correspond to an increase in the extracellular concentration of the neurotransmitter dopamine, particularly in the nucleus accumbens.

The intuitive notion that dopamine signals subjective pleasure is probably overly simplistic (Robinson and Berridge, 1993). Schultz and colleagues have clarified the physiological function of ventral midbrain dopamine neurons by recording their activity extracellularly in behaving primates (reviewed by Schultz, 1998). Dopamine neurons of SNpc and VTA were found to respond to particularly important, or salient, stimuli, with a burst of action potentials after a latency of ~100-200 ms and lasting ~100-200 ms. These include unconditioned rewards and aversive stimuli, conditioned stimuli predicting rewards or aversive stimuli, and high-intensity, surprising, or novel stimuli. However, appetitive stimuli (rewards and conditioned stimuli predicting rewards) are more effective than aversive stimuli, which elicit smaller responses in many fewer cells relative to appetitive stimuli (Mirenowicz and Schultz, 1996). Dopamine neurons are therefore preferentially activated by appetitive stimuli.

The activation of dopamine neurons by appetitive stimuli is not uniform, but rather is sensitive to learning. Initially, dopamine cells are unaffected by a neutral stimulus, but increase their activity in response to an unpredicted primary reward such as juice. With

repeated pairings of a neutral stimulus with a primary reward, the response of dopamine neurons is conditioned such that the formerly neutral stimulus now elicits a response, but the primary reward is no longer effective (Ljungberg et al., 1992). If the conditioned stimulus is itself predicted by a prior stimulus, so that it has no further predictive value, then it is no longer effective, whereas the first predictive stimulus is effective (Schultz et al., 1993). Furthermore, if the predicted reward is omitted, then the firing of dopamine neurons is suppressed at the expected time of reward (Schultz et al., 1993). It is therefore concluded that dopamine neurons signal an error in the prediction of reward. They are activated when a reward is better than predicted at a given moment, remain uninfluenced by events that are as good as predicted, and are depressed by events that are worse than predicted (Schultz et al., 1997; Schultz, 1998). More recently, it has been reported that dopamine neurons code for the time of reward as well as its occurrence (Hollerman and Schultz, 1998), being activated by rewards that occur slightly before or after the predicted time. It is not known which synaptic inputs mediate these phasic burst responses in dopamine neurons.

The response profile of dopamine neurons fits very well with learning theory and suggests a role for dopamine in the learning of conditioned stimuli (Montague et al., 1996; Schultz et al., 1997; Suri and Schultz, 1998). In addition, this body of work promotes the hypothesis that an increased activity of dopamine neurons is more closely related to craving or motivation for rewards and not necessarily to the hedonic response to rewards themselves (Robinson and Berridge, 1993). Additional evidence that dopamine does not mediate the hedonic response to rewarding stimuli comes from a recent study of brain-stimulation reward. Once rats had learned to lever press for electrical stimulation of the VTA, self-stimulation was maintained, but was no longer effective in evoking dopamine release in the nucleus accumbens (Garris et al., 1999).

## Mechanisms of Drug Addiction

Although repeated administration of addictive drugs causes many changes in the brain, most changes are relatively short-lived, disappearing within a few days after discontinuation of the drug. However, some changes are long-lasting and may be essentially permanent. At the behavioral level, sensitization of the locomotor stimulant effects of addictive drugs is long-lasting and may underlie the persistent craving that characterizes addiction and relapse to drug use (Robinson and Berridge, 1993). Sensitization may also play a causative role in psychosis induced by repeated use of psychostimulants (Robinson and Becker, 1986). The mechanisms underlying behavioral sensitization have been particularly well-characterized for cocaine and amphetamine. It is established that the increase in extracellular dopamine in the nucleus accumbens produced by psychostimulants is augmented weeks after their repeated administration (Robinson et al., 1988; Kalivas and Duffy, 1990; reviewed by Kalivas and Stewart, 1991; Robinson and Berridge, 1993). It is likely that this sensitization of the mesolimbic dopamine system is necessary, and possibly sufficient, for the expression of behavioral sensitization, which is known to be dopamine-dependent.

Repeated treatment with cocaine or amphetamine cross-sensitizes the stimulant response to the other psychostimulant, suggesting that sensitization to both agents works through similar mechanisms (Kalivas and Stewart, 1991). It has been established that amphetamine-induced or depolarization-induced dopamine release is enhanced in slices of nucleus accumbens from amphetamine-pretreated animals, and cocaine may have a similar effect (Kalivas and Stewart, 1991). Because slices of accumbens do not contain dopamine cell somata, it is presumed that changes have occurred in dopamine terminals to enable sensitized dopamine release. Recently, it has been found that cocaine pretreatment alters the mechanism by which amphetamine releases dopamine (Pierce and Kalivas, 1997). The release of dopamine in response to local application of amphetamine in the nucleus

accumbens *in vivo* was augmented in cocaine-pretreated rats, and the augmented component of release was found to be $Ca^{2+}$-dependent, but independent of vesicular exocytosis. It is not known whether repeated psychostimulant treatment alters the activity of mesolimbic dopamine neurons, and if so, to what extent this might contribute to an increased dopamine release in the nucleus accumbens in response to systemic administration of psychostimulants.

A persistent change at the cellular level produced by repeated psychostimulant treatment is an increased sensitivity to D1 receptor-mediated inhibition of medium spiny neurons in the nucleus accumbens. This has been observed both in brain slices (Higashi et al., 1989) and in vivo in anesthetized rats (Henry and White, 1991) after repeated administration of psychostimulants. This effect appears to result from an enhanced activation of voltage-independent K+ channels, and is mimicked by activation of adenylyl cyclase or a cAMP analogue (Higashi et al., 1989). Through an apparently similar mechanism, the ability of D1 activation to inhibit $Ca^{2+}$ spikes is augmented by repeated methamphetamine treatment (Higashi et al., 1989). These effects are likely to result both from direct phosphorylation of channels by protein kinase A, and by inhibition of protein phosphatase 1 by phosphorylated DARPP-32 (Fienberg et al., 1998). The D1 supersensitivity induced by cocaine persists for greater than one month and its decay coincides with the decay of locomotor sensitization (Henry and White, 1995). However, numerous studies have failed to find a sustained alteration in D1 receptor number by chronic psychostimulant treatment (Kalivas and Stewart, 1991). Thus, it appears that psychostimulants produce a long-lasting change in the signal transduction pathway activated by D1 receptors.

As discussed above, D1 receptors augment $GABA_B$ IPSPs in dopamine neurons (Cameron and Williams, 1993), and this is presumably mediated by D1 receptors

on terminals originating in the nucleus accumbens. It was therefore of interest to investigate the effects of repeated cocaine treatment on D1 modulation of $GABA_B$ IPSPs in dopamine neurons. Bonci and Williams (1996) found that the effect of adenylyl cyclase activation, directly or by D1 agonists, was opposite in drug-treated guinea pigs as compared to saline-treated controls. D1 agonists and forskolin inhibited the IPSP in drug-treated animals, while D1 antagonists augmented it. However, in the presence of adenosine A1 receptor antagonists or drugs disrupting the transport or metabolism of cAMP, forskolin and D1 ligands had the same effect in drug-treated and control animals. Furthermore, agents preventing activation of adenosine receptors produced a greater enhancement of the IPSP by themselves in drug-treated animals, and this effect was dependent on tonic D1 receptor activation by endogenous dopamine. However, the sensitivity of the IPSP to inhibition by an A1 agonist was unaltered in drug-treated animals, indicating that extracellular adenosine levels must be elevated by repeated cocaine treatment. Identical effects were observed with repeated morphine treatment.

It has been shown that cAMP can be transported out of cells and metabolized to adenosine (Rosenberg et al., 1994), which can then activate adenosine receptors. It is therefore hypothesized that repeated cocaine or morphine treatment increases the transport of cAMP out of the terminals or increases the metabolism of cAMP to adenosine. In either case, the result is that the $GABA_B$ IPSP is tonically inhibited by dopamine activation of D1 receptors, production of cAMP, transport and metabolism of cAMP to extracellular adenosine, and activation of A1 receptors (Bonci and Williams, 1996).

## Course of thesis research

My original intention was to further study the effect of repeated drug treatment on the regulation of $GABA_B$ IPSPs by the dopamine / adenosine interaction outlined above.

Because the long-term effects of chronic drug exposure have been best characterized in rats, I decided to perform these studies in rats rather than guinea pigs. The course of my research took a sharp turn with the discovery of a slow IPSP mediated by mGluR1. This IPSP differs from those previously characterized both in its postsynaptic mechanism and in its mediation by glutamate.

Following the characterization of the mGluR IPSP, I returned to studying the effects of repeated cocaine treatment. However, as the slow IPSP was now known to have both glutamate- and GABA-mediated components, the synapse-specificity of the increased adenosine tone was investigated. It was found that cocaine treatment increased the adenosine tone on glutamate but not GABA terminals. In addition, tonic adenosine selectively suppressed the mGluR inhibitory component of the synaptic glutamate response.

# Glutamate mediates an inhibitory postsynaptic potential in dopamine neurons

Christopher D. Fiorillo and John T. Williams

John T. Williams

Vollum Institute

Oregon Health Sciences University

3181 S.W. Sam Jackson Park Road

Portland, Oregon 97201

Phone: (503) 494-5465

FAX: (503) 494-6972

email: williamj@ohsu.edu

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Rapid information transfer within the brain depends on chemical signalling between neurons that is mediated primarily by glutamate and GABA (γ-aminobutyric acid), acting at ionotropic receptors to cause excitatory or inhibitory postsynaptic potentials (EPSPs or IPSPs), respectively. In addition, synaptically released glutamate acts on metabotropic receptors to excite neurons on a slower timescale through second-messenger cascades, including phosphoinositide hydrolysis (Pin and Duvosin, 1995). We now report a unique IPSP mediated by the activation of metabotropic glutamate receptors. In ventral midbrain dopamine neurons, activation of metabotropic glutamate receptors (mGluR1) mobilized calcium from caffeine/ryanodine-sensitive stores and increased an apamin-sensitive potassium conductance. The underlying potassium conductance and dependence on calcium stores set this IPSP apart from the slow IPSPs described so far (Surprenant and Williams, 1987; Pan et al., 1989; Otis et al., 1993). The mGluR-induced hyperpolarization was dependent on brief exposure to agonist, because prolonged application of exogenous agonist desensitized the hyperpolarization and caused the more commonly reported depolarization (Mercuri et al., 1993; Shen and Johnson, 1997; reviewed by Pin and Duvosin, 1995). The rapid rise and brief duration of synaptically released glutamate in the extracellular space can therefore mediate a rapid excitation through activation of ionotropic receptors, followed by inhibition through the mGluR1 receptor. Thus the idea that glutamate is solely an excitatory neurotransmitter must be replaced with a more complex view of its dual function in synaptic transmission.

Intracellular recordings were made in dopamine neurons of the ventral tegmental area (VTA) and substantia nigra. In the presence of ionotropic receptor antagonists, a train of stimuli (10 at 66 Hz) elicited a hyperpolarization that had two overlapping components of similar amplitude. The early component was mediated by $GABA_B$ receptors (Fig. 1a) as previously reported (Johnson and North, 1992). The late component was insensitive to $GABA_B$ antagonists (Fig. 1a,b) and reversibly blocked by TTX (300 nM, 91±2%, n=4), suggesting that it might also be a slow IPSP.

The kinetics of this late hyperpolarization suggested that it was mediated by a mechanism distinct from the $GABA_B$-mediated IPSP. The mGluR1-subtype is present on dopamine cells (Martin et al., 1992; Testa et al., 1994), is coupled to phosphoinositide (PI) hydrolysis, and is known to release calcium from intracellular stores in other cells (Pin and Duvosin, 1995). Calcium could activate a calcium-sensitive potassium conductance (rSK3) present in dopamine cells that is sensitive to the bee venom toxin, apamin (Shepard and Bunney, 1991; Kohler et al., 1996). Apamin irreversibly blocked the late hyperpolarization (by 86±3%, n=7, Fig. 1a), but did not affect the $GABA_B$ IPSP. Thus the late hyperpolarization was dependent on a rise in intracellular $[Ca^{2+}]$ and activation of a $K^+$ current.

The mGluR antagonists, (S)-MCPG (0.5-1.0 mM), (S)-4-CPG (500 μM), and (RS)-AIDA (100-500 μM), reduced the amplitude of the late hyperpolarization by 75±5% (n=6, Fig. 1b,c), 73±7% (n=4), and 44±8% (n=6) respectively. This pharmacological profile suggests that the late hyperpolarization is an IPSP mediated by mGluR1 (Brabet et al., 1995; Moroni et al., 1997; reviewed by Conn and Pin, 1997) and the mGluR1d splice variant has been specifically demonstrated in dopamine cells (Kolinski et al., 1998). The inhibition caused was selective for the IPSP since (S)-MCPG and (S)-4-CPG did not affect

the amplitude of the apamin-sensitive component of the afterhyperpolarization (AHP) following the action potential ((S)-MCPG, 0±3%, n=5; (S)-4-CPG, 11±5 decrease, n=5).

The mGluR-mediated IPSP had variable kinetics relative to the $GABA_B$ IPSP (Fig 1d). The peak latency ranged from 320-740 ms, with a mean value of 477±13 ms (n=52). IPSPs were frequently observed that had a long latency to onset (up to ~300 ms), but then rose to peak more rapidly than the $GABA_B$ IPSP. The mGluR IPSP was also unique in that agents which altered the activation of mGluRs also altered the peak latency. The mGluR1 antagonists MCPG and AIDA delayed the time to peak by 163±38 ms (n=5) and 48±15 ms (n=6), respectively. A μ-opioid (DAMGO, 1 μM, n=6) and a 5-HT1 (5-CT, 100 nM, n=5) agonist reduced the peak amplitude by 35±7% and 23±4%, and delayed the peak latency by 24±7 ms and 32±11 ms, respectively. D1 receptor activation (SKF 82958, 1 μM), which enhances glutamate release in the VTA both in vivo (Kalivas and Duffy, 1995) and in vitro (Peter Kalivas and Don Cameron, personal communication), augmented the IPSP (32±5%, n=5) and reduced the peak latency (46±12 ms). None of these agonists had any effect on the membrane potential of dopamine cells. Agents which modulate the $GABA_B$ IPSP in the same way do not alter its wave form (Johnson et al., 1992; Cameron and Williams, 1993).

While recording IPSPs, current was injected to prevent spontaneous action potentials, which normally allow calcium influx. In 11 of 25 neurons, the amplitude of the IPSP was augmented (74±23%, 19 - 275%) and the peak latency reduced (79±18 ms) by removing the holding current for 20 to 50 seconds between stimuli (Fig. 2). The facilitation often lasted for more than one minute. Augmentation of the mGluR IPSP by prior depolarization may result from loading of intracellular $Ca^{2+}$ stores that were partially depleted during prolonged hyperpolarization. We therefore tested agents known to disrupt the mobilization of $Ca^{2+}$ from intracellular stores (reviewed by Kostyuk and Verkhratsky,

1994; Ehrlich et al., 1994).

Ryanodine (10 μM), which blocks (McPherson et al., 1991) or reduces (Rousseau et al., 1987) the conductance of the ryanodine receptor and prevents its activation, irreversibly reduced the mGluR IPSP by 75±12% (n=6, Fig. 3a) after 15-20 minutes, while it had no effect on the $GABA_B$ IPSP (4±8% decrease, n=4, Fig. 3a). Caffeine (10 mM), which activates the ryanodine receptor and may also interfere with $IP_3$ receptor activation (Ehrlich et al., 1994), rapidly and reversibly inhibited the mGluR IPSP (69±4% after 4-6 minutes, n=9, Fig. 3b), while it more slowly facilitated the $GABA_B$ IPSP (70±18% after 6-10 minutes, n=4, Fig. 3b). Thapsigargin (10 μM) and cyclopiazonic acid (10 μM), which deplete intracellular $Ca^{2+}$ stores by blocking the ATPase that mediates $Ca^{2+}$ uptake (Thastrup et al., 1990; Seidler et al., 1989) blocked the mGluR IPSP by 19±5% (n=5) and 98±4% (n=6, Fig. 3c) respectively, after 15-20 minutes. The $GABA_B$ IPSP was not altered by thapsigargin (5±5% increase, n=3) or cyclopiazonic acid (6±7% decrease, n=4, Fig. 3c).

Following the mGluR IPSP a slow EPSP was occasionally observed. The slow EPSP had a longer peak latency (~800 ms) and duration, and was evoked with a greater number of stimuli than the mGluR IPSP (Fig. 4a). The slow EPSP was blocked by mGluR antagonists (MCPG or 4-CPG, n=4), confirming reports of mGluR-mediated slow postsynaptic excitations in dopamine (Shen and Johnson, 1997) and other neurons (Pin and Duvosin, 1995). Thus, depending on the stimulation protocol, synaptic activation of mGluR1 can mediate both hyperpolarizing and depolarizing responses in the same cell.

Application of exogenous mGluR agonists was performed by superfusion or pressure ejection. Superfusion of the selective mGluR 1/5 agonists (S)-DHPG (1-50 μM, n=16) and quisqualate (2 μM, n=5) always depolarized the membrane, confirming

previous studies showing that mGluR agonists depolarize dopamine (Mercuri et al., 1993) and other neurons (Charpak et al., 1990; Pin and Duvosin, 1995). One potential explanation for this apparent contradiction is that prolonged activation of mGluRs desensitizes the hyperpolarizing response. Agonists were therefore applied rapidly by pressure ejection to avoid desensitization.

Pressure ejections (5-800 ms) of L-glutamate (100 μM) or L-aspartate (1 mM; Charpak et al., 1990; Masu et al., 1991) caused both hyperpolarizations and depolarizations (Fig. 4a). Depolarizations were readily observed and blocked by mGluR antagonists, whereas hyperpolarizations were observed with more difficulty. In contrast to depolarizations, hyperpolarizations were only observed at lower concentrations of agonist and when applied closer to the soma. In all cells in which hyperpolarizations were observed (n=15), they were elicited with shorter duration pulses than depolarizations (Fig. 4a). Hyperpolarizing responses were blocked by MCPG (1 mM, 80±9%, n=4, Fig. 4b), caffeine (10 mM, 90±3%, n=4, Fig 4c) and apamin (100 nM, 92±1%, n=4), but were resistant to TTX (300 nM, n=5).

Desensitization of the hyperpolarization was examined with the combination of pressure ejection of aspartate and superfusion of DHPG (1 μM) at a concentration expected to occupy only a fraction of mGluR1 receptors. DHPG (1 μM) caused a sustained depolarization (3.4±0.6 mV) and reduced the amplitude of the hyperpolarization induced by pressure ejection of aspartate by 80±11% (n=4, Fig. 4b), but did not affect the apamin-sensitive AHP (8±6% increase, n=6). Superfusion of DHPG also caused an identical inhibition of the mGluR IPSP (81±5%, n=5, Fig. 1c). This suggests that continuous, low receptor occupancy rapidly desensitizes the hyperpolarizing response to mGluR activation downstream of the receptor itself.

In both acutely dissociated hippocampal CA1 neurons and cultured cerebellar granule cells, activation of mGluRs mobilizes intracellular calcium (Shirasaki et al., 1994;

Irving et al., 1992) and activates a potassium conductance (Shirasaki et al., 1994; Fagni et al., 1991). In both cell types, the mGluR response was blocked by caffeine and ryanodine, and appeared to depend on activation of $IP_3$ receptors (Shirasaki et al., 1994; Irving et al., 1992). It is proposed that the mGluR-mediated IPSP results from activation of a mGluR1 which causes $IP_3$-induced $Ca^{2+}$ release, to trigger $Ca^{2+}$-induced $Ca^{2+}$ release through ryanodine receptors. The mGluR-mediated IPSP is therefore unlike previously characterized slow IPSPs mediated by a membrane delimited pathway and the activation of an inwardly rectifying potassium conductance (Surprenant and Williams, 1987; Pan et al., 1989; Otis et al., 1993). Not surprisingly, these two mechanisms give rise to IPSPs with distinct kinetic properties (Fig. 1d). It is unlikely that mGluRs are unique in evoking this form of synaptic inhibition, as we have observed similar hyperpolarizations mediated by PI-coupled muscarinic acetylcholine receptors (unpublished results).

Like other PI-coupled receptors, mGluR receptor activation has generally been found to be excitatory (Charpak et al., 1990; Pin and Duvosin, 1995). Whereas most previous studies have used slow application of agonist, this study indicates that inhibition requires rapid application of agonist. The present results show that the activation of mGluR1 by synaptically released glutamate can induce $Ca^{2+}$ mobilization in the absence of depolarization. In dopamine cells, this causes a pure inhibition by activation of a $Ca^{2+}$-dependent potassium conductance. Prolonged synaptic activation of mGluRs may result in suppression of the IPSP as well as evoking a slow EPSP. Thus, depending on the frequency and pattern of afferent input to dopamine neurons, glutamate can mediate inhibition or excitation by activation of the same receptor.

## Methods

**Slice preparation.** Intracellular recordings were made in horizontal slices (300 μm) of ventral midbrain from male Wistar rats (150-200 gm). Details of the method of slice preparation and recording have been published (Cameron and Williams, 1993). Recordings were made from submerged slices in a chamber (0.5 ml) superfused with physiological saline at a rate of 1.5 ml/min and maintained at 35°C. The solution was equilibrated with 95% $O_2$/ 5% $CO_2$ and contained (mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.4 $NaH_2PO4$, 25 $NaHCO_3$, and 11 D-glucose.

**Recordings.** Microelectrodes (50-80 MΩ) were filled with 2 M KCl. Recordings were made from dopamine neurons, identified by their electrical properties, in VTA (61) and substantia nigra (16). No differences were observed between neurons of the VTA and substantia nigra; the data was pooled. The membrane potential was held between -60 and -70 mV to prevent spontaneous action potentials.

**Synaptic potentials.** Synaptic potentials were evoked with bipolar tungsten stimulating electrodes with a tip separation of 300-1000 μM. A train of 10 stimuli of 400 μs at 0.5 - 1.5 mA was delivered at 66 Hz every 60 seconds. Stimulating electrodes were placed within 1 mm rostral or caudal of the recording site.

All synaptic potentials were recorded in the presence of picrotoxin (100 μM, $GABA_A$), strychnine (1 μM, glycine), eticlopride (100 nM, D2), and CNQX (10 μM, AMPA) or NBQX (5 μM, AMPA). Slices were treated with MK-801 (50-100 μM) at the start of all experiments to block NMDA receptor-mediated currents. In all experiments on

the mGluR IPSP, the solution also contained the $GABA_B$ antagonist CGP 35348 (100-300 μM), CGP 52432 (500-1000 nM) or CGP 56999a (100-1000 nM). It is noteworthy that the $GABA_A$ receptor antagonist bicuculline methiodide suppresses both the apamin-sensitive AHP (Johnson and Seutin, 1997) and the mGluR IPSP in the presence of picrotoxin.

**Drugs**. All drugs were applied by superfusion, except pressure ejections of L-glutamate (100 μM) or L-aspartate (1 mM), which were performed at 20 psi with a Picospritzer II using glass pipettes (1 μm tip diameter).

**Data analysis** . Values are given as arithmetic means ± standard error of the mean. All differences that are stated in the text are statistically significant (paired t-tests, $p<0.05$). The amplitude of the afterhyperpolarization (AHP) following a single action potential was determined by measuring the difference in membrane potential between 20 ms before and 50 ms after the initiation of the action potential. Using this measurement, apamin blocked 63±4% (n=8) of the AHP, indicating the presence of one or more additional conductances underlying this potential change.

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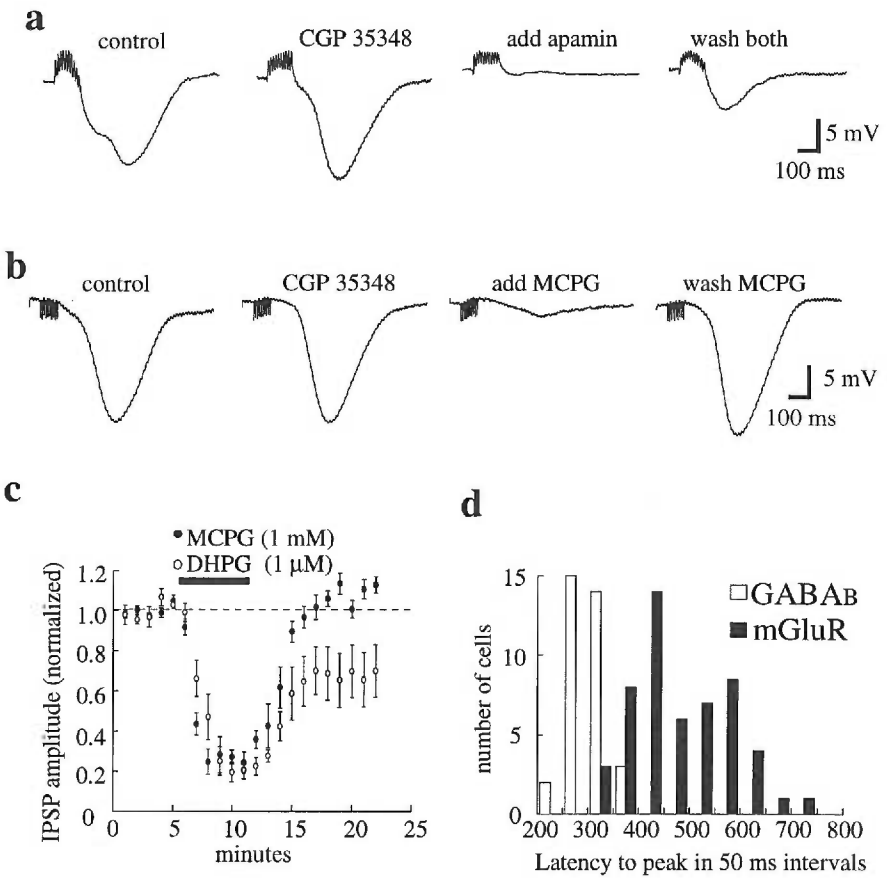


**Figure 1** The late component of the IPSP is mediated by mGluRs and activation of an apamin-sensitive potassium conductance. **a**, Consecutive averaged traces showing that the early component of the IPSP is selectively blocked by the GABAB antagonist CGP 35348 (100 μM), while the late component is selectively and irreversibly blocked by apamin (100 nM). Wash of apamin and CGP 35348 restores only the GABAB IPSP. **b**, The late component of the IPSP is reversibly blocked by the mGluR antagonist (S)-MCPG (1 mM). **c**, The mGluR IPSP is blocked by (S)-MCPG (0.5 - 1 mM, n=6) and is desensitized by a low concentration of the mGluR agonist (S)-DHPG (1 μM, n=5). DHPG also caused a sustained depolarization of about 3 mV (see text). In all figures, each point represents the average of IPSPs from several cells, each of which was normalized to the average amplitude of the five IPSPs preceding drug application (MCPG, -18.9±1.3 mV, n=6; DHPG, -17.8±3.1 mV, n=5). **d**, Histogram of the peak latency for GABAB (-12.9±0.8 mV, n=34) and mGluR (-13.5±0.7 mV, n=52) IPSPs. The peak latency was measured from the start of the stimulus train, which consisted of 10 stimuli and lasted 135 ms.

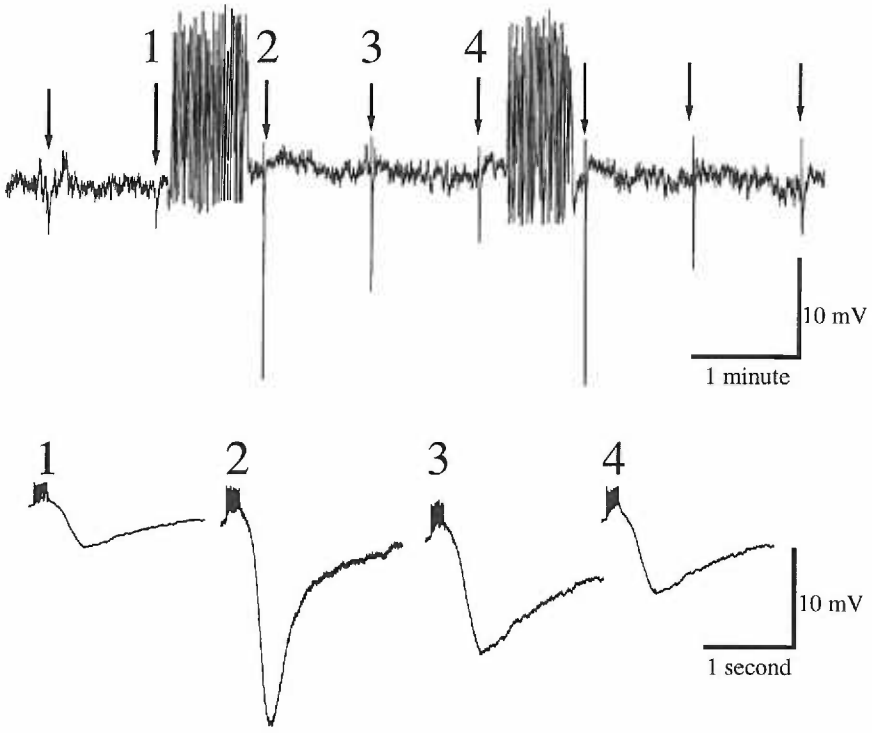


**Figure 2** The amplitude of mGluR IPSPs is increased by prior depolarization of the membrane potential. Removing the holding current between stimuli allowed spontaneous action potentials and increased the amplitude of the IPSP. The full extent of the action potentials and the following afterhyperpolarizations are not shown. Arrows indicate points of stimulation. The downward deflections below the arrows represent IPSPs. The traces below show, on an expanded time scale, the IPSPs corresponding to the numbered arrows in the upper portion.

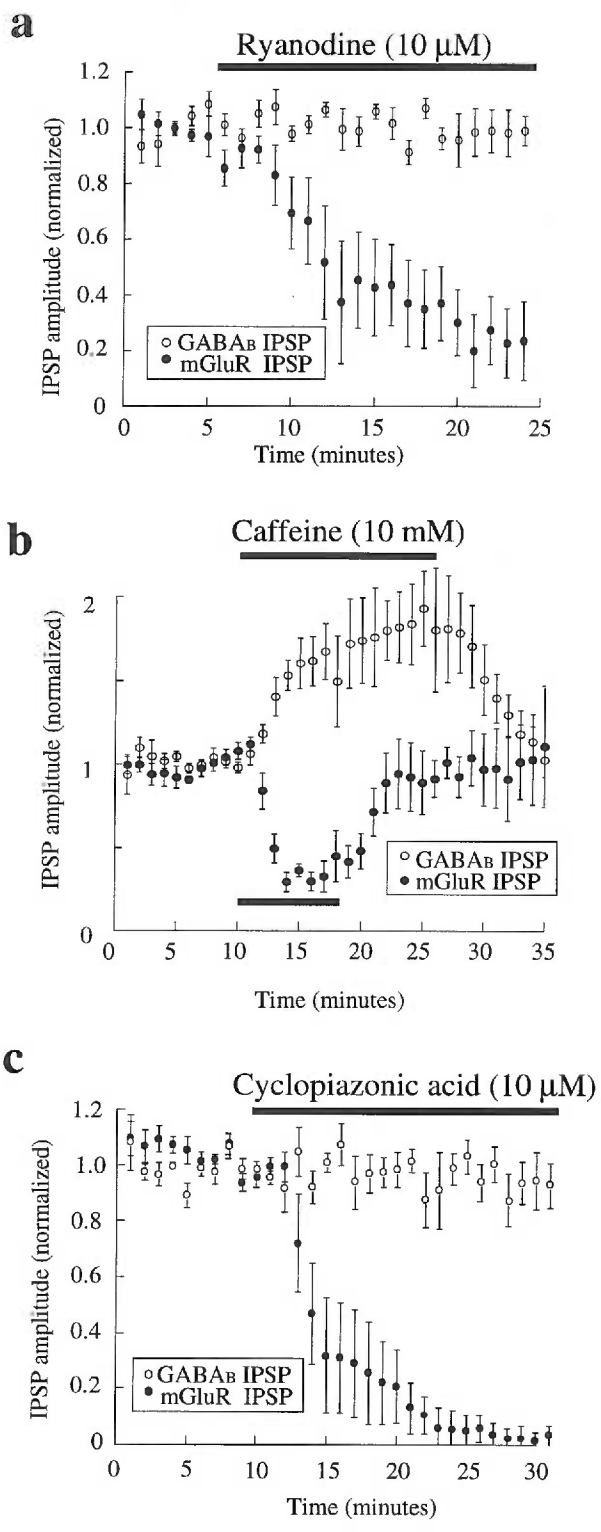


**Figure 3** The mGluR-mediated IPSP is dependent on intracellular Ca2+ stores. **a**, Ryanodine (10 μM) blocked the mGluR IPSP (control = -12.9±2.0 mV, n=6) but had no effect on the GABAB IPSP (control = -14.5±2.3 mV, n=4). **b**, Caffeine (10 mM) blocked the mGluR IPSP (control = -13.2±1.9 mV, n=9) while it facilitated the GABAB IPSP (control = -11.2±1.1 mV, n=4). Caffeine was applied for a longer time on the GABAB IPSP than on the mGluR IPSP, as indicated by the bars. **c**, Cyclopiazonic acid (10 μM) abolished the mGluR IPSP (control = -16.0±1.8, n=6) without altering the GABAB IPSP (control = -16.5±3.7, n=4).

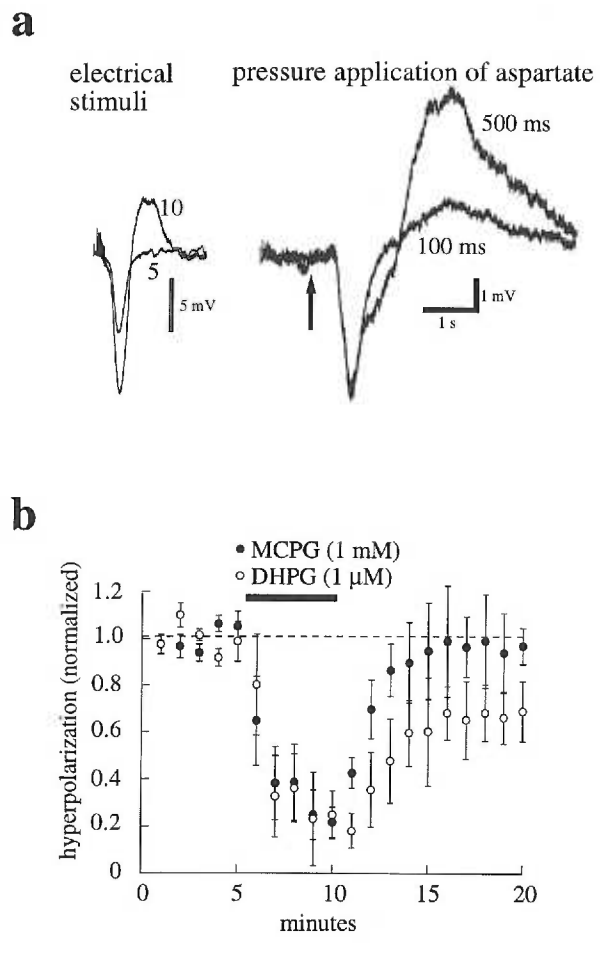


**Figure 4** Brief exogenous application of aspartate applied by pressure ejection mimics the IPSP. **a**, Left, in this cell, a train of 5 stimuli evokes only an IPSP, while a train of 10 stimuli elicits an EPSP following the IPSP. Right, in another cell, pressure ejection of aspartate (1 mM) for 100 ms produced primarily a hyperpolarization, while a 500 ms ejection also produced a depolarization. The time scale is the same for traces on the left and right. **b**, The mGluR antagonist MCPG (1 mM) blocked and a low concentration of the agonist DHPG (1 μM) desensitized the hyperpolarizations produced by aspartate (1 mM) or glutamate (100 μM) (MCPG control = -9.0±1.5 mV, n=4; DHPG control = -8.7±1.8 mV, n=4). **c**, Caffeine (10 mM) also blocked the hyperpolarizations produced by aspartate (control = -9.4±1.3 mV, n=4). Shortly after washing out the caffeine, the response was transiently enhanced.

## Appendix to manuscript 1

Because the first manuscript was condensed for publication in Nature, some relevant data was not reported. This data is presented here.

When applying drugs to disrupt intracellular $Ca^{2+}$ stores, slow AHPs were recorded as well as slow IPSPs. These drugs reduced the amplitude of the apamin-sensitive component of the AHP as well as the mGluR IPSP (Fig 5*). Caffeine (31±2%, n=10), ryanodine (26±6%, n=4), and cyclopiazonic acid (23 ± 7%, n=5) each suppressed the sAHP (measured 50 ms after the action potential; fig. 5*, b-d; paired t-tests, $p<0.01$). Apamin (100 nM) reduced the amplitude of the AHP, measured in the same way, by 63 ± 4% (n=8, fig 5*a). This suggests that intracellular $Ca^{2+}$ stores amplify the $Ca^{2+}$ transient following a single action potential. Most likely this occurs through $Ca^{2+}$-induced $Ca^{2+}$ release at ryanodine receptors.

As quantified in the main text of the manuscript, a low concentration of the mGluR1 agonist DHPG (1 μM) blocked the mGluR hyperpolarization (fig. 4), but did not alter the AHP. It also caused a small but sustained depolarization, suggesting it did not desensitize the receptor. These results are interpreted to mean that the desensitization of the hyperpolarizing response occurs downstream of the receptor, but upstream of the ryanodine-sensitive $Ca^{2+}$ stores. Most likely this is due to depletion of $Ca^{2+}$ from $IP_3$-sensitive stores.

The L-type $Ca^{2+}$ channel has been found to be coupled to both mGluR1 and ryanodine receptors (Chavis et al., 1996) and to SK channels in neurons (Marrion and Tavalin, 1998), in addition to its well-known role in the activation of ryanodine receptors in skeletal and cardiac muscle. It is conceivable that $Ca^{2+}$ entry through L channels could be

contributing to the mGluR IPSP. However, the L-type antagonist nimodipine (3 μM) reduced the mGluR IPSP by only 24±11% (n=4, p=0.16). This slight reduction could be due to a presynaptic effect (Bonci et al., 1998). Therefore, the mGluR hyperpolarization is not dependent on L-type $Ca^{2+}$ channels.

Another PI-coupled receptor, common to many types of neuron and known to mediate responses very similar to mGluR1, is the muscarinic acetylcholine receptor. This receptor is present in dopamine neurons, and like mGluR1, produces a large depolarization with superfusion of agonist in a slice preparation (Lacey et al., 1990). Dopamine neurons receive a cholinergic innervation, and it seemed likely that muscarinic receptors also mobilize $Ca^{2+}$. We therefore tested the ability of the muscarinic antagonist scopolamine (1 μM) to block slow IPSPs. Only 1 of 15 IPSPs (evoked with caudal stimulation) was blocked by scopolamine (fig. 6*a). This IPSP had a short latency relative to the mGluR IPSP. Since that time, scopolamine has been tested on IPSPs with short latencies that are not blocked by $GABA_B$ antagonists, and two of these were also blocked by scopolamine (fig 6*a). In addition, pressure ejection of acetylcholine (3 mM) hyperpolarized the membrane (n=4). The hyperpolarization was reduced by caffeine (57±8%, n=3, fig. 6*b) and apamin (74±21%, n=3, fig. 6*c). With longer applications of acetylcholine, a slow depolarization was produced. It is concluded that muscarinic receptors hyperpolarize dopamine neurons through a pathway similar to that activated by mGluR1.

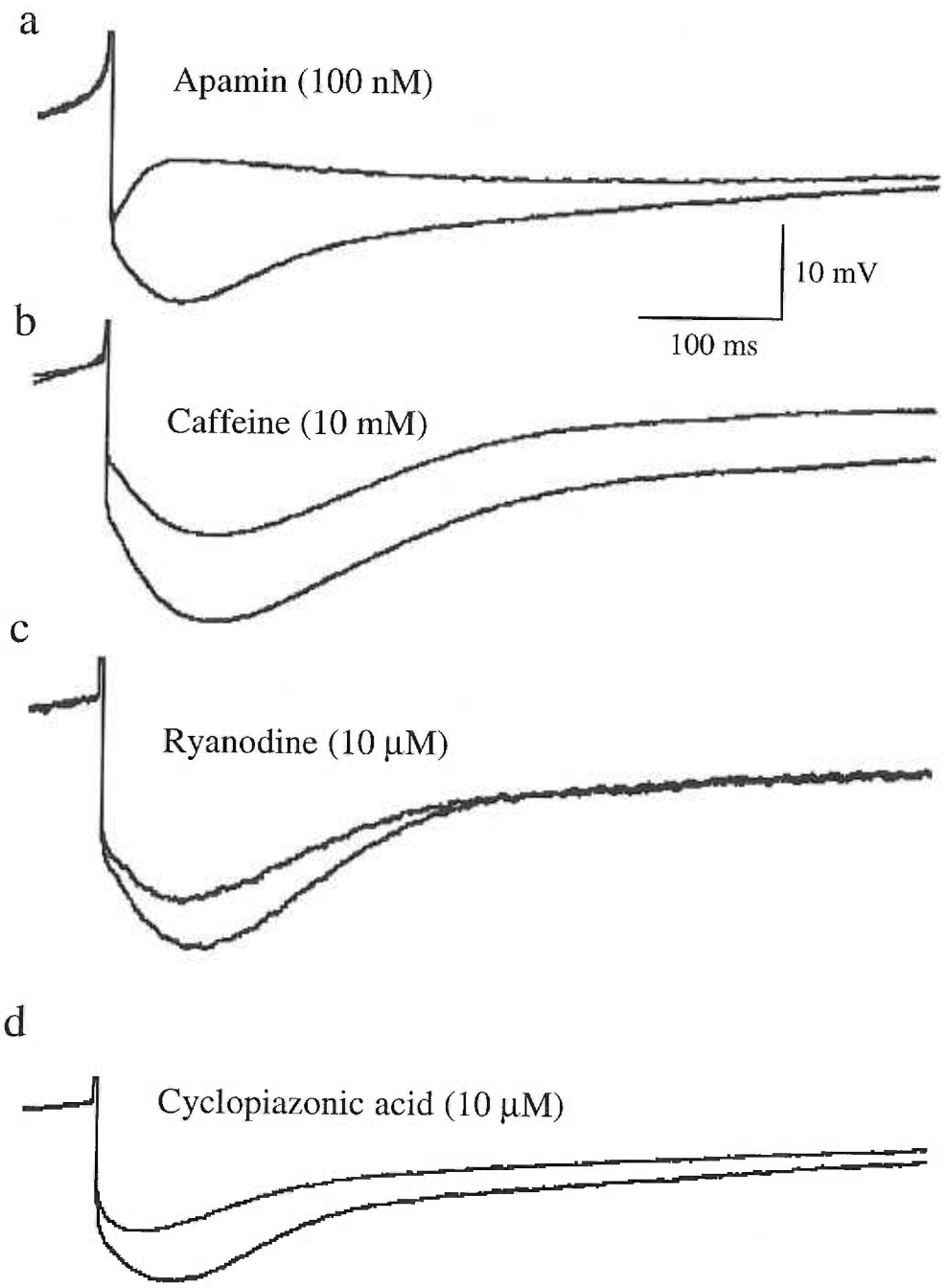


**Figure 5***. Apamin and drugs that alter intracellular Ca2+ stores reduce a component of the AHP in dopamine neurons. Neurons were allowed to fire spontaneous action potentials, with enough negative current injected so as cause the neuron to fire at a slow rate (<1 Hz). **a**, Apamin (100 nM) completely blocks a component of the AHP. **b**, The apamin-sensitive component of the AHP is depressed but not blocked by caffeine (10 mM), **c**, ryanodine(10 µM), and **d**, CPA (10 µM).

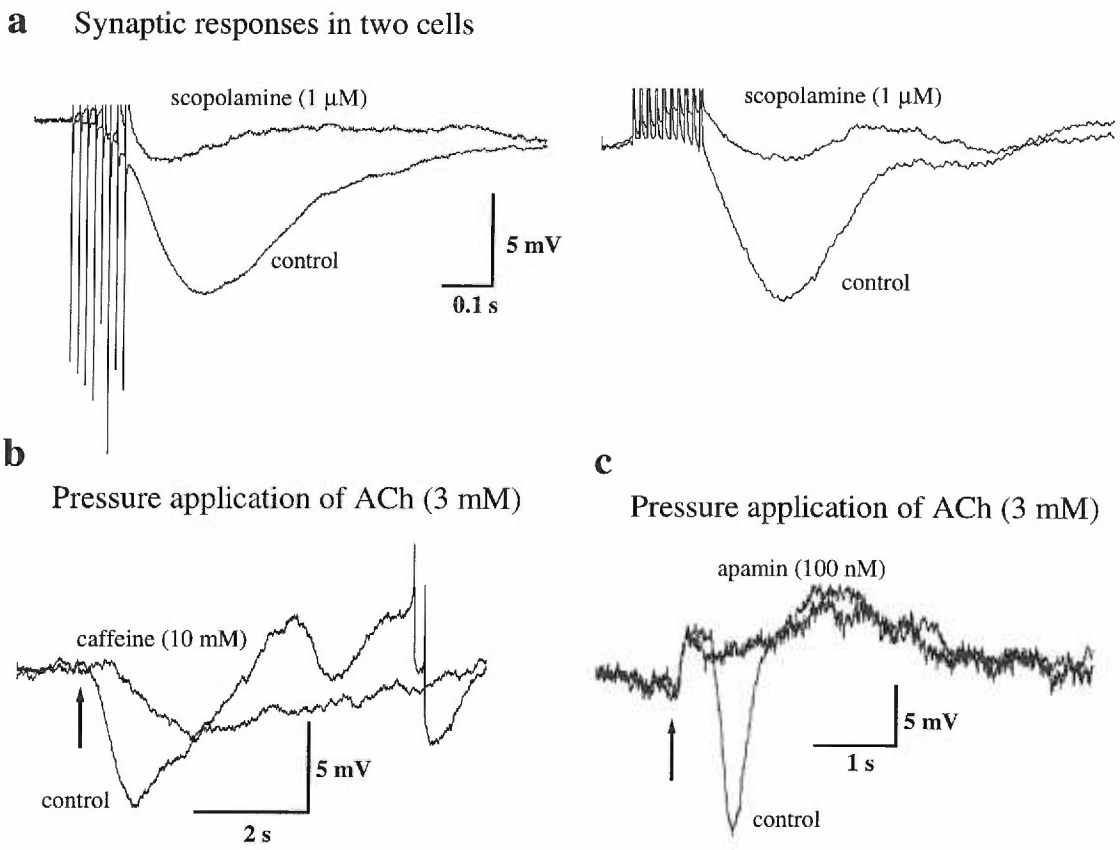


**Figure 6***. Inhibitory cholinergic responses in dopamine neurons. **a**, two examples of muscarinic IPSPs. Traces are shown in the absence and presence of scopolamine. **b**, Caffeine (10 mM) suppresses the hyperpolarizing response to acetylcholine (3 mM, 60 ms pressure ejection). In **b** and **c**, hexamethonium (400 μM) was superfused to block nicotinic responses. Acetylcholine was applied at the time indicated by the arrows. **c**, Apamin (100 nM) blocks the acetylcholine-induced hyperpolarization (300 ms pressure ejection), but leaves a depolarization. The depolarization was blocked by scopolamine (1 μM, not shown).

**Repeated cocaine selectively increases tonic adenosine inhibition of mGluR-mediated IPSPs in dopamine neurons of the ventral tegmental area**

Christopher D. Fiorillo and John T. Williams

(not yet submitted)

The mesolimbic dopamine system is a common target of addictive drugs and is central to their reinforcing properties. With repeated exposure to psychostimulants such as cocaine and amphetamine, long-lasting changes occur in the mesolimbic dopamine system that are thought to underlie continued drug-seeking and relapse. It was previously found that increased adenosine tone results in inhibition of slow inhibitory postsynaptic potentials (IPSPs) in dopamine neurons of guinea pigs 7-10 days withdrawn from cocaine or morphine. We examined the synapse specificity of the increased adenosine tone in slices from the ventral tegmental area of rats 10-20 days withdrawn from repeated cocaine treatment. The selective A1 receptor antagonist, DPCPX, selectively enhanced the slow, mGluR-mediated IPSP in slices from cocaine-treated animals. DPCPX was without effect on fast, glutamate receptor-mediated EPSCs. However, in the presence of a low, physiologically relevant concentration of amphetamine, DPCPX did augment fast EPSCs in cocaine-treated rats. Although DPCPX increased slow $GABA_B$-mediated IPSPs, this was not changed by cocaine pretreatment. Even in the presence of amphetamine, cocaine pretreatment was without effect on the modulation by DPCPX of $GABA_B$ IPSPs. This suggests that the elevated adenosine tone acts preferentially or exclusively on glutamate terminals. Furthermore, because endogenous adenosine caused greater inhibition of the glutamate IPSP than of the glutamate EPSC, it may be speculated that glutamate afferents more effectively elicit burst firing in cocaine-treated rats, a phenomenon known to enhance dopamine release.

## INTRODUCTION

The mesolimbic dopamine system, originating in the ventral tegmental area (VTA), is thought to play a central role in reward, in incentive learning, and in motivational processes. Consistent with this, the mesolimbic dopamine system is the target of addictive

drugs, many or all of which increase extracellular dopamine levels, particularly in the nucleus accumbens (Di Chiara and Imperato, 1988). The addictive drugs best studied with respect to the dopamine system are the psychostimulants, especially cocaine and amphetamine (reviewed by Pierce and Kalivas, 1997). Repeated use of psychostimulants by humans can result in the development of profound craving for the drug, as well as sensitization to the drug's psychotomimetic effects. These effects of drug use are sustained even after long periods of abstinence. In rodents, repeated administration of cocaine or amphetamine results in a long-lasting sensitization to the locomotor stimulant effects of the drug. This sensitization is mediated at least in part by an enhanced ability of the stimulant to increase dopamine levels in the nucleus accumbens. The sensitization of the mesolimbic dopamine system may render it hypersensitive not only to psychostimulants, but also to stress and to environmental stimuli associated with drug use, all of which are known to promote craving and relapse to drug use (Robinson and Berridge, 1993).

Although the behavioral consequences of repeated psychostimulant administration are reasonably well understood, far less is known about the underlying cellular and molecular changes. Perhaps the best established, persistent change at the cellular level is an alteration in signal transduction by dopamine D1 receptors, which are positively coupled to adenylyl cyclase. This has been shown postsynaptically in the nucleus accumbens both *in vitro* (Higashi et al., 1989) and *in vivo* (Henry and White, 1991, 1995), and presynaptically in the VTA *in vitro* (Bonci and Williams, 1996).

D1 receptors in the VTA are found on afferent terminals from the nucleus accumbens and ventral pallidum containing GABA (Mansour et al., 1992; Lu et al., 1997b), and glutamatergic afferents from the prefrontal cortex (PFC) express D1 receptor mRNA (Lu et al., 1997a). Stimulation of D1 receptors in drug-naive animals enhances release of both GABA (Cameron and Williams, 1993) and glutamate (Kalivas and Duffy, 1995; Fiorillo and Williams, 1998) onto dopamine neurons. In drug-naive guinea pigs, D1

receptor agonists enhance the slow inhibitory postsynaptic potential (IPSP). However, after 7-10 days withdrawal from repeated injections of cocaine or morphine, D1 agonists inhibited the IPSP (Bonci and Williams, 1996). It was found that in treated animals, the cAMP produced by D1 activation was metabolized to extracellular adenosine, which then inhibited transmitter release through activation of adenosine A1 receptors. As a result, the adenosine tone was elevated in slices from drug-treated animals, and was entirely dependent on tonic activation of D1 receptors. After blocking A1 receptors or the metabolism of cAMP, the modulation of transmitter release by D1 receptor agonists or antagonists was identical at all concentrations in slices from saline- and drug-treated animals (Bonci and Williams, 1996). This suggests that the metabolism of cAMP to adenosine has been increased by repeated cocaine treatment, but that D1 receptors and their coupling to effectors have not been changed.

More recently, it has been reported that the slow IPSP in dopamine neurons of rats consists of two components, an early component mediated by $GABA_B$ receptors, and a late component mediated by metabotropic glutamate receptors (mGluRs, Fiorillo and Williams, 1998). Furthermore, it is known that D1 receptors are present on glutamate as well as GABA terminals, so the observed changes could be present on either or both sets of terminals. It was therefore of interest to examine the synapse specificity of the increased adenosine tone in drug-treated animals. The present study focused on adenosine tone in cocaine-treated rats. Certain experiments were also performed in rats treated with amphetamine or morphine.

## Methods

*Treatment protocol.* Naive, male Wistar rats weighed 150-200 grams. Treated rats received injections of 1 ml/kg of 0.9% NaCl solution, with or without 20 mg/kg cocaine

HCl, once daily for 14 days in their home cages. Other rats were given 2 mg/kg amphetamine sulfate every third day for 15 days (5 injections) or 10 mg/kg morphine sulfate every other day for 14 days (7 injections). The animals were then withdrawn for 10-20 days before sacrifice, at which time they weighed 300-350 grams.

*Slice preparation.* Intracellular or whole cell patch recordings were made in horizontal slices (250-300 μm for intracellular recordings, 200 μm for patch recordings) of ventral midbrain. Details of the method of slice preparation and recording have been published (Williams et al., 1984). Recordings were made from submerged slices in a chamber (0.5 ml) superfused with physiological saline at a rate of 1.5 ml/min and maintained at 35°C. The solution was equilibrated with 95% $O_2$/ 5% $CO_2$ and contained (mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.4 $NaH_2PO4$, 25 NaHCO3, and 11 D-glucose.

*Recordings.* Recordings were made with an Axoclamp 2A amplifier from dopamine neurons, identified by their electrical properties (Johnson and North, 1992), in the VTA. The VTA was defined as the area medial and rostral, but not lateral or caudal, of the medial terminal nucleus of the accessory optic tract. While some of these neurons are found close to the MT in the area of dense cell bodies termed the "compacta", they are likely to be mesolimbic (Fallon and Loughlin, 1995). Microelectrodes (50-80 MΩ) were filled with 2 M KCl. The membrane potential was adjusted to between -60 and -70 mV to prevent spontaneous action potentials. For whole-cell patch recordings, cells were visualized using an upright microscope with infrared illumination and Nomarski optics. Patch electrodes (2-5 MΩ) contained (in mM) 125 KCl, 1 $MgCl_2$, 1 EGTA, 5 HEPES, 1 ATP, and 0.3 GTP. For recordings of NMDA EPSCs, electrodes contained 125 Cs-gluconate, 10 NaCl, 1 $MgCl_2$, 10 HEPES, 1 EGTA, 0.3 $CaCl_2$, 1 ATP, and 0.3 GTP. A junction

potential of approximately -10 mV with gluconate-containing electrodes was not corrected. The membrane potential was clamped between -70 and -80 mV for recording AMPA EPSCs, and at +30 mV for recording NMDA EPSCs. The access resistance was monitored with each stimulus by applying a 10 mV hyperpolarizing step. Membrane potential or holding current was recorded continuously at a slower sampling frequency in all experiments.

*Synaptic potentials.* Synaptic potentials were evoked with bipolar tungsten stimulating electrodes with a tip separation of 300-1000 μM. For all synaptic responses, a train of 8-10 stimuli of 400 μs at 0.3 - 1.5 mA was delivered at 66 Hz (15 ms interval) every 60 seconds, except for NMDA EPSCs, which were evoked at 10 Hz. Stimulating electrodes were placed within 1 mm rostral of the recording site. By stimulating rostrally, descending afferents may be preferentially activated, many of which are presumed to originate in the prefrontal cortex (PFC) and nucleus accumbens.

The following antagonists were used to isolate the desired synaptic response. Picrotoxin (100 μM, $GABA_A$), strychnine (1 μM, glycine), and eticlopride (100 nM, D2) were present in all experiments. NBQX (5 μM, AMPA) and MK-801 (50-100 μM, NMDA, pretreatment only) were used to isolate all slow IPSPs. The $GABA_B$ antagonist CGP 35348 (100-300 μM) or CGP 56999a (100-1000 nM) was used to isolate mGluR IPSPs. $GABA_B$ IPSPs were studied after treating the slice with apamin (100 nM, SK channels) or in cases in which a $GABA_B$ antagonist completely blocked the slow IPSP. Pretreatment with MK-801 was performed before studying AMPA EPSCs, and NBQX was present in experiments on the NMDA EPSC.

An mGluR-mediated slow EPSP can also be evoked in dopamine neurons and overlaps with the IPSP (Shen and Johnson, 1997). The slow EPSP is infrequently

observed with microelectrode recording, and when present, requires more stimuli than the IPSP (Fiorillo and Williams, 1998). The depolarizing response to mGluR activation requires a more prolonged activation of the receptor than does the hyperpolarizing response, while the hyperpolarizing response desensitizes with prolonged receptor activation. It is therefore possible that a drug which increases glutamate release might enhance the EPSP and thereby mask or desensitize the IPSP. For this reason, after DPCPX was applied to mGluR IPSPs, apamin was superfused to reveal a slow EPSP if present. In the few cases when an EPSP was present, the data were discarded.

*Drugs*. All drugs were applied to the slice by superfusion. The majority of drugs used, including DPCPX, had no effect on membrane potential or holding current. CPA at high concentrations sometimes caused a hyperpolarization of up to 5 mV. Amphetamine (3 μM, in the presence of eticlopride) consistently produced a small depolarization (~3 mV). CGP 35348 (200 μM) and CGP 56999a (100-1000 nM) consistently caused depolarizations of several mV, suggesting tonic activation of postsynaptic $GABA_B$ receptors.

Adenosine, adenosine trisphosphate (ATP), S(+)-amphetamine sulfate, apamin, guanosine trisphosphate (GTP), picrotoxin, and strychnine were from Sigma (St. Louis, MO). $N^6$-cyclopentyladenosine (CPA), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), S(-)-eticlopride, and MK-801 were from Research Biochemicals International (Natick, MA). 1,2,3,4-tetrahydro-6-nitro-2,3,-dioxo-benzo[f]quinoxaline (NBQX) was from Tocris Cookson (St. Louis, MO). Cocaine HCl and morphine sulfate were from the National Institute on Drug Abuse (Rockville, MD). CGP 35348 and CGP 56999a were a gift from Novartis Pharmaceuticals (Switzerland).

*Data analysis*. Each specific experiment was performed only once per rat. Values are

given as arithmetic means ± standard error of the mean. The percent change produced by a drug was calculated as the mean amplitude of five to ten synaptic responses after equilibrium had been reached (7-20 minutes) relative to the mean of five responses before drug superfusion. To construct concentration-response curves, two concentrations of CPA were superfused sequentially before reversal with DPCPX. To estimate the $EC_{50}$ and maximal response, concentration-response curves were fit with a least squares regression using the logistic equation. Statview software was used for performing statistical tests; $p<0.05$ was considered as a significant difference. One- and two-way ANOVAs were performed with Fisher's post hoc test. Unpaired comparisons between two groups were made with a Mann-Whitney U test, while paired comparisons were made using a Wilcoxan signed rank test.

## RESULTS

### Identification of VTA dopamine neurons

Dopamine neurons in the VTA of horizontal slices were identified by their electrical properties (Johnson and North, 1992). With intracellular, KCl-containing electrodes, these cells exhibited slow, spontaneous firing (~1-3 Hz), broad action potentials followed by large after hyperpolarizations, and a depolarizing "sag" in response to hyperpolarizing current injection. With patch electrodes, dopamine neurons were identified by their firing pattern in the cell-attached mode, and the presence of a large time- and voltage-dependent inward rectification in the current-voltage relation (H current) measured in the whole-cell mode. As in previous studies, these neurons were found to respond to electrical field stimulation of the slice with both fast and slow synaptic responses mediated by both glutamate and GABA.

## The mGluR IPSP is augmented by DPCPX

We first measured the increase in slow IPSP amplitude in response to superfusion of the adenosine A1 receptor selective antagonist DPCPX (Fig. 1). In saline-treated animals, DPCPX (200 nM) did not cause a significant increase in IPSP amplitude (7.6±6.6%, n=9). However, in cocaine- or amphetamine-treated rats, DPCPX caused a 52.1 ± 12.7% (n=16) and 91.0±24.0% (n=6) increase (Fig. 1B, C), respectively (one-way ANOVA, main effect of treatment, $F[2,29] = 6.2$, $p=0.006$; effect of cocaine, $p=0.028$, and amphetamine, $p=0.002$). This confirms in rats previous results obtained in guinea pigs (Bonci and Williams, 1996), showing that cocaine pretreatment potentiates the augmentation of slow IPSPs by A1 receptor antagonists.

In the above analysis of "slow IPSPs," responses were selected in which the mGluR-mediated IPSP was present, as determined pharmacologically or by visual inspection. A $GABA_B$-mediated IPSP was present in most but not all cases. The increase produced by DPCPX was observed to be primarily due to an increase in the late (mGluR) component of the IPSP (Fig. 1A). This is illustrated in figure 1C, in which the effect of DPCPX on the IPSP is shown at two time points chosen to discriminate as much as possible between the early ($GABA_B$) and late (mGluR) components of the IPSP (Fig. 1C).

The effect of DPCPX was also examined on isolated mGluR IPSPs. The mGluR IPSP, in the presence of a $GABA_B$ antagonist (CGP 35348,100-300 μM or CGP 56999a, 0.1-1 μM) was not significantly augmented by DPCPX in either saline- or cocaine-treated rats (7.0 ± 14.0%, n=5 and 20.6 ± 13.4%, n=8, respectively, Fig. 2). It appears that $GABA_B$ antagonists prevent the augmentation of glutamate release by DPCPX.

## DPCPX has no effect on fast glutamate-mediated transmission

The adenosine tone was also examined on glutamate-mediated fast excitatory postsynaptic currents (EPSCs). AMPA-mediated EPSCs were evoked using the same protocol as used to evoke slow IPSPs. DPCPX was without effect on EPSCs in slices from both saline- and cocaine-treated rats (Fig. 3; 0.3 ± 4.5%, n=10, and 0.9 ± 6.9%, n=10, respectively, percent increase in amplitude of the first EPSC in the train). Another recent study in our lab has also found a lack of adenosine tone on AMPA EPSCs in dopamine neurons of the VTA (Manzoni and Williams, in press)

It is generally thought that the same glutamate terminals release glutamate onto both AMPA and NMDA subtypes of glutamate receptor. However, because of the unique role that NMDA receptors are thought to play in the burst firing of dopamine neurons (Johnson et al., 1992b; reviewed by Overton and Clark, 1997), as well as the possible presence of "NMDA-only" synapses, we also examined adenosine tone on NMDA-mediated EPSCs, studied at +30 mV to relieve $Mg^{2+}$ blockade. DPCPX had no effect or caused a small decrease in the amplitude of NMDA EPSCs in both saline- and cocaine-treated rats (-9.7 ± 2.4%, n=6, and -9.7 ± 4.5%, n=7, respectively). Therefore, while adenosine tone is present on mGluR synaptic responses in cocaine-treated rats, it is absent on synaptic responses mediated by ionotropic glutamate receptors (iGluRs).

## An effect of DPCPX on EPSCs is revealed by amphetamine

Having examined adenosine tone under resting conditions, we next investigated adenosine tone in the presence of a low concentration of amphetamine (3 μM, Clausing et al., 1995). By releasing dopamine and activating D1 receptors, amphetamine may increase adenosine tone, as implied by Bonci and Williams (1996). Amphetamine (3 μM) did not have a clear effect on AMPA EPSCs in slices from saline- or cocaine-treated rats after 10-15 minutes of superfusion (-0.6 ± 13%, n=4 and -1.5 ± 5.0%, n=12, respectively).

In the presence of amphetamine, DPCPX was without effect on AMPA-mediated EPSCs in slices from saline-treated animals (0.0 ± 3.2%, n=10), but augmented the EPSC by 31 ± 9.7% (n=10) in slices from cocaine-treated animals (Fig. 3; two-way ANOVA, effect of pretreatment, F[1,36] = 5.6, p=0.024; amphetamine, F[1,36] = 4.9, p=0.033; and a pretreatment X amphetamine interaction, F[1,36] = 4.7, p=0.037). Although the first EPSC in the train was significantly augmented, the third and tenth EPSCs were not (14.±11%, n=10, and 14±14%, n=8, respectively). It is predicted that amphetamine would cause an equal or even more pronounced increase in the adenosine tone on mGluR IPSPs in slices from cocaine-treated rats. However, at present this has not been adequately investigated due to a postsynaptic inhibitory effect of amphetamine on the mGluR IPSP, mediated at least in part by activation of $\alpha_1$-adrenergic receptors (unpublished observations).

## Tonic activation of $GABA_B$ receptors may be necessary for adenosine tone

As stated above, in slices from cocaine-treated rats, DPCPX significantly augmented mGluR IPSPs in the absence, but not in the presence, of $GABA_B$ antagonists. We therefore examined the effect of a $GABA_B$ antagonist (CGP 56999a, 0.3-1.0 μM) on amphetamine-induced adenosine tone on AMPA EPSCs in slices from cocaine-treated rats. CGP 56999a was itself without significant effect on the amplitude of the AMPA EPSCs in naive or cocaine-treated rats, similar to previous results in naive- and morphine-treated rats (Manzoni and Williams, in press). In the presence of CGP 56999a, DPCPX was without effect on the amplitude of EPSCs in the absence or presence of amphetamine (0.4 ± 5.8%, n=7 and 2.4 ± 4.0%, n=10, respectively, not shown). This suggests that tonic activation of $GABA_B$ receptors is necessary for adenosine tone on glutamate terminals.

The mechanism by which CGP 56999a blocks adenosine tone was further investigated by measuring EPSC inhibition by exogenous adenosine in slices from cocaine-treated rats. Initially adenosine (50 μM) produced an inhibition of -20 ± 7.0% (n=5). After superfusion of CGP 56999a (0.5 - 1.0 μM), adenosine caused a similar inhibition of -28 ± 4.5%. This indicates that blockade of $GABA_B$ receptors does not decrease the sensitivity of presynaptic A1 receptors. It may therefore be the case that tonic activation of $GABA_B$ receptors is necessary for adenosine production, though it is not clear through what mechanism this could occur.

## The facilitation of $GABA_B$ IPSPs by DPCPX is not altered by cocaine pretreatment

Isolated $GABA_B$ IPSPs were studied after exposure of the slice to apamin (100 nM), or in cases in which the entire IPSP could be blocked by CGP 35348 (100 - 300 μM). DPCPX augmented the isolated $GABA_B$ IPSP by 19.3 ± 3.8% (n=8, Wilcoxan signed-rank test, p=0.008, Fig. 4). However, the facilitation of $GABA_B$ IPSPs by DPCPX was unchanged by cocaine pretreatment (20.4 ± 2.8%, n=10, Fig. 4). This is in apparent contradiction to previously published results in cocaine-treated guinea pigs (Bonci and Williams, 1996). However, in the present study it was found that in slices taken from rats 10 or more days withdrawn from repeated morphine injections, DPCPX produced a substantial increase in the amplitude of $GABA_B$ IPSPs (46.1 ± 8.6%, n=4). This is significantly greater than its effect in slices from saline-treated rats (p=0.02, Mann-Whitney U test), and is similar to results from guinea pigs (Bonci and Williams, 1996).

Although cocaine-pretreatment does not elevate adenosine tone on GABA terminals

in slices under resting conditions, there may be adenosine tone in the presence of amphetamine. Unexpectedly, amphetamine reduced the augmentation produced by DPCPX in both treatment groups (Fig. 4). However, in the presence of amphetamine, the effect of DPCPX was not different between slices from saline- and cocaine-pretreated rats ($6.1 \pm 2.0\%$, n=4 and $9.6 \pm 3.7\%$, n=10, respectively, two-way ANOVA, effect of amphetamine only, $F[1,28] = 10.2$, $p=0.004$). Although the interpretation of this result is complicated by an effect of amphetamine that is not understood, it supports the hypothesis that the increased adenosine in the VTA caused by repeated cocaine treatment is restricted to glutamate terminals.

## Sensitivity of synaptic responses to A1 receptor inhibition

It has been reported that repeated cocaine treatment does not alter the inhibition by CPA (a metabolically stable, A1 receptor-selective analog of adenosine) of either $GABA_B$ IPSPs in the VTA (Bonci and Williams, 1996) or glutamate EPSPs in the nucleus accumbens (Manzoni et al., 1998). However, the potency of adenosine in the nucleus accumbens (at least at room temperature) is decreased following cocaine withdrawal due to its enhanced uptake (Manzoni et al., 1998). In the present study an approximately half-maximal concentration of adenosine (50 μM) caused an inhibition of the AMPA EPSC of $-32 \pm 9.8\%$ (n=6) in slices from saline-treated rats, $-35 \pm 2.4\%$ (n=5) in cocaine-treated rats, and $-33 \pm 4.9\%$ (n=7) in amphetamine-treated rats (Fig. 5c). This suggests that in the VTA, repeated psychostimulant administration does not produce long-lasting changes in the uptake of adenosine or in the sensitivity of glutamate terminals to adenosine inhibition.

The inhibition by CPA of the various synaptic inputs to dopamine neurons was measured in slices from naive rats. The sensitivity and efficacy of CPA in inhibiting AMPA, mGluR, and $GABA_B$ synaptic responses was similar. The potency and maximal

inhibition were, respectively, 96±13 nM and -65±2% for the GABAB IPSP, 79±45 nM and -55±6% for the AMPA EPSC, and 19±6 nM and -54±4% for the mGluR IPSP (Fig. 5B). Considering the presence of endogenous adenosine tone on the $GABA_B$ IPSP that was not corrected for in these experiments, it appears that the efficacy of A1 receptor-mediated inhibition is greatest on the $GABA_B$ IPSP, as previously suggested (Wu et al., 1995). However, the mGluR IPSP may be the most sensitive to A1 inhibition (Fig. 5B). The disparity between the A1 inhibition of AMPA and mGluR synaptic responses may be particularly large considering that while the inhibition by CPA of the first AMPA EPSC in the train is similar to the inhibition of the mGluR IPSP (Fig. 5B), CPA did not cause consistent inhibition of either the third (-6±21%, n=7, 1 μM) or the tenth EPSC (-15±13%, n=7, 1 μM) at any dose (Fig. 5A). However, the latter stimuli in the train do make a significant contribution to the mGluR IPSP (Fig. 6). The lack of efficacy of A1 receptors in inhibiting the latter EPSCs in a train is very similar to previous studies of other inhibitory presynaptic receptors, particularly the $GABA_B$ receptor, which can in fact augment the latter EPSCs in a train of sufficiently high frequency (Brenowitz et al., 1998).

## Discussion

### Cocaine treatment selectively increases adenosine tone on glutamate terminals

The present study confirms previous work showing that repeated cocaine treatment increases adenosine tone in the VTA (Bonci and Williams, 1996). The present results indicate that the increase in adenosine tone selectively inhibits glutamate release. While there was no adenosine tone on fast or slow glutamate-mediated synaptic responses in

control animals, cocaine treatment resulted in substantial adenosine tone on slow, mGluR-mediated IPSPs. In the presence of a physiologically relevant concentration of amphetamine (3 μM, Clausing et al., 1995), adenosine tone was also present on fast EPSCs in cocaine but not saline treated animals. However, cocaine pretreatment did not alter adenosine tone on $GABA_B$ IPSPs in the presence or absence of amphetamine.

The lack of increase in adenosine tone on $GABA_B$ IPSPs resulting from cocaine treatment in the present study is in apparent contradiction with previously published results (Bonci and Williams, 1996). Because the presence of an mGluR IPSP was not yet known at the time of the earlier study, it is possible that the effects observed on the slow IPSP were due to changes in glutamate release rather than GABA. A species difference could also account for the disparate results. For instance, if adenosine is produced in glutamate terminals, it could diffuse far enough to reach GABA terminals in guinea pigs but not in rats. Experiments are currently underway in guinea pigs to resolve this issue.

The present study did not address the mechanism by which the adenosine tone is increased. Presumably, it is the result of dopamine activation of D1 receptors, production of cAMP, and subsequent metabolism of cAMP to adenosine (Bonci and Williams, 1996; Shoji et al., 1999). In support of such a mechanism, adenosine tone on fast EPSCs in cocaine-pretreated rats was increased by amphetamine, which enhances dopamine release. Furthermore, inhibition of EPSCs by exogenous adenosine was not altered by cocaine or amphetamine pretreatment, suggesting that increased adenosine tone is dependent on enhanced adenosine production (Bonci and Williams, 1996).

D1 receptors are present on glutamate terminals of afferents from the prefrontal cortex (Lu et al., 1997), as well as GABA terminals of afferents from the nucleus accumbens and ventral pallidum (Mansour et al., 1992, Lu et al., 1997b). In light of the present results, it is likely that the increased adenosine produced after cocaine pretreatment

derives from D1 receptor activation on glutamate-containing terminals from the prefrontal cortex. While D1 receptors on GABA terminals produce more adenosine than those on glutamate terminals in slices from control rats, this transduction mechanism appears unaltered by repeated cocaine treatment. The present results also suggest that extracellular adenosine can be quite highly localized, as adenosine tone inhibited only GABA release in slices from control animals, and was increased only on glutamate terminals by cocaine pretreatment.

## Adenosine tone selectively inhibits the mGluR component of the glutamate synaptic response

Repeated cocaine treatment resulted in adenosine tone on the mGluR IPSP, but not on the AMPA- or NMDA receptor-mediated EPSCs. The reason for this selectivity of adenosine action is not known. Concentration-response curves to the metabolically stable A1 receptor agonist CPA suggest that the mGluR IPSP may be slightly more sensitive than the AMPA EPSC to A1 inhibition. It is possible that either the difference in glutamate concentration and kinetics necessary for receptor activation, or the very different postsynaptic transduction mechanisms, could account for the slightly different sensitivities to A1 inhibition. A postsynaptic effect of A1 receptors on the mGluR IPSP is possible but unlikely, as the maximal inhibition by CPA was the same for AMPA and mGluR synaptic responses, and neither adenosine nor DPCPX modulated mGluR-mediated hyperpolarizations by aspartate in guinea pig VTA (J.T. Williams, unpublished observations). In any case, the small difference in sensitivity to A1 inhibition is unable to account for the substantial difference in adenosine tone on iGluR and mGluR synaptic responses .

One explanation for the difference in adenosine tone is that different populations of

terminals mediate the iGluR and mGluR synaptic responses. Although there is no evidence for "mGluR only" synapses, it is quite possible that a large proportion of terminals releasing glutamate onto AMPA and NMDA receptors do not release glutamate onto mGluRs. It seems that the vast majority of terminals in the VTA releasing GABA onto $GABA_A$ receptors on dopamine cells in response to local stimulation do not activate postsynaptic $GABA_B$ receptors, as only the $GABA_B$ IPSP is sensitive to presynaptic regulation by D1 (Cameron and Williams, 1993) and 5-HT1 agonists (Johnson et al., 1992a). However, the degree of modulation by maximally effective concentrations of A1, μ-opioid, 5-HT1, and D1 receptor agonists is similar for both AMPA and mGluR synaptic responses (present results; Manzoni and Williams, 1999; Fiorillo and Williams, 1998; unpublished observations), so at present there is little evidence for a difference in the pool of glutamate terminals responsible for the iGluR and mGluR synaptic responses.

If mGluRs were activated by only a subset of glutamate terminals, then adenosine tone in treated animals might similarly suppress glutamate release from these terminals onto both iGluRs and mGluRs. However, even under this scenario, it is likely that adenosine would effectively inhibit only the first few EPSPs in a train (Figs. 3A, 5A). Thus, with a short train of presynaptic action potentials, adenosine would cause a greater inhibition of the IPSP than of the sum of the EPSPs. The net result would be a greater excitation of dopamine neurons for a given presynaptic fiber volley in cocaine-treated rats.

## Implications for the mesolimbic dopamine system

Because of the difference in kinetics of the iGluR and mGluR synaptic potentials, it is predicted that the mGluR IPSP acts to limit the duration of the burst of action potentials driven by the iGluR EPSP (particularly the NMDA component of the EPSP). A selective inhibition of the mGluR IPSP would therefore be expected to prolong the duration of burst

events. It has been reported that at 10 days withdrawal from repeated amphetamine treatment of rats, stimulation of prefrontal cortex more reliably elicits bursts in dopamine neurons of the VTA (Tong et al., 1995). The present results, with both cocaine and amphetamine treatment, suggest a mechanism which may account, at least in part, for the enhanced excitation of dopamine neurons after psychostimulant treatment.

A recent study showed that systemic cocaine evokes greater glutamate release in the VTA of rats 21 days withdrawn from daily cocaine treatment (Kalivas and Duffy, 1998). The glutamate release was blocked by prior infusion of a D1 antagonist into the VTA. However, the enhanced increase in glutamate release in response to cocaine was very transient and may have been due to a conditioned response of the behaving animal rather than a pharmacological action of cocaine.

It is well established that conditioned cues are a primary trigger of relapse to drug use in both human addicts and other species. It has been hypothesized that sensitization of the mesolimbic dopamine system may underlie the powerful craving elicited by external stimuli associated with drug effects (Robinson and Berridge, 1993). The potential role of dopamine neurons in such a process has been advanced by studies of dopamine cell activity during the learning of cues predicting reward in monkeys (Ljungberg et al., 1992; Schultz et al., 1993; reviewed by Schultz, 1998). Initially, dopamine cells are unaffected by a neutral stimulus, but respond to an unpredicted natural reward, such as juice, with a burst of action potentials. With repeated pairings of the neutral stimulus with reward, the response of dopamine neurons is conditioned such that the formerly neutral stimulus now elicits a response, but the natural reward is no longer effective. Dopamine neurons therefore respond to errors in the prediction of reward. This finding promotes the theory that an increased activity of dopamine neurons underlies the craving and anticipation of rewards and not necessarily the response to rewards themselves (Robinson and Berridge, 1993). It is not known which input(s) to dopamine cells mediates this excitation, but the

glutamatergic inputs from the PFC are a likely candidate. If this is the case, then selective inhibition of the mGluR IPSP by adenosine might be one mechanism by which a conditioned cue could elicit a greater dopamine response, and presumably greater craving for drug, in a cocaine-experienced animal.

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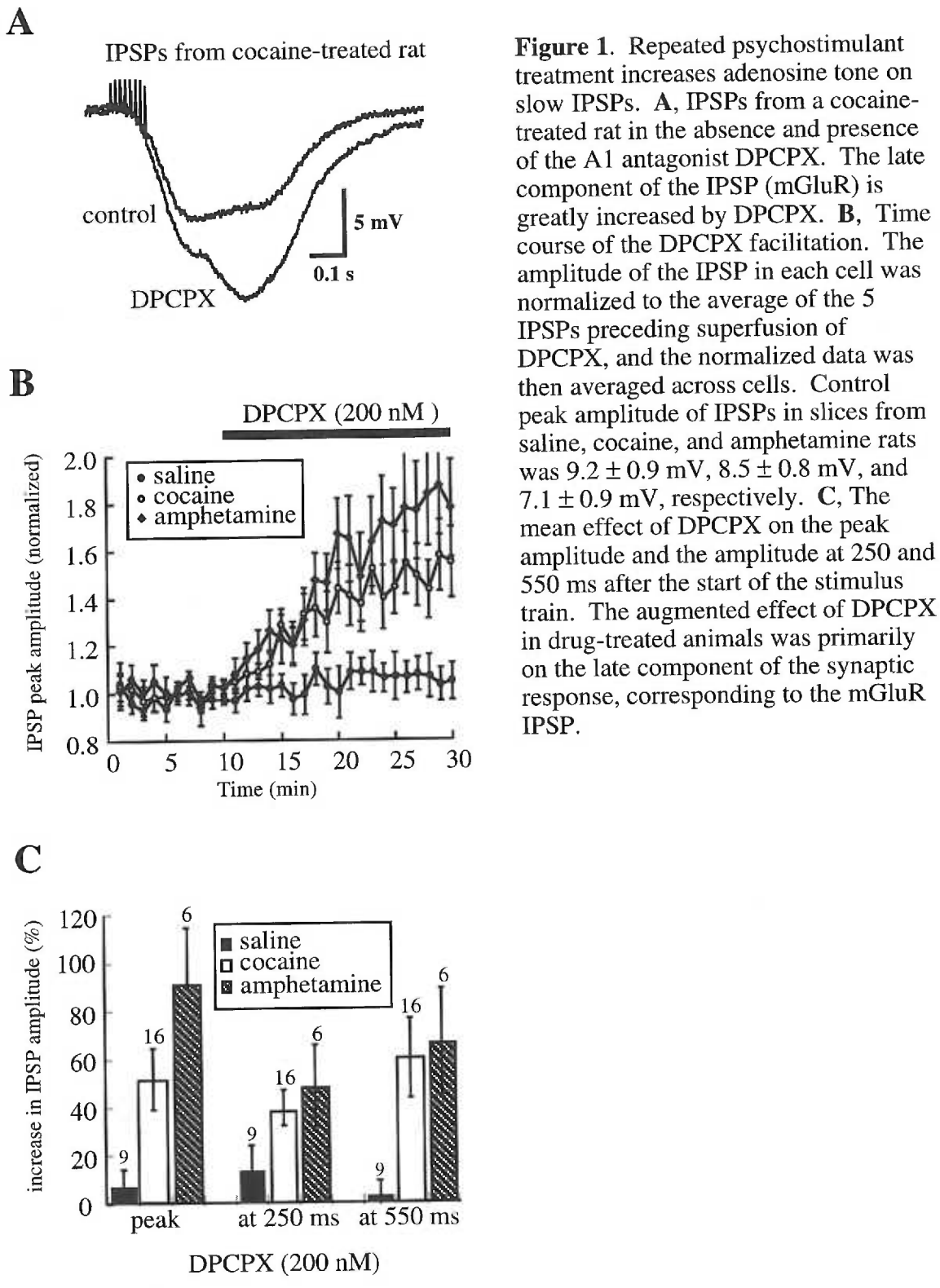


**Figure 1**. Repeated psychostimulant treatment increases adenosine tone on slow IPSPs. **A**, IPSPs from a cocaine-treated rat in the absence and presence of the A1 antagonist DPCPX. The late component of the IPSP (mGluR) is greatly increased by DPCPX. **B**, Time course of the DPCPX facilitation. The amplitude of the IPSP in each cell was normalized to the average of the 5 IPSPs preceding superfusion of DPCPX, and the normalized data was then averaged across cells. Control peak amplitude of IPSPs in slices from saline, cocaine, and amphetamine rats was $9.2 \pm 0.9$ mV, $8.5 \pm 0.8$ mV, and $7.1 \pm 0.9$ mV, respectively. **C**, The mean effect of DPCPX on the peak amplitude and the amplitude at 250 and 550 ms after the start of the stimulus train. The augmented effect of DPCPX in drug-treated animals was primarily on the late component of the synaptic response, corresponding to the mGluR IPSP.

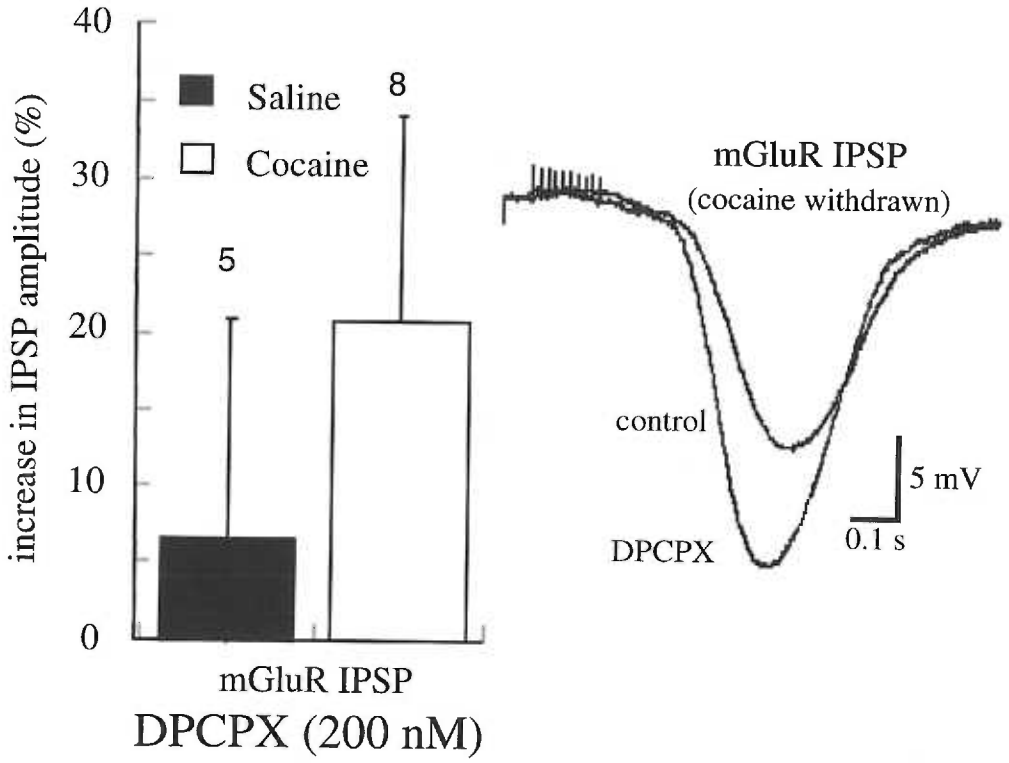


**Figure 2.** Cocaine treatment does not significantly alter adenosine tone on mGluR IPSPs isolated in the presence of the GABAB antagonists CGP 35348 (100-300 μM) or CGP 56999a (100-1000 nM). The bar graph on the left shows the average increases in amplitude of mGluR IPSPs in response to DPCPX (200 nM) in saline- and cocaine-pretreated rats. Control peak amplitudes were 9.8 ± 0.9 mV for IPSPs in saline-treated rats and 9.7 ± 0.9 mV in cocaine-treated rats. Numbers above the error bars indicate the number of rats in which each experiment was performed. At the right are mGluR IPSPs from a cocaine-treated animal in the absence and presence of DPCPX.

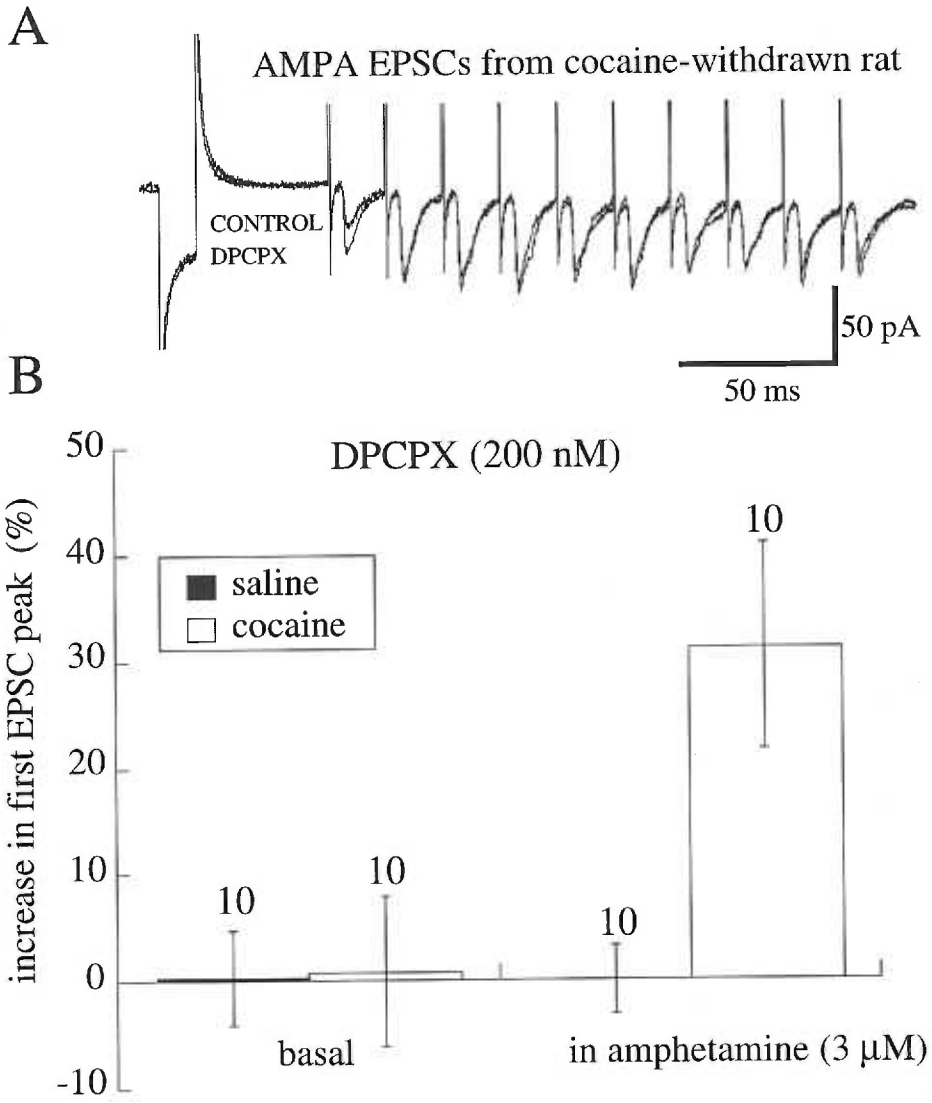


**Figure 3**. Adenosine tone is absent on AMPA EPSCs, but is present in slices from cocaine-treated rats in the presence of amphetamine. **A**, Traces from cocaine-treated rat. DPCPX (200 nM) augmented the first EPSC in the train in the presence of amphetamine (3 μM). Traces are normalized to the 5 ms before the first stimulus. The downward current deflection at the left is the response to a 10 mV hyperpolarizing step. **B,** Averaged data from the first EPSC in the train, showing that DPCPX is only effective in increasing the amplitude in slices from cocaine-treated rats in the presence of amphetamine (3 μM).

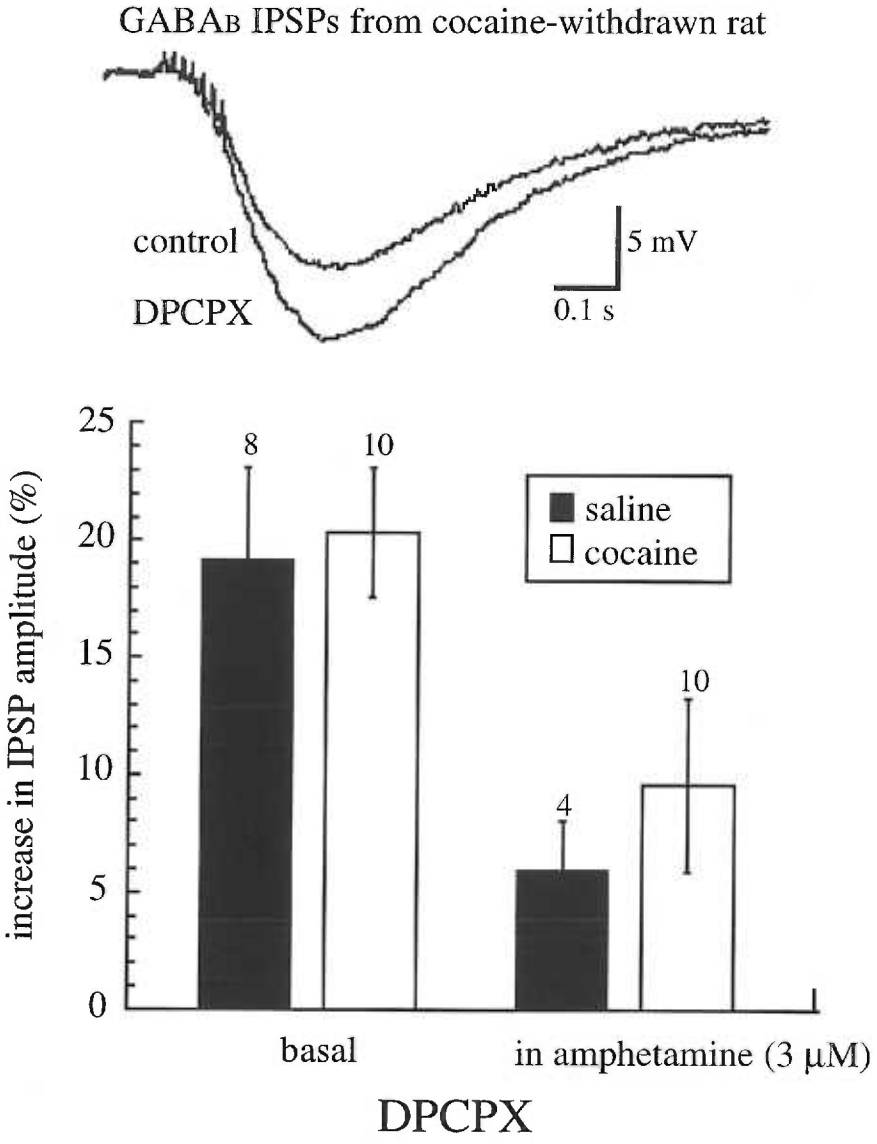


**Figure 4.** Adenosine tone on GABAB IPSPs is not altered by cocaine pretreatment. Top, GABAB IPSPs from a cocaine-treated rat in the absence and presence of the adenosine A1 receptor antagonist DPCPX (200 nM). Bottom, graph showing the mean augmentation of GABAB IPSP peak amplitude by DPCPX in slices from saline- and cocaine-treated animals, in the presence or absence of amphetamine (3 μM). Numbers above each error bar indicate the number of animals in which each experiment was performed. Control peak amplitudes were 11.3 ± 1.3 mV in saline-treated rats, 8.6 ± 1.1 mV in cocaine-treated rats, 10.4 ± 0.6 mV in saline-treated rats in the presence of amphetamine, and 8.4 ± 0.9 in cocaine-treated rats in the presence of amphetamine.

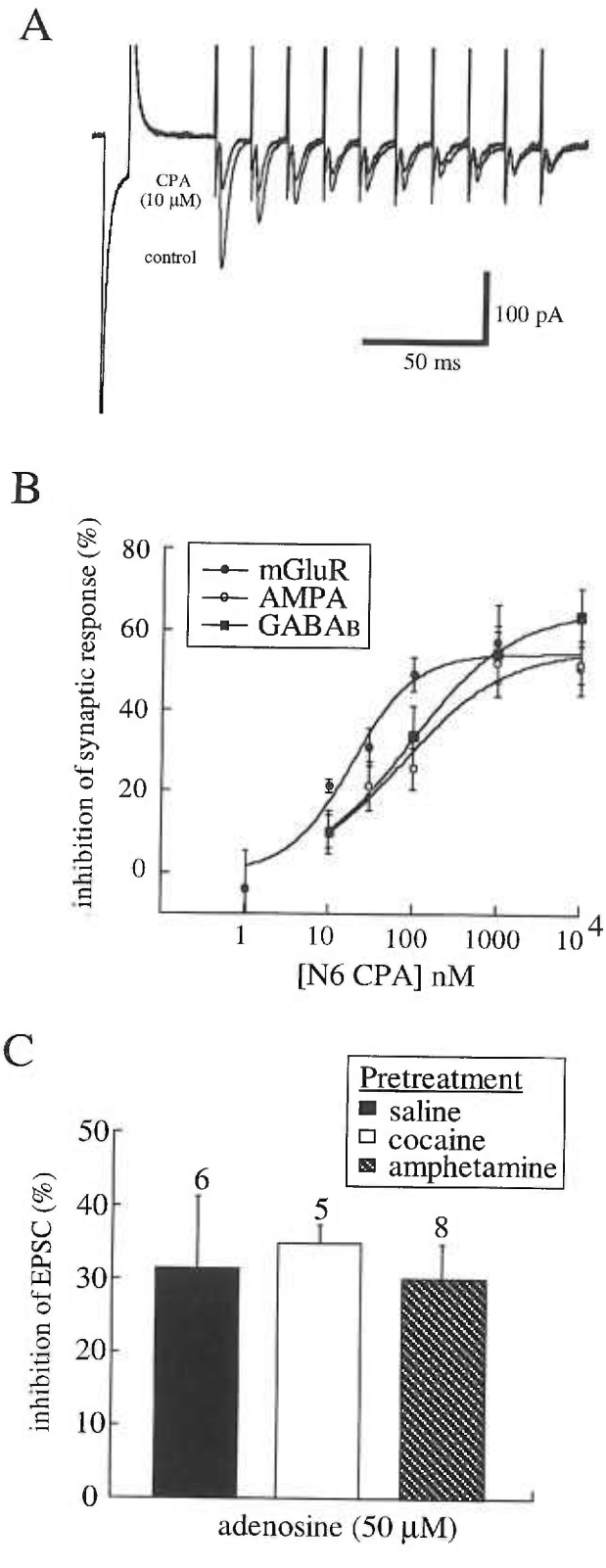


**Figure 5**. Inhibition of synaptic responses by exogenous A1 agonists. **A**, Traces from a naive rat in the presence and absence of CPA. CPA (10 μM) inhibits the first EPSC but is ineffective on the latter EPSCs. Traces were normalized to the 5 ms preceding the first stimulus. **B**, Concentration-response curves to CPA. AMPA and mGluR synaptic responses were recorded in the presence of the GABAB antagonist CGP 56999a (100-500 nM). Each point is the average of 4-8 experiments. Inhibition of AMPA-mediated responses was measured for the first EPSC in a train of 10. **C**, Adenosine (50 μM) is equally effective in inhibiting AMPA EPSCs in slices from saline, cocaine, and amphetamine-treated rats.

Dependence of mGluR IPSP on number of stimuli

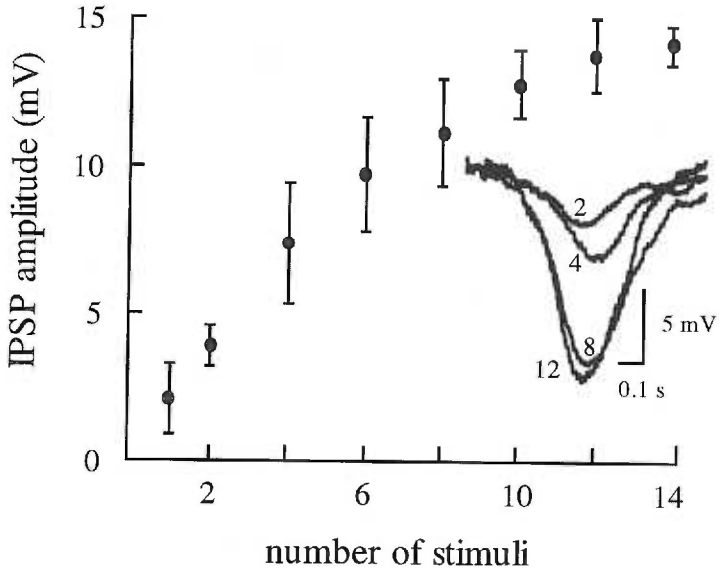


**Figure 6**. The mGluR IPSP is sensitive to the latter stimuli in a train of ten at 66 Hz. The plot is based on the average of four cells. The inset shows raw traces of mGluR IPSPs in a single cell, each corresponding to the number of stimuli shown.

## Discussion

*Synaptic inhibition by glutamate*

The mGluR IPSP characterized in the first manuscript is unique in several fundamental ways. First, it demonstrates an inhibitory postsynaptic potential mediated by glutamate in the mammalian central nervous system. In a previous demonstration of postsynaptic inhibition by glutamate, the receptor mediating the response was not known (Nicoll and Alger, 1981). Also in that report, the inhibition always followed an EPSP or inward current, and may therefore have been dependent on the preceding excitation.

Glutamate has a unique inhibitory effect on retinal ON bipolar cells of the tiger salamander, where it suppresses a cGMP-activated cation conductance (Nawy and Jahr, 1990; Nawy, 1999). It is accepted that this mechanism is present in mammalian retina as well and mediates the physiological response of these cells to light. However, this response may not occur outside the retina. Unlike conventional synaptic transmission by glutamate, it is observed following changes in tonic extracellular glutamate levels and is mediated by the retina-specific mGluR6. By contrast, the IPSP mediated by group I mGluRs, or the $Ca^{2+}$ transient underlying it, is likely to be common to many types of neuron.

*Mechanism of the mGluR IPSP*

The second unique aspect of the mGluR IPSP in dopamine neurons is its mediation by a PI-coupled receptor and dependence on intracellular $Ca^{2+}$ stores. This sets it apart from previously characterized slow IPSPs, which are mediated by direct, membrane-delimited activation of an inwardly rectifying $K^+$ channel (Surprenant and Williams, 1987; Pan et al., 1989; Otis et al., 1993; Calabresi et al., 1998). In contrast, the mGluR IPSP is mediated by one or more diffusible second messengers. Although it was

not shown in dopamine neurons, mGluR1 is presumably working through activation of phospholipase C (PLC) and $IP_3$, as has been shown in numerous other cell types. $Ca^{2+}$ released by $IP_3$ is presumably greatly amplified by $Ca^{2+}$ induced $Ca^{2+}$ release (CICR) from ryanodine receptors. Most of the $Ca^{2+}$ directly activating the $K^+$ channels apparently is released from ryanodine receptors, as ryanodine blocked most or all of the mGluR IPSP. However, in some cells the mGluR IPSP was not entirely blocked by ryanodine, suggesting that $Ca^{2+}$ may be coming from another source, perhaps $IP_3$ receptors. The recent discovery of a membrane permeable, selective $IP_3$ receptor antagonist (Xestospongin C, Gafni et al., 1997) should aid in determining the involvement of $IP_3$ receptors in the future.

In cultured cerebellar granule neurons, mGluR1 has been shown to activate L-type $Ca^{2+}$ channels directly through ryanodine receptors by a mechanism reminiscent of E-C coupling in skeletal muscle (Chavis et al., 1996). This effect was independent of all second messengers tested and was preserved in excised patches, suggesting a direct protein interaction. The facilitation of $Ca^{2+}$ currents was not mimicked by a muscarinic receptor agonist, although the muscarinic receptor also couples to the PI pathway in these same cells. Such a mechanism is unlikely to be involved in the mGluR IPSP, as the mGluR IPSP was lost in the whole-cell recording mode, was only blocked by 24% by the L-type $Ca^{2+}$ channel antagonist nimodipine (3 μM, appendix 1), and muscarinic receptors mediate a similar response to mGluRs. In addition, the lack of efficacy of nimodipine is particularly instructive in light of recent evidence that, in hippocampal pyramidal neurons, small-conductance $Ca^{2+}$ activated $K^+$ channels are opened specifically by $Ca^{2+}$ entry

through L-type $Ca^{2+}$ channels and not by $Ca^{2+}$ entry through other voltage-gated $Ca^{2+}$ channels (Marrion and Tavalin, 1998).

*The inhibitory junction potential of smooth muscle*

Much of what is known about the physiology of neurons was first discovered in studies of muscle. The neuromuscular junction (with skeletal muscle) provided the first insights into fast neurotransmission in mammals. Likewise, the study of cardiac myocytes has yielded a great deal of information about the actions of G protein-coupled receptors, as well as CICR, which is only just beginning to be understood in neurons. Though pioneering work has also been done on smooth muscle, these studies seem to have been relatively forgotten today in the field of neuroscience. In fact, the mGluR IPSP bears more resemblance to the inhibitory junction potential (IJP) of smooth muscle, discovered over 30 years ago (Bennett et al., 1966), than to any previously characterized IPSP. Though described in many types of smooth muscle, the IJP has been best characterized in the taenia caeci of guinea pig. It closely resembles the mGluR IPSP in its kinetics (Bennett et al., 1966; Bridgewater et al., 1995) and in its mediation by apamin-sensitive K+ channels (Vladimirova and Shuba, 1978; Banks et al., 1979; Bridgewater et al., 1995). Various neurotransmitters mediate IJPs in smooth muscles through activation of PI-coupled receptors, that presumably release $Ca^{2+}$ from intracellular stores. However, the neurotransmitter mediating the IJP in taenia caeci, as well as many other muscles, is still not certain (e.g. Bridgewater et al., 1995). In guinea pig choroidal arterioles, muscarinic acetylcholine receptors mediate an IJP through activation of both $SK_{Ca}$ and $BK_{Ca}$ (Hashitani et al., 1998). It is interesting that in these same muscles, other PI-coupled receptors mediate excitatory junction potentials (Hashitani et al., 1998; Bridgewater et al., 1995).

*$Ca^{2+}$ signalling in dopamine neurons*

Characterization of the mGluR IPSP and subsequent work has revealed multiple physiological functions of $Ca^{2+}$ signalling in dopamine neurons. Pharmacological agents that interfere with $Ca^{2+}$ stores not only block the mGluR IPSP, but also greatly reduce the amplitude of the slow, apamin-sensitive AHP (appendix 1). This suggests that CICR amplifies the increase in $[Ca^{2+}]$ resulting from influx through voltage-gated $Ca^{2+}$ channels during the action potential, as demonstrated in a number of other types of neuron (e.g. Cohen et al., 1997; reviewed by Berridge, 1998; Sah, 1996).

Spontaneous release of $Ca^{2+}$ from $IP_3$ or ryanodine receptors, and activation of K+ channels, has been demonstrated in many cell types (Berridge, 1997). Elementary, spontaneous release events are mediated by a small number of $Ca^{2+}$ release channels and lead to highly localized increases in $[Ca^{2+}]$ (Nelson et al., 1995; Horne and Meyer, 1997). Spontaneous, transient activation of apamin-sensitive channels occurs in dopamine neurons, and is particularly apparent in slices from young animals (Seutin et al., 1998). These events are not due to release of endogenous neurotransmitter, but are dependent on intracellular $Ca^{2+}$ stores (Vincent Seutin, personal communication). Though clearly dependent on ryanodine receptors, it is not known whether these spontaneous events are also dependent on $IP_3$ receptors. However, they are blocked by superfusion of an mGluR agonist (C. Paladini and J. Williams, personal communication), whereas the AHP is not. This could suggest that spontaneous events, but not AHPs, depend on $IP_3$ receptors, and the $IP_3$-sensitive $Ca^{2+}$ store is depleted by an mGluR agonist. Supporting this, the firing

of spontaneous action potentials transiently enhances both the frequency and amplitude of spontaneous hyperpolarizing events, in the presence or absence of an mGluR agonist (C. Paladini and J.T. Williams, personal communication). Mobilization of $Ca^{2+}$ from intracellular stores in dopamine neurons can thus arise spontaneously, in response to action potentials, or following activation of PI-coupled receptors.

*Postsynaptic modulation of the mGluR IPSP*

As previously stated, the mGluR IPSP has a very complex postsynaptic mechanism relative to other synaptic potentials. This provides numerous targets for postsynaptic regulation. For instance, chronic morphine treatment upregulates the mGluR IPSP through a postsynaptic mechanism (Yoshihisa Shoji and John Williams, unpublished observations). Two potential mechanisms of postsynaptic regulation are suggested by the present results. First, it was found that removing the holding current to allow spontaneous action potentials could augment the mGluR IPSP in about half the cells tested. The difference between cells, which very likely reflects a difference in the degree of filling of $Ca^{2+}$ stores, could be related to the health of the cells. Depolarization has been found to enhance $[Ca^{2+}]$ in the intracellular stores of many different types of cells, but these results have been obtained *in vitro*, often with nonphysiological release by agents such as caffeine and with disruption by recording pipettes. If the augmentation occurred in unhealthy cells, it may not occur *in vivo*, where stores may be filled by normal activity. However, spontaneous firing routinely augments the frequency and amplitude of spontaneous, apamin-sensitive hyperpolarizations in slices from neonatal rats recorded in the perforated-patch mode (Carlos Paladini, personal communication). It is very likely that these neurons are healthier than those in slices from adults after impalement with intracellular pipettes. It may therefore be the case *in vivo* that the degree of filling of the $Ca^{2+}$ stores is dynamically

regulated, thus endowing neurons with a 'memory' of recent activity (Berridge, 1998). This could provide an associative mechanism for integrating synaptic inputs occurring up to several minutes apart (Batchelor and Garthwaite, 1997).

A second postsynaptic mechanism to regulate the mGluR IPSP was suggested by the finding that superfusion of a low concentration of a group 1 mGluR agonist not only depolarized the neuron but caused a very rapid and complete desensitization of the mGluR-mediated hyperpolarizing response. This may occur *in vivo* during intense activity of glutamatergic afferents. In addition, it is likely that other neurotransmitter receptors couple to release from the same $Ca^{2+}$ stores. As already discussed, brief activation of muscarinic acetylcholine receptors produces hyperpolarization through a similar or identical mechanism to that of mGluR1 (unpublished observations). It has recently been found that superfusion of agonists of muscarinic, $\alpha_1$-adrenergic, and 5-$HT_2$ receptors also block or attenuate mGluR IPSPs (J.T. Williams, unpublished observations). All of these receptors are known to couple to PI hydrolysis, and to depolarize dopamine neurons with sustained activation (Grenhoff et al., 1995; Pessia et al., 1994; Lacey et al., 1990). Inhibition of mGluR IPSPs most likely occurs through depletion of shared, $IP_3$-sensitive $Ca^{2+}$ stores. It is not known to what extent this cross-desensitization occurs *in vivo*.

*Conditions necessary for observing hyperpolarization by mGluRs*

There are several reasons that the mGluR IPSP and inhibition by PI-coupled receptors have gone undetected. To begin with, the receptor must be activated rapidly and briefly. Slow, prolonged activation of the receptor, even with low receptor occupancy, causes rapid desensitization of the hyperpolarizing response without producing any hyperpolarization. Any effect of an increase in $G_k$ that might be present at early timepoints

could be masked by a receptor-mediated depolarization that is present in many cell types. Even without desensitization, it is likely that much of the $Ca^{2+}$ transient underlying the IPSP is dependent on a sudden activation of receptors (Hernandez-Cruz et al., 1997). This is because CICR is a regenerative process. A large, $Ca^{2+}$-mediated hyperpolarization would therefore not be observed with superfusion of agonists in a slice preparation, just as an action potential is not observed with a sufficiently slow, controlled depolarization of the membrane potential. In addition, just as $Na^+$ channels undergo relatively slowly developing, voltage-dependent inactivation, $Ca^{2+}$ release channels may display more slowly developing, $Ca^{2+}$-dependent inactivation. All of these factors would prevent the observation of large increases in $[Ca^{2+}]$ or $G_k$ by slow application of agonist. The present results illustrate the importance of kinetic considerations and the limitations of equilibrium measurements in neurophysiological studies.

Most studies of synaptic transmission apply only one or two stimuli at a time at low frequency. Although this causes a large and rapid increase in glutamate concentration in the synapse that is sufficient to activate ionotropic receptors, it is probably insufficient to significantly activate postsynaptic mGluRs due to their perisynaptic localization (Baude et al., 1993). A train of stimuli was necessary to activate mGluRs in the present study (manuscript 2, fig. 6) as well as in a previous study in dopamine neurons (Shen and Johnson, 1997).

A second major reason the hyperpolarizing response to mGluR activation has seldom been observed is its sensitivity to the recording configuration. In the present work the mGluR IPSP was studied with intracellular pipettes containing 2M KCl. Hyperpolarization by mGluRs is not observed with patch pipettes in the whole-cell recording mode, but can be maintained with perforated-patches in CA1 and CA3 pyramidal

neurons of the hippocampus (Shirasaki et al., 1994; Harata et al., 1996) and in astrocytes (Chen et al., 1997) as well as in dopamine neurons (unpublished observations). The apamin-sensitive AHP, which is largely dependent on intracellular $Ca^{2+}$ stores in dopamine neurons, runs down dramatically during the first minute after achieving the whole-cell configuration (J.T. Williams, unpublished observations). Both apamin-sensitive AHPs and spontaneous hyperpolarizations are preserved with the perforated patch but lost in the whole-cell mode (C. Paladini and J.T. Williams, personal communication). The role of intracellular $Ca^{2+}$ stores in the physiology of neurons may prove more important than previously recognized as neuronal function is probed by techniques less invasive than whole-cell patch pipettes.

A third reason that hyperpolarizing responses may not have been observed is that certain $Ca^{2+}$-activated $K^+$ channels are blocked by bicuculline methiodide (Johnson and Seutin, 1997; Seutin et al., 1997). Bicuculline methiodide is a competitive antagonist of $GABA_A$ receptors that has long been used to isolate synaptic responses mediated by glutamate and other neurotransmitters. In dopamine neurons, it blocks both the mGluR IPSP (unpublished observations) and the apamin-sensitive AHP (Johnson and Seutin, 1997; Seutin et al., 1997) in the presence of the noncompetitive $GABA_A$ antagonist picrotoxin, and at concentrations routinely used to block $GABA_A$ receptors. The apamin-sensitive $K^+$ channel present in the dopamine cell region is SK3 (Kohler et al., 1996); it is not known which other potassium channels are blocked by bicuculline.

*mGluR synaptic responses in other neurons*

It is uncertain how many other types of neurons possess an mGluR IPSP. Although many neurons express group one mGluRs, a number of other constituents may

need to be present in the proper subcellular arrangement, including $Ca^{2+}$-activated $K^+$ channels and ryanodine receptors. It is therefore likely that some types of neuron do not express mGluR-mediated IPSPs, but may express mGluR EPSPs. However, previous reports of mGluR EPSPs have generally not met the conditions outlined above, due to use of either bicuculline or whole-cell patch pipettes (Charpak and Gahwiler, 1991; Glaum and Miller, 1992; Batchelor et al., 1994; Pozzo Miller et al., 1995; Batchelor et al., 1996; Congar et al., 1997; Batchelor and Garthwaite, 1997; Shen and Johnson, 1997). Although it is likely that hippocampal CA1 pyramidal neurons do express an mGluR IPSP (Shirasaki et al., 1994; Jaffe and Brown, 1994), it may have gone undetected, in part because of the lack of availability of a potent antagonist for mGluR5 (Brabet et al., 1995; Kingston et al., 1995).

Although mGluR IPSPs may not be present in all neurons possessing group 1 mGluRs, the $IP_3$ pathway and $Ca^{2+}$ stores are likely to be ubiquitous. When the mGluR IPSP was blocked by apamin, no synaptic potential was left in most dopamine neurons, though $Ca^{2+}$ release probably still remained. With longer trains of stimuli or with superfusion of agonist, mGluR-mediated depolarization was always observed, as it is in many other types of neuron. This indicates that brief activation of mGluRs can mobilize $Ca^{2+}$ without causing depolarization. The selective mobilization of $Ca^{2+}$ from intracellular stores is likely to be a phenomenon that generalizes to many PI-coupled receptors in many neuronal types. Evidence that this is indeed the case has since come from imaging of $[Ca^{2+}]$ in cerebellar Purkinje cells, in which synaptic activation of mGluR1 by a short train of stimuli resulted in transient, highly localized elevations in $[Ca^{2+}]$ in the absence of changes in membrane potential (Finch and Augustine, 1998; Takechi et al., 1998). However, an mGluR-mediated slow EPSP is present in Purkinje neurons (Batchelor et al.,

1994; Batchelor and Garthwaite, 1997), presumably with stronger stimulation protocols (Takechi et al., 1998). It is therefore likely that in many types of neuron, mGluR1 couples more efficiently to mobilization of $Ca^{2+}$ than to depolarizing mechanisms.

*Implications of the mGluR IPSP for synaptic plasticity*

One of the cell types that is likely to have an mGluR IPSP is the hippocampal CA1 pyramidal neuron (Shirasaki et al., 1994; Jaffe and Brown, 1994). These neurons, perhaps more than any other in the mammalian CNS, have provided a cellular model of associative learning known as long-term potentiation (LTP; Kandel, 1991). LTP is expressed as an enhanced efficacy of excitatory transmission at a previously active subset of afferent fibers. Its induction depends on $Ca^{2+}$ entry into the postsynaptic neuron through the NMDA subtype of glutamate receptor. The NMDA receptor is unique among ligand-gated ion channels in that it only opens at depolarized potentials, due to $Mg^{2+}$ blockade at more hyperpolarized potentials. LTP, and perhaps learning, occurs when NMDA receptors are sufficiently activated by the coincidence of depolarization and glutamate binding to the NMDA receptor. The time course of the glutamate-mediated EPSP will therefore determine the window of time during which an additional glutamate input can effectively activate NMDA receptors to initiate LTP. The mGluR IPSP would be expected to terminate the EPSP and thereby restrict this time window (Pin, 1998). It is interesting in this respect that manipulations which modulate the amplitude of the IPSP, pre- or postsynaptically, also modulate its latency. Though not quantified, addition of the NMDA antagonist MK-801 not only blocked the slow, NMDA-mediated EPSP, but also delayed by approximately 100 ms the peak latency of the mGluR IPSP (unpublished observations). This may be due to facilitation of mGluR-mediated $Ca^{2+}$ release by $Ca^{2+}$ entry through NMDA receptors. In addition, $Ca^{2+}$ released by mGluRs could have effects on synaptic

plasticity and other $Ca^{2+}$-dependent processes independent of effects on membrane potential, as may occur in long-term depression of parallel fiber EPSPs in cerebellar Purkinje cells (Finch and Augustine, 1998; reviewed by Linden and Connor, 1995).

*Function of the mGluR IPSP in dopamine neurons*

Burst firing of dopamine neurons greatly facilitates dopamine release in projection areas (Gonon, 1989; Suaud-Chagny et al., 1992), and is thought to be dependent on activation of NMDA receptors (Chergui et al., 1993). A burst of action potentials in dopamine neurons *in vivo*, whether occurring in behaving monkeys or in response to stimulation of PFC in anaesthetized rats, appears to be frequently followed by a quiescent period (Schultz et al., 1993; Tong et al., 1995). If the burst is evoked by a glutamate EPSP, then the quiescent period occurs at the latency expected for the mGluR IPSP. This is consistent with the mGluR IPSP terminating the EPSP and burst events in dopamine neurons. Other mechanisms may participate as well, including direct $Ca^{2+}$ activation of $K^+$ channels and activation of the $Na^+/K^+$ ATPase (Johnson et al., 1992).

The pattern of activity of glutamate afferents to dopamine neurons in a behaving animal is not known. When glutamate is released *in vitro* in response to a train of 10 stimuli at 66 Hz, a dopamine neuron resting just below firing threshold (due to hyperpolarizing current injection) responds with two to three action potentials at approximately 20 Hz riding on a glutamate-mediated EPSP (unpublished observations). This is very similar to naturally occurring bursts *in vivo* (Grace and Bunney, 1984b; Freeman et al., 1985; Schultz, 1998). The mGluR IPSP follows the EPSP and is likely to shorten the duration of postsynaptic burst events elicited by a presynaptic burst of activity.

A persistent activation of NMDA receptors can also evoke bursting of dopamine neurons, and blockade of $Ca^{2+}$-activated $K^+$ channels with apamin greatly enhances this

burst firing (Johnson et al., 1992; Seutin et al., 1993). Although *in vitro* this is likely to result from inhibition of the AHP by apamin, it is very probable that *in vivo* mGluR activation of this same conductance counteracts burst firing. However, if extracellular glutamate were to be present for a sufficiently prolonged period *in vivo*, it is expected that the mGluR-mediated activation of K+ channels would desensitize and NMDA-dependent bursts would be enhanced. It is not known to what extent this occurs under normal physiological conditions.

Similarly, it is not clear under what conditions mGluR-mediated depolarizations will be produced. Sustained activation of mGluRs in dopamine neurons always causes depolarization (Mercuri et al., 1993; Shen and Johnson, 1997; present results). Slow mGluR EPSPs can be evoked with only a few more stimuli than the IPSP in some cells (manuscript 1, fig. 4a). However, in the vast majority of cells, 10 stimuli at 66 Hz evokes a mGluR-mediated synaptic potential that is entirely blocked by apamin (fig. 1a). An mGluR EPSP could not be reliably elicited even with high intensity trains of up to 25 stimuli, whether the stimulating electrodes were placed rostral or caudal of recording electrode. However, in a few cells, a short latency mGluR EPSP evoked by only four stimuli overcame a small IPSP, indicating the control of mGluR EPSPs by unknown and uncontrolled factors. With whole-cell patch recordings, mGluR EPSCs can be readily evoked with caudal stimulation (Shen and Johnson, 1997). This suggests that disruption of the internal milieu with whole-cell patch recording could enhance mGluR depolarizations, beyond its clear ability to prevent mGluR hyperpolarizations. In addition, cyclopiazonic acid, which blocks $Ca^{2+}$ uptake into endoplasmic reticulum, may enhance mGluR EPSPs. Cyclopiazonic acid converted the IPSP into an mGluR EPSP (5 of 6 cells). It is unlikely that an EPSP was already present but masked by the IPSP, as apamin rarely revealed an EPSP (1 of 7 cells studied in manuscript 1). This could potentially result

from a prolonged elevation in cytosolic $Ca^{2+}$, in the absence of $Ca^{2+}$ uptake into intracellular stores, and activation of a cation conductance (Congar et al., 1997) or the $Na^{+}/Ca^{2+}$ exchanger (Lee and Boden, 1997). In any case, these results suggest that disruption of cytosolic $Ca^{2+}$ dynamics may enhance the mGluR EPSP at the expense of the mGluR IPSP.

*The modulation of the mGluR IPSP by cocaine and amphetamine*

As shown in the second manuscript, repeated administration of cocaine or amphetamine causes a selective inhibition of the mGluR IPSP by endogenous adenosine through a presynaptic mechanism. When stimulated by amphetamine, adenosine tone was also present on fast glutamate-mediated EPSCs. However, cocaine pretreatment did not alter adenosine tone on $GABA_B$ IPSPs, whether measured in the presence or absence of amphetamine.

In apparent contradiction to the present results, it was previously reported that adenosine tone is elevated on $GABA_B$ IPSPs following cocaine pretreatment (Bonci and Williams, 1996). There are two major differences between these studies that are likely to account for the disparate results: the species difference (guinea pig vs. rat) and the recognition in the present study of the two components of the slow IPSP. The simplest explanation is that the increased adenosine tone on the slow IPSP in cocaine-treated guinea pigs was in fact on the mGluR component of the IPSP, which is present in guinea pigs but was not yet recognized at the time of the previous study. It should be noted that the late (mGluR ) component of the IPSP is more similar kinetically to the $GABA_B$ IPSP in guinea pigs than it is in rats, and is therefore not as easily distinguished in guinea pigs without the use of pharmacological agents. However, adenosine tone is increased on the $GABA_B$

IPSP in rats after long-term withdrawal from repeated morphine (present results), as Bonci and Williams (1996) reported in guinea pigs. Adenosine tone is also increased on $GABA_B$ IPSPs during acute morphine withdrawal in guinea pigs (Shoji et al., 1999). Experiments are currently underway to examine the adenosine tone on isolated $GABA_B$ IPSPs in cocaine-treated guinea pigs.

As discussed above, the selective suppression of the mGluR IPSP is likely to enhance bursting of dopamine neurons induced by excitatory afferents. There is in fact evidence that burst firing *in vivo* is more readily evoked by stimulation of PFC in amphetamine-treated rats (Tong et al., 1995). It is speculated (in discussion of manuscript 2) that this could contribute to relapse in addicts triggered by conditioned stimuli, in accordance with the theory of drug addiction proposed by Robinson and Berridge (1993).

Increased adenosine tone in the VTA could also play a role in locomotor sensitization to psychostimulants, which is long-lasting and involves increased dopamine release in the nucleus accumbens (Kalivas and Stewart, 1991). However, it is not clear that there is any increase in the activity of dopamine neurons in sensitized animals, and sensitized dopamine release could occur in the absence of any changes in the VTA (Kalivas and Stewart, 1991). In addition, there may be no substantial glutamatergic drive to dopamine neurons under normal physiological conditions (Westerink et al., 1996), so it is not clear that inhibition of the mGluR IPSP could lead to a sustained increase in dopamine levels in the nucleus accumbens. Finally, for increased adenosine tone in the VTA to be a mechanism of sensitization it would have to be activated by psychostimulants. This may be the case, as it is likely to be dependent on activation of D1 receptors (Bonci and Williams, 1996) and is augmented by a low concentration of amphetamine. Although the increase in adenosine tone produced by amphetamine was demonstrated on glutamate EPSCs, which would be expected to reduce the excitatory drive to dopamine neurons, it is expected that

amphetamine would cause an even greater adenosine-dependent reduction in the mGluR IPSP. Therefore, the net effect of amphetamine on glutamate terminals might be shifted by cocaine treatment towards greater excitatory drive of dopamine neurons. However, the contribution of this change to stimulant-induced dopamine release in the nucleus accumbens and to behavioral sensitization might be minor.

While trying to measure adenosine tone on the mGluR IPSP in the presence of amphetamine, it was discovered that amphetamine potently inhibits the IPSP (JT Williams, unpublished observations). This effect was found to be mediated by $\alpha_1$-adrenergic receptors, which presumably deplete postsynaptic $Ca^{2+}$ stores when tonically activated. Cocaine could have a similar action through 5-$HT_2$ receptors. The acute effect of these drugs on the mGluR IPSP could therefore be dominated by postsynaptic mechanisms. It is hypothesized that by inhibiting the mGluR IPSP acutely, amphetamine and cocaine may enhance burst firing. Consistent with this idea, there is evidence that tonic activation of $\alpha_1$-adrenergic receptors contributes to burst firing in dopamine neurons *in vivo* (Grenhoff and Svensson, 1993), but not *in vitro* (Grenhoff et al., 1995).

## Conclusions

The mGluR IPSP characterized in the first manuscript represents the first description of a slow IPSP mediated by release of $Ca^{2+}$ from intracellular stores. The various molecular components thought to underlie the IPSP are present in many types of neuron throughout the central nervous system, and it is very likely that IPSPs mediated by mGluRs and other PI-coupled receptors will prove common once their function is studied under appropriate conditions. However, because this IPSP appears to require the presence

of many molecular components, and it is likely that these components must be tightly colocalized, it may be weak or nonexistent in other types of neuron. As the same PI-coupled receptors can mediate excitation in dopamine and other neurons, it will be important for future studies to uncover the mechanisms that determine which of these competing actions predominates. The present results indicate that the kinetics of receptor activation are a crucial determinate of whether mGluR1 produces inhibition or excitation.

Dopamine neurons are critical in mediating addiction to various drugs. The second manuscript suggests that repeated cocaine administration may lead to a long-lasting suppression of the mGluR IPSP by adenosine. Though other synaptic potentials in dopamine neurons are also sensitive to adenosine inhibition, only the mGluR IPSP is suppressed by adenosine under resting conditions. This is likely to facilitate glutamate-induced burst firing, and could potentially play a role in relapse to drug use in addicts. While this effect is thought to be presynaptic, preliminary evidence suggests that amphetamine, and possibly cocaine, can acutely inhibit the mGluR IPSP postsynaptically. The mGluR IPSP in dopamine neurons may therefore prove to be an important target of both the acute and chronic effects of psychostimulants.

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# Opioid Desensitization: Interactions with G Protein-coupled Receptors in the Locus Coeruleus

Christopher D. Fiorillo and John T. Williams

Address The Vollum Institute, L474
Oregon Health Sciences University
3181 SW Sam Jackson Park Rd
Portland, OR 97201

Phone (503)-494-5465

FAX (503)-494-6972

e-mail williamj@ohsu.edu

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In rat locus coeruleus (LC) neurons, $\alpha_2$-adrenoceptors, μ-opioid and somatostatin receptors all activate the same potassium conductance. Chronic treatment with morphine results in a loss of sensitivity that is specific to the μ-opioid response, with no change in the $\alpha_2$-adrenoceptor-mediated response. Acute desensitization induced by opioid, somatostatin, and $\alpha_2$-adrenoceptor agonists was studied in brain slices of rat LC using intracellular recording. A supramaximal concentration of the opioid agonist [Met$^5$]-enkephalin (ME) induced a profound homologous desensitization, but little heterologous desensitization to an $\alpha_2$-adrenoceptor agonist (UK14304) or somatostatin. All desensitized currents showed partial recovery. A supramaximal concentration of UK14304 caused a relatively small amount of desensitization. While little interaction was observed among inhibitory G protein-coupled receptors, activation of an excitatory receptor had marked effects on inhibitory responses. Muscarinic agonists, which produce an inward current in LC neurons, reduced the magnitude of agonist-induced outward currents and increased both the rate and amount of opioid desensitization. Muscarinic activation did not alter desensitization of $\alpha_2$-adrenoceptor responses. Acute desensitization shares several characteristics with the tolerance induced by chronic morphine treatment of animals.

## Introduction

Desensitization of receptor-mediated responses due to prolonged or excessive exposure to agonist is a widespread phenomenon. Desensitization is of particular interest with respect to the development of tolerance to drugs, where it can limit the therapeutic utility of many

substances. Because of the great efficacy of morphine in the treatment of pain, tolerance to opioids has been the subject of much research. Both the acute and chronic actions of opioids on neurons of the LC have been intensely studied. LC cells express the μ-subtype of opioid receptor, which activates a potassium conductance (Pepper and Henderson,1980; Williams et al.,1982, 1988) and inhibits adenylate cyclase (Duman et al.,1988; Beitner et al.,1989) through a pertussis toxin-sensitive G protein (Aghajanian and Wang,1986). The LC has been proposed to play a central role in the behavioral phenomena of tolerance and withdrawal to opiates (Maldonado et al.,1992; Koob et al.,1992).

In rats treated chronically with morphine, the dose-response curves for full opioid agonists in slices of LC are shifted to the right and the maximal current for the partial agonist normorphine is reduced (Christie et al., 1987). Responses show no recovery over several hours in the slice in the absence of agonist. The decreased opioid response due to chronic morphine treatment is homologous, as $\alpha_2$-adrenoceptor activation of the same potassium current is unchanged. While the number of opioid receptor binding sites in the central nervous system may be marginally reduced by chronic opiate treatment, downregulation does not appear to be responsible for tolerance (Tao et al.,1987; Werling et al.,1989). It therefore appears that the decline in response after chronic morphine treatment is not the result of a change in the number of receptors or potassium channels.

Results in the LC are consistent with studies in cell lines naturally expressing opioid receptors, in which chronic opioid treatment produces a homologous desensitization of opioid inhibition of activated adenylyl cyclase. These studies have generally concluded that desensitization results from uncoupling of opioid receptors from their G proteins, and that downregulation of receptor number is limited and occurs only after uncoupling (Law et al.,1983, 1991; Puttfarken et al.,1988).

In addition to the tolerance that develops in the LC with prolonged exposure to

μ-opioid agonists, acute desensitization occurs in the presence of high concentrations of agonist. It was previously reported that supramaximal concentrations of μ-opioid agonists produced a hyperpolarization that declined over five minutes to 70% of its peak value and recovered over about 20 minutes (Harris and Williams,1991). This desensitization was found to be primarily homologous with respect to the $\alpha_2$-adrenoceptor mediated hyperpolarization.

The recent cloning of opioid receptors, as well as a G protein-coupled potassium channel, has made possible coexpression studies. These studies provide an opportunity for examining mechanisms of desensitization under carefully controlled conditions. An understanding of the differences between homologous and heterologous desensitization in the LC may aid in the interpretation of results from more artificial preparations. The purpose of the present study is to more fully characterize the two processes. By measuring drug-induced currents under voltage clamp, we have better quantified desensitization than is possible through voltage measurements. Our results clearly illustrate the homologous nature of μ-opioid desensitization. While little interaction was observed among $G_i$ coupled receptors, activation of muscarinic receptors had marked effects on agonist-induced outward currents. In particular, we found that μ-opioid desensitization, but not $\alpha_2$-adrenoceptor desensitization, is enhanced by muscarinic agonists.

## Materials and Methods

*Subjects*. Male Wistar rats (150-200 gm), housed according to NIH guidelines, were used for all experiments.

*Tissue preparation and recordings*. Exact details of the method of slice preparation and recording have been previously published (Williams *et al.*, 1984). To summarize, rats

were anesthetized with halothane and killed. Horizontal slices (300 μm thick) containing the LC were cut using a vibratome and stored in an oxygenated chamber at 35°C. For recording, a hemisected slice was submerged in a volume of 0.5 ml of artificial cerebrospinal fluid (ACSF), which was superfused at a temperature of 35° at a rate of 1.5 ml/min. The solution was equilibrated with 95% $O_2$/ 5% $CO_2$ and contained (mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.4 $NaH_2PO_4$, 25 $NaHCO_3$, and 11 D-glucose. Microelectrodes were filled with 2 M KCl and had resistances of 25-50 MΩ. Measurement of current and voltage were made with an Axoclamp 2A amplifier. Membrane currents were recorded in discontinuous voltage-clamp mode with a switching frequency of 3.5 - 4.5 kHz. The potential at the headstage was monitored with a second oscilloscope. Detailed descriptions of the electrical properties of LC neurons in a slice preparation identical to that used here have been previously published (Williams et al.,1984).

*Drugs.* DL-2-amino-5-phosphonovaleric acid (APV), bestatin, [$Met^5$]-enkephalin (ME), (-) scopolamine, somatostatin, and DL-thiorphan, were obtained from Sigma (St. Louis); acetylcholine chloride (ACh), carbachol, hexamethonium chloride, idazoxan, (+/-) muscarine chloride, and UK-14,304 from Research Biochemicals Incorporated (Natick, MA); (S)-a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) from Tocris Neuramin (Bristol, UK).

*Drug application.* Drugs were applied by superfusion. In all experiments, ME was used in combination with the peptidase inhibitors bestatin (20 μM) and thiorphan (2 μM). ME was chosen because it washes (or is degraded) quickly even at high concentrations. ME has been shown to act exclusively at μ-opioid receptors on LC neurons (Williams and North.,1984), and we observed identical results with the selective μ-opioid agonist DAMGO in several cells. The nicotinic antagonist hexamethonium (400 μM) was applied in experiments with carbachol or ACh. In experiments with AMPA, antagonists were used

to block the actions of released endogenous transmitters. These included the NMDA receptor antagonist APV (30 μM), the $\alpha_2$-adrenoceptor antagonist idazoxan (1 μM), and the muscarinic antagonist scopolamine (1 μM). None of the above antagonists has a measurable effect when applied alone.

*Data analysis* Values are given as arithmetic means±SEM. For statistical analysis, paired and unpaired t-tests were performed. Differences in data for which $p<.05$ were taken to be significant. Each experiment of a given protocol was performed on a separate animal. All time constants were obtained by fitting the data to a single exponential using iterations of the sum of squared errors.

## Results

*Desensitization of opioid responses*

Superfusion of [Met$^5$]-enkephalin (ME, 30 μM) caused a peak outward current of 417±19 pA (n=72). The current decayed with a time constant of 3.1±0.2 minutes (n=17) to a stable level of 53±2% (n=17) of the peak amplitude (Fig. 1A). No correlation was found between the maximal amplitude of the current and either the rate or amount of desensitization. Multiple applications of ME could be applied to a single cell with little change in the rate or amount of desensitization.

In order to examine the recovery of the outward current, an approximately half-maximal concentration of ME (0.3-1 μM) was perfused until a steady state current (214±21 pA) was reached (Fig 2). The high concentration of ME (30 μM) was then perfused for 5 minutes before switching back to the low concentration. The current peaked and declined to 67±2% (n=19) of the peak value at the end of the 5 minutes. Immediately after washing of the high concentration, the current produced by the low concentration of ME was reduced to 14±4% (n=19) of its initial level. With continuous perfusion of the

low concentration of ME, the current recovered to 64±4% (n=19) of control twenty minutes after washout of the high concentration of ME. ME was then completely washed out. Subsequent applications of the low concentration of ME, 10-30 minutes later, showed greater recovery (77±3%, n=13) and were reproducible over time. Full recovery was never observed to low concentrations, though subsequent application of the high concentration (30 μM) produced currents that were 95% or more of the original peak in 9 of 21 cells, with an average of 86±3% after 20 minutes or more. This is significantly greater recovery than that observed to low concentrations (paired t-test, $p<.05$, n=8), suggesting a long-lasting rightward shift in the dose-response curve.

Time constants of recovery were calculated for those cells in which the current induced by the low concentration of ME reached a plateau before washout (Fig 2). The average time constant for these cells was 7.5±1.1 minutes (n=11). In eight other cells, the recovering current did not reach a clear plateau before the ME was washed approximately 20 minutes after washing the high concentration of ME. Prolonged application (10 minutes or more) of a low concentration of ME revealed a slight rundown (see *Desensitization to low concentrations of agonist*).

*Desensitization of $\alpha_2$-adrenoceptor responses*

In order to examine the degree to which opioid desensitization is heterologous, the same experiment as above was carried out substituting a submaximal concentration of UK14304, a selective $\alpha_2$-adrenoceptor agonist, for the low concentration of ME (Fig 2). UK14304 (100 nM) initially caused an outward current of 329±49 pA (n=9). A five minute application of ME (30 μM) in the continued presence of UK14304 produced a peak current of 454±56 pA (measured from the original holding current) which declined to 81±3% (n=9) of the peak after 5 minutes. After the ME had washed out, the UK14304 current

was reduced to 86±3% (n=9) of its initial value, and after twenty minutes it had recovered to 96±2% (n=7). For those cells in which the rate of recovery could be calculated the time constant was 4.5±1.0 (n=7) minutes. Prolonged application of low concentrations of UK14304 alone revealed a slight decay of the current (see *Desensitization to low concentrations of agonist*).

Desensitization to a high dose of UK14304 was also studied. UK14304 (3 μM) produced a peak current of 373±43 pA which declined to 78±3% (n=12) of the peak after 10-15 minutes (Fig 1A). The current decayed with a time constant of 7.2±1.3 (n=7) minutes in those cells in which the desensitizing current reached a plateau, compared to 3.1±0.2 minutes (n=17) for ME (30 μM). The UK14304 response was terminated by superfusion with the selective $\alpha_2$-adrenoceptor antagonist idazoxan (1 μM). Reversal of the response with an antagonist was necessary because of the slow washout of UK14304 and precluded any further experiments with $\alpha_2$-adrenoceptor agonists in that slice. Once the current had returned to basal levels (about 15 minutes after perfusion of idazoxan) a low concentration of ME gave a response that was 76±9% (n=5) of the pre-UK14304 level. Subsequent exposures to ME in 3 cells showed no further change.

*Desensitization of somatostatin responses*

Somatostatin activates the same potassium current as μ-opioid and $\alpha_2$-adrenoceptor agonists in the LC. Somatostatin (3-6 μM) produced a maximal peak outward current of 313±48 pA. The decay of the current was variable, but averaged 62±9% (n=6) of the peak after about 10 minutes, and had a time constant of 1.6±0.6 minutes (n=3, data not shown). Application of ME (30 μM) immediately following desensitization to somatostatin revealed little reduction in the peak response and no apparent occlusion of ME desensitization in

three cells, suggesting that somatostatin desensitization is primarily homologous with respect to the opioid response. This is in agreement with results in guinea pig submucosal neurons (Shen and Surprenant,1993).

Heterologous desensitization of the somatostatin response by ME was also examined. Somatostatin (300-600 nM) induced a current of 152±12 pA that was reduced to 77±4% (n=12) immediately after a five minute application of ME (30 μM, Fig 3). The somatostatin current recovered from heterologous desensitization in three cells, suggesting that the smaller response after ME was not due solely to homologous desensitization to somatostatin. In any case, heterologous desensitization of the somatostatin response by opioids is much less than the homologous desensitization to opioids.

*Desensitization to low concentrations of agonist*

A small amount of desensitization was also observed in the continuous presence of low concentrations of both ME and UK14304 (data not shown). ME (300 nM) produced a peak current of 292±50 pA which declined to 87±4% (paired t-test, $p<.02$, n=5) of the peak after 10 minutes. UK14304 (25-100 nM, a half-maximal current is produced by about 25 nM) resulted in a peak current of 333±43 pA which declined to 88±2% (paired t-test, $p<.01$, n=8) of the peak after approximately 20 minutes. This is significantly less desensitization than was observed to the high concentration of UK14304 (3 μM, unpaired t-test, $p<.05$) It appears that desensitization can occur at lower concentrations, but the rate and/or the maximum degree of desensitization is less. The decline in both the opioid and $\alpha_2$-adrenoceptor currents is therefore dependent on the concentration of agonist, even at concentrations greater than that needed to induce a maximal current.

*Desensitization corresponds to decline in potassium conductance*

Current/voltage relations were examined at the peak current and after desensitization to high concentrations of ME (30 μM) and UK14304 (3 μM). Both were qualitatively similar, showing a decrease in conductance with desensitization, but little shift in reversal potential (Fig 1B). The ME-induced slope conductance between -60 and -90 mV declined from a maximum of 4.1±0.4 nS to a stable level of 2.2±0.3 nS (7-15 min.), with an average decline of 1.9±0.2 nS (n=17). The conductance induced by UK14304 was initially 3.0±0.5 nS and was reduced to 2.4±0.3 nS after 10-15 minutes of perfusion, with an average decrease of 0.6±0.2 nS (n=7). The decline in conductance was significantly greater for ME than for UK14304 (unpaired t-test, $p<.001$). The difference in the reversal potentials before and after desensitization is almost certainly due to the poor voltage control in horizontal slices. No shift in reversal potential occurs with opioid desensitization in coronal slices (Harris and Williams,1991), in which voltage control is significantly greater than in horizontal slices (Travagli et al., in press).

*Interactions with muscarinic agonists*

Having examined interactions among receptors that couple to activation of $K^+$ channels, we next investigated interactions of these receptors with an excitatory G protein-coupled receptor. Muscarine produces an inward current in LC neurons at all potentials between -40 and -140 mV (Fig 4B; Shen and North,1992). The inward current is accompanied by little change in conductance over this potential range, and it has been proposed to result from an increase in a non-selective cation conductance and a decrease in potassium conductance (Shen and North,1992). However, the G protein and second messenger systems involved remain unknown.

Muscarine (10 μM), carbachol (10-20 μM), or acetylcholine (1.0-1.5 mM) were used to elicit currents ranging from -40 to -234 pA, with a mean of -103±12 (n=20). The I-V curve between -60 and -140 mV (Fig 4B) was well fit by a straight line with a slope of

1.0±0.2 nS (n=19). Muscarinic agonists decreased the peak amplitude of opioid-induced currents (Fig 4A) to 67±7% of control (paired t-test, $p<.01$, n=10) and UK14304-induced currents to 65% of control (unpaired t-test, $p<.05$, n=8). Internal controls could not be performed with UK14304 because of its very slow rate of washing from the tissue. However, carbachol-induced inward currents were found to be approximately twice as large as control in the presence of UK14304 (3 μM) in two cells.

Muscarinic activation was found to increase both the rate and amount of μ-opioid desensitization (Fig 4A) without affecting $\alpha_2$-adrenoceptor desensitization. In the presence of muscarinic agonists, the ME-induced current declined to 36±4% (n=12) of the peak value versus 57±2% (n=12) of the peak in control (unpaired t-test, $p<.001$; paired t-test, $p<.02$, n=6). The current decayed with a time constant of 1.9±0.2 minutes (n=10) in the presence of muscarinic agonists compared to 3.4±0.4 minutes (n=10) in control (unpaired t-test, $p<.01$). As in control, the ME-induced desensitization observed at -60 mV in the presence of muscarinic agonists resulted exclusively from a decline in conductance that reversed polarity near $E_k$ (Fig. 4B). The peak ME conductance measured from -60 to -90 mV was 3.5±0.7 nS and the desensitized conductance was 1.6±0.3 nS, with an average change of -2.0±0.6 nS (n=7). In the presence of muscarinic agonists the current measured after a 10-15 minute exposure to UK14304 (3 μM) was 83±3% (n=8) of the peak (data not shown), unchanged from control.

Because voltage control is known to be poor in horizontal slices of LC (Travagli et al.,1995; Oleskevich et al.,1993), we considered the possibility that the effects of muscarinic agonists were secondary to depolarization of poorly clamped dendrites. To test this, ME desensitization was examined in the presence of the selective glutamate agonist AMPA. Concentrations of AMPA ranging from 200-350 nM elicited an average current of -151±20 pA (n=8). In the presence of AMPA, neither the peak amplitude (paired t-test,

p=.14, n=5), the amount of desensitization to ME (30 μM) (paired t-test, p>.6, n=5) nor the time constant (paired t-test, p>.6, n=3; unpaired, p>.6, n=6 control, n=5 in AMPA) was significantly different from control (data not shown). The amount of desensitization in muscarine relative to control in the same cell (n=6) was significantly greater (unpaired t-test, p<.05) than that in AMPA relative to internal control (n=5). Furthermore, if the effect on desensitization was voltage dependent, then the amount and rate of desensitization should correlate with the size of the inward current. No correlation was found between the amplitude of the muscarine or AMPA induced currents and the size of the effect on the time constant or amount of ME desensitization, or the peak current.

## Discussion

Voltage-clamp recordings were used to study acute desensitization to high concentrations of μ-opioid and $\alpha_2$-adrenoceptor agonists. A supramaximal concentration of the μ-opioid agonist ME induced an outward current that declined to 50% of the peak, while the current produced by an analogous concentration of the $\alpha_2$-adrenoceptor agonist UK14304 declined to only 80% of the peak. Muscarinic agonists increased both the rate and amount of desensitization to ME but did not alter desensitization to UK14304. Desensitization to ME was primarily homologous. Both the homologous and heterologous components of desensitization recovered over 10-30 minutes. The lack of cross-desensitization of the somatostatin response provides particularly compelling evidence for the homologous nature of opioid desensitization considering that somatostatin receptors are closely related in primary structure to opioid receptors. More convincing evidence comes from studies in undifferentiated SH-SY5Y human neuroblastoma cells, which express both μ- and

∂-opioid receptors yet show only homologous desensitization (Prather et al.,1994).

*Mechanism of Opioid Desensitization*

The mechanism of acute opioid desensitization in the LC has been previously examined, with few positive results. The phosphatase inhibitor microcystin was found to prolong the time course of recovery from desensitization (Osborne and Williams, 1995). ß-chlornaltrexamine (ß-CNA), an irreversible opioid receptor antagonist, reduced the maximum hyperpolarization but did not reduce the relative amount of desensitization (Harris and Williams,1991). This suggests that desensitization occurs locally at the level of single receptors and does not depend on the number of functional receptors or the magnitude of the response.

Results in the LC as well as in cell lines show that the amount of desensitization of opioid receptors depends on the concentration of agonist, its affinity, and its intrinsic efficacy (Law et al.,1983). The amount of desensitization is therefore correlated with the amount of time the receptor spends in an active state. The same is true for homologous desensitization of the $\beta_2$-adrenoceptor (reviewed by Lohse,1993; Dohlman et al.,1991). In its active conformation, the $\beta_2$-receptor is a substrate for phosphorylation by a G protein-coupled receptor kinase (GRK). Phosphorylation allows the protein β-arrestin to bind to the receptor and thereby prevent coupling to G proteins. Opioid desensitization also results from a functional uncoupling of receptors from G proteins (Law et al.,1991; Puttfarcken et al.,1988).

Multiple GRKs and arrestins have been discovered and are known to act on other receptors *in vitro* (Premont et al.,1995; Craft and Whitmore.,1995; Gurevich et al.,1995). In particular, the $m_2$ muscarinic receptor, which couples to the same intracellular effectors

as opioid receptors, undergoes agonist-dependent desensitization and phosphorylation by a GRK *in vitro* and *in vivo* (Kameyama et al.,1994; Tsuga et al.,1994). It was recently shown that the uncoupling of atrial $m_2$ muscarinic receptors from $K^+$ channels, which is very similar to opioid desensitization in both time constant and extent, is absent in patch clamp configurations in which the intracellular solution is lost (Shui et al.,1995). Desensitization is reestablished in outside-out patches when GRK2 and ATP are included in the pipette.

*Mechanism of $\alpha_2$-Adrenoceptor Desensitization*

It seems unlikely that $\alpha_2$-adrenoceptor desensitization in the LC occurs by a GRK-mediated mechanism. Adrenoceptor desensitization is heterologous, as both the opioid and the $\alpha_2$-adrenoceptor responses are reduced to a similar degree. The most notable feature of $\alpha_2$-adrenoceptor desensitization in the LC is its slow and limited nature. In guinea pig submucosal neurons the $\alpha_2$-adrenoceptor-activated outward potassium current does not desensitize (Shen and Surprenant, 1993). While some subtypes of cloned $\alpha_2$-adrenoceptors are known to desensitize quite extensively, others do not (Kurose and Lefkowitz,1994).

*Coexpression Studies in Xenopus Oocytes*

Several recent studies have described the coupling of coexpressed μ-opioid receptors and GIRK1 potassium channels in *Xenopus* oocytes; homologous desensitization was not

observed (Mestek et al.,1995; Kovoor et al.,1995; Chen and Yu,1994). Kovoor et al. (1995) reported the slow inactivation ($\tau$=15 minutes) of the potassium current coupled to coexpressed $\mu$-opioid and 5-$HT_{1A}$ receptors in *Xenopus* oocytes. The current decayed with the same time constant whether induced by agonist, GTP$\gamma$S, or high potassium, and was resistant to manipulations of calcium and kinase activity. Slow inactivation of potassium channels could underlie the heterologous component of desensitization in the LC. However, Kovoor et al reported that extrapolation of the decay exponential to t = infinity revealed complete loss of agonist-induced current. In the LC, currents induced by supramaximal concentrations of opioids always decayed to steady state levels of about 50% of the peak current, while $\alpha_2$-adrenoceptor-induced currents decayed to a plateau of 80% of the peak after 15 minutes. In addition, while Kovoor et al. found that the rate of decay of the current was independent of the size of the current or the concentration of agonist, we found that both opioid and $\alpha_2$-adrenoceptor desensitization was dose dependent. It is therefore unlikely that the same potassium channel inactivation observed in oocytes accounts for a significant amount of the heterologous component of agonist-induced desensitization in the LC.

*Enhancement of Opioid Desensitization by Muscarine*

Muscarine causes an inward current in LC neurons through activation of a receptor of uncertain pharmacology (Egan and North,1985; Shen and North, 1992; reviewed by Caulfield,1993). The inward current appears to result from activation of a non-selective cation conductance and possibly inhibition of a potassium conductance (Shen and North,1992). Neither buffering of internal calcium (Shen and North,1992; Osborne and Williams,1995) nor application of phorbol esters altered or mimicked opioid desensitization

or the muscarine current (G.C. Harris and J.T. Williams, unpublished observations).

The βγ subunits of G proteins are involved in targeting of some GRKs to their substrates (Kameyama et al.,1993; Pitcher et al.,1992; Haga and Haga,1992). If opioid desensitization involves a receptor kinase, then the βγ subunits freed by activation of muscarinic receptors might enhance receptor desensitization by increasing the effective concentration of GRKs at the receptor. If this is the case, activation of many if not all G protein-coupled receptors would be expected to enhance desensitization of opioid receptors and perhaps other receptors.

The observed facilitation of opioid desensitization by muscarine does not necessarily indicate an effect on the actual process of agonist-induced homologous receptor desensitization. Due to the receptor reserve, a large number of functional opioid receptors must be uncoupled from $K^+$ channels before a reduction in the maximal current is observed (Osborne and Williams,1995; Christie et al.,1987). Much of the receptor desensitization has therefore already occurred by the time a decline in current is measured. If muscarine acts to uncouple opioid receptors independent of opioid agonists, thereby decreasing the number of functional receptors and the receptor reserve, it is possible that a greater decay in the opioid-induced current would be observed in the presence of muscarine without a change in the amount of homologous receptor desensitization. We found that muscarine decreases the magnitude of the outward current induced by both opioid and $\alpha_2$-adrenoceptor agonists. However, decreasing the coupling efficiency would not be expected to change the observed rate of decay. The faster time constant therefore suggests that muscarinic activation facilitates the agonist-induced functional uncoupling of opioid receptors from their effectors.

*Comparison of Acute and Chronic Desensitization*

The acute desensitization examined here shares a number of characteristics with chronic tolerance to opiates in the LC. Both are primarily homologous, with the opioid dose-response curves shifted to the right by opioid treatment and the maximum response to the partial agonist normorphine significantly reduced. The acute desensitization protocol used here was estimated to produce a greater loss of functional receptors (90% after 5 minutes, Osborne and Williams, 1995) than the chronic morphine treatment (75%, Christie et al.,1987). This may be due in part to the greater efficacy of full agonists in producing desensitization (Harris and Williams,1991; Law et al.,1983).

The only marked difference between the acute and chronic treatments is the rate of recovery. While acutely treated cells show recovery of the opioid response over 20-30 minutes even in the presence of low concentrations of agonist, cells from chronically treated animals show no recovery over several hours in the slice in the absence of agonist (Christie et al.,1987). However, recovery from acute desensitization is not complete. While the response to high concentrations of ME sometimes showed full recovery, the response to low concentrations never approached full recovery. This is consistent with a long-lasting rightward shift in the dose-response curve, as observed in chronically treated animals. It seems doubtful that this shift, measured over about 45 minutes, resulted exclusively from an agonist-independent rundown of responses. Harris and Williams (1991) found that less acute desensitization is observed after either acute desensitization or chronic morphine treatment. It therefore appears that acute desensitization may produce long-lasting changes in the response to opioids that are at least superficially similar to the changes seen in chronically treated animals. Considering the incomplete recovery from acute desensitization and the lesser amount of desensitization produced by chronic morphine, it is not clear that there is any qualitative difference in desensitization resulting from acute treatment of cells and chronic treatment of animals.

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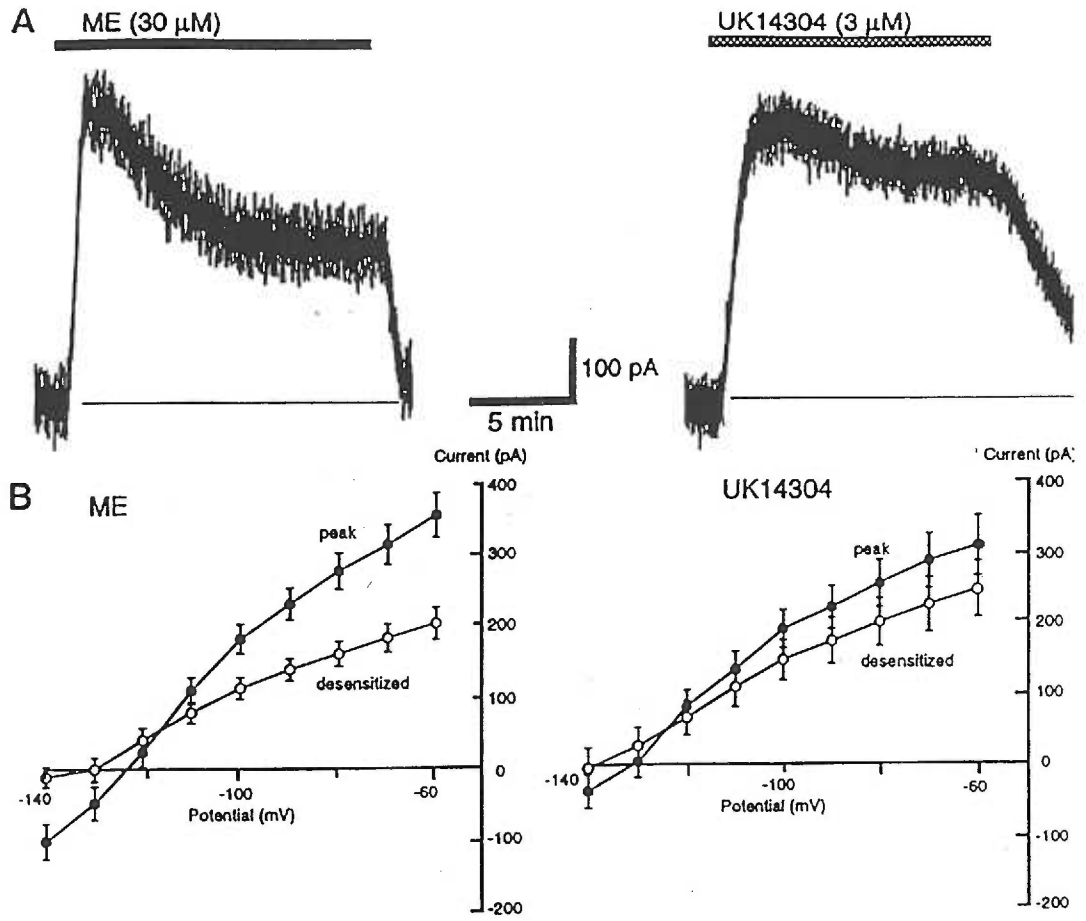


**Figure 1**. Desensitization induced by μ-opioid and $\alpha_2$-adrenoceptor agonists. **A**, Supramaximal concentrations of the μ-opioid agonist ME and the $\alpha_2$-adrenoceptor agonist UK14304 both induced outward currents that declined with time. The decline in the ME-induced current was always much greater than the decline in the UK14304-induced current. Idazoxan (1 μM) was superfused to reverse the current induced by UK14304. In all cells, currents were recorded while clamping the membrane at -60 mV. Holding currents ranged from 30 to -200 pA in the absence of drugs. **B**, The decline in both ME- and UK14304-induced currents was accompanied by a decline in conductance (ME, n=17; UK14304, n=7). Current-voltage (I-V) plots were created at the peak of the current and after its decay by subtracting the raw I-V curve in the absence of drug from the raw I-V curve in the presence of drug. I-V curves were generated by 200 ms steps of the membrane voltage from a holding potential of -60 mV to potentials ranging from -60 mV to -140 mV.

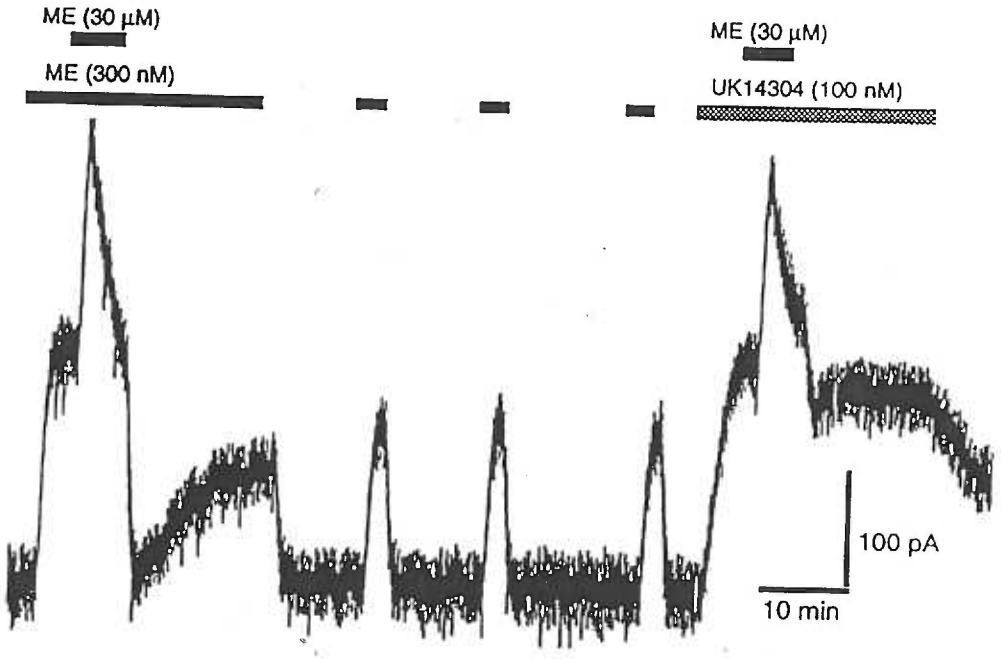


**Figure 2.** Homologous and heterologous desensitization by ME. The response to a half-maximal concentration of ME was decreased on average to 14% of its initial value after a five minute exposure to a supramaximal concentration of ME. By contrast, the same protocol only reduced the response to the $\alpha_2$-adrenoceptor agonist UK14304 to 86% of control. Both desensitized currents recovered.

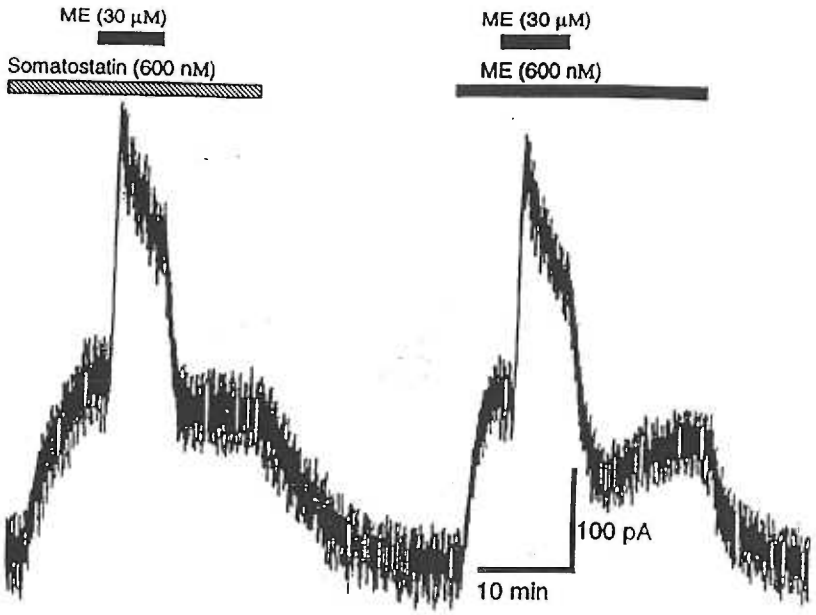


**Figure 3.** ME causes little cross-desensitization of somatostatin responses. The response to low concentrations of somatostatin was reduced to an average of 77% of control following a five minute application of a supramaximal concentration of ME.

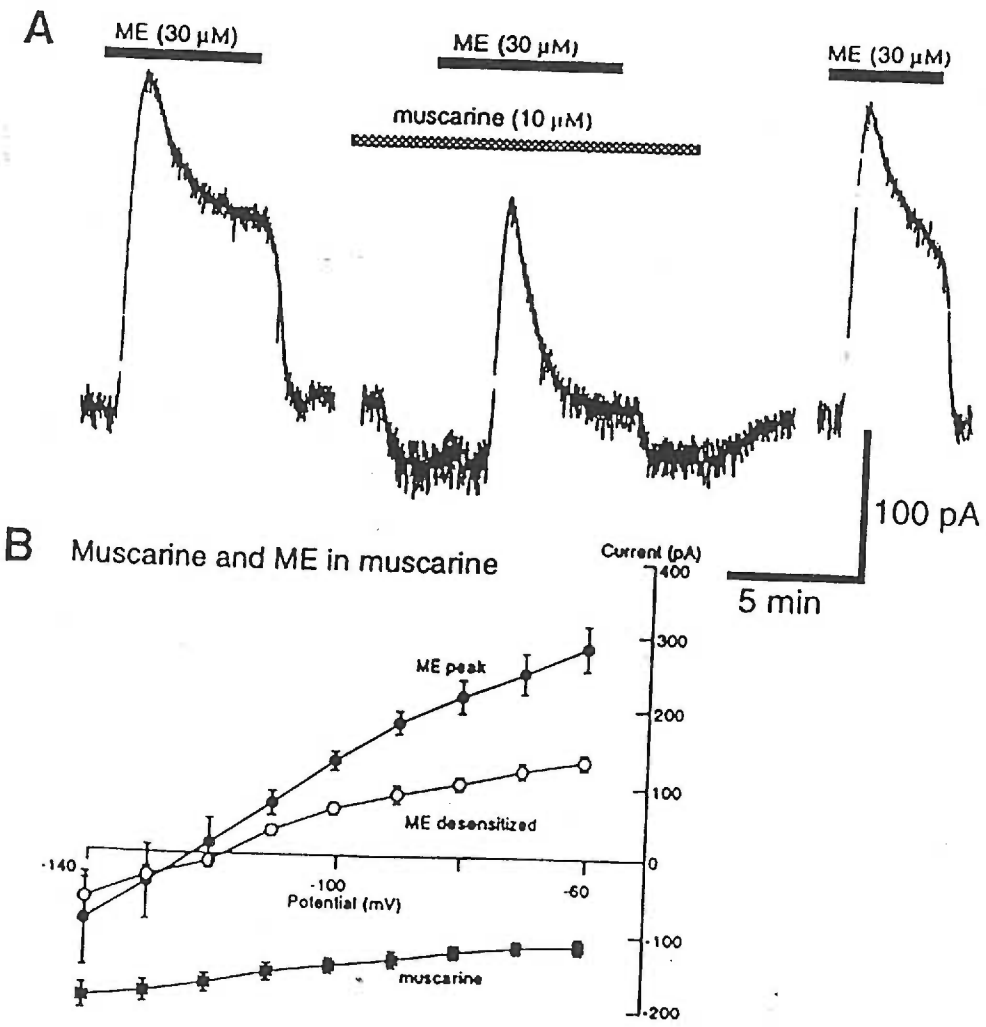


**Figure 4.** Muscarine reduces the magnitude of agonist-induced currents while specifically enhancing opioid desensitization. **A**, Muscarine decreases the magnitude of ME-induced currents, and increases the rate and amount of desensitization. All traces were obtained from the same cell in the order shown, with the applications of ME separated by about 25 minutes. Carbachol and acetylcholine had the same effect as muscarine. Muscarinic agonists also decreased the magnitude of currents induced by the $\alpha_2$-adrenoceptor agonist UK14304, but did not affect desensitization to UK14304 (not shown). **B**, Current-voltage relations of the conductance activated by muscarinic agonists (n=19), as well as the peak and desensitized conductance induced by ME in the presence of muscarinic agonists (n=7).