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INTRODUCTION

The ventral tegmental area (VTA) contributes to the motivational actions of natural rewards and a variety of drugs including opioid agonists. Both κ opioid receptors (KOP-R) (Arvidsson et al. 1995; Mansour et al. 1996) and μ opioid receptors (MOP-R) (Garzon and Pickel 2001; Svingos et al. 2001) are present in significant density in the VTA. Furthermore, microinjections of both KOP-R and MOP-R agonists directly into the VTA produce robust behavioral responses. The KOP-R agonist U50488H produces conditioned place aversion (Bals-Kubik et al. 1993). In contrast, the MOP-R agonist [D-Ala^2, N-Me-Phe^4, Gly-ol^5]-Enkephalin (DAMGO) produces conditioned place preference (Bals-Kubik et al. 1993; Nader and van der Kooy 1997; Phillips and LePiane 1980).

Glutamate transmission within the VTA is required for the motivational properties of opioids (e.g., Cornish et al. 2001; Harris and Aston-Jones 2003; Xi and Stein 2002). In the VTA, glutamatergic inputs are derived from neurons in the medial prefrontal cortex (mPFC), subthalamic nucleus (STN), and the pedunculopontine nucleus (PPN) (Charara et al. 1996; Christie et al. 1985; Groenewegen and Berendse 1990; Sesack and Pickel 1992). There is also indirect evidence that lateral hypothalamic projections to the VTA contain glutamate (Chou et al. 2001; Rosin et al. 2003). Under physiological conditions, glutamate can induce phasic firing in dopaminergic neurons through the activation of *N*-methyl-D-aspartate (NMDA) receptors (Chergui et al. 1993; Johnson et al. 1992; Overton and Clark 1997), and this effect is facilitated by group 1 metabotropic glutamate receptor activation (Zheng and Johnson 2002). There is also evidence that glutamate activation of 2-amino-3(3-hydroxy-5-methyl-4-isoxazolyl) propionic acid (AMPA) receptors in the VTA can increase extracellular dopamine (DA) in the nucleus accumbens (NAc) (Karreman et al. 1996; Schilstrom et al. 1998).

Essential to determining how KOP and MOP receptor agonists in the VTA produce their behavioral actions is elucidating their synaptic effects at the cellular level in all VTA cell types. VTA neurons are classified as principal, secondary, or tertiary according to their electrophysiological and pharmacological properties (Cameron et al. 1997). Both principal and tertiary neurons exhibit the hyperpolarization-activated nonspecific cation current (I_h) and have relatively long duration action potentials. Secondary cells lack an I_h and have shorter duration action potentials. They are directly hyperpolarized by MOP-R agonists and are GABA neurons. Secondary cell inhibition by MOP-R agonists has been proposed to disinhibit principal neurons through local circuitry (Johnson and North 1992; Margolis et al. 2003). Tertiary neurons differ from principal cells in that they are directly hyperpolarized by MOP-R agonists and serotonin. While most principal neurons are dopaminergic (~80%), <40% of tertiary neurons are dopaminergic. KOP-R agonists postsynaptically inhibit a subset of both principal and tertiary neurons, an effect limited to dopaminergic neurons of each class (Margolis et al. 2003).

Despite the evidence that VTA glutamatergic transmission is critical for reward and motivation, our understanding of presynaptic control of glutamate release by opioids is incomplete. We therefore examined the effects of both KOP and MOP receptor agonists on glutamate release onto each VTA cell type. We directly compared KOP and MOP effects within and across individual neurons and neuron types. Because both KOP and MOP effects were observed, we addressed the issue of

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whether KOP and MOP receptor agonists act on the same terminals by testing whether the effects of the two agonists occluded.

METHODS

Twenty- to 36-day-old male Sprague-Dawley rats were anesthetized with isofluorane, and their brains were removed. Horizontal slices (200 μm thick) containing the VTA were prepared using a vibratome (Leica Instruments). Slices were submerged in artificial cerebrospinal fluid (ACSF) solution containing (in mM) 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 1.0 NaH_2PO_4, 2.5 $CaCl_2$, 26.2 $NaHCO_3$, and 11 glucose, saturated with 95% O_2-5% CO_2 and allowed to recover at 32°C for ≥1 h.

Individual slices were visualized under a Zeiss Axioskop with differential interference contrast optics and infrared illumination. Whole cell patch-clamp recordings were made at 31°C using 2.5- to 4-MΩ pipettes containing (in mM) 123 K-gluconate, 10 HEPES, 0.2 EGTA, 8 NaCl, 2 MgATP, and 0.3 Na_3GTP (pH 7.2, osmolarity adjusted to 275).

Recordings were made using an Axopatch 1-D, filtered at 2 kHz, and collected at 5 kHz using IGOR Pro (Wavemetrics, Lake Oswego, OR). I_h was recorded by voltage clamping cells and stepping from −60 to −120 mV. Series resistance and input resistance were sampled throughout voltage-clamp experiments with 4-mV, 200-ms depolarizing steps once every 10 s. For purposes of classification, every neuron was tested for postsynaptic actions of either the MOP-R selective agonist DAMGO (3 μM) or the 5-HT_1 agonist 5-carboxamidotryptamine (5-CT; 500 nM) in current clamp following the voltage-clamp experiment. Cells were recorded in voltage-clamp mode ($V = -70$ mV) while measuring excitatory postsynaptic currents (EPSCs). All EPSCs were measured in the presence of picrotoxin (100 μM). Stimulating electrodes were placed 60–150 μm rostral to the patched cell. In neurons where paired pulses were administered, two pulses 50 ms apart were delivered once every 10 s. The EPSC amplitude was calculated by comparing a 2-ms period around the peak to a 2-ms interval just before stimulation. The paired-pulse ratio (PPR) was calculated by dividing the amplitude of the second EPSC by that of the first, trial by trial, and averaging across trials. Spontaneous events were identified in a subset of experiments by searching the smoothed first derivative of the data trace for values that exceeded a set threshold, and these events were visually confirmed. Experiments with baseline sEPSC frequencies <0.25 Hz were excluded from drug effect analyses because too few events were detected to reliably measure changes in frequency.

Results are presented as means ± SE where appropriate. Summary comparisons were made between the average of the 4 min of baseline just preceding each respective drug application to 4 min of stable drug effect. The significance of drug effects was tested across all VTA neurons and within individual cell types using the two-way repeated-measures ANOVA followed by the Student-Newman-Keuls (SNK) method for multiple comparisons. Differences in effect sizes between neuron populations were tested with one-way ANOVA and the SNK method where appropriate. The significance of effects within individual neurons was tested with the Student's *t*-test, comparing the last 4 min of baseline to the last 4 min of drug application. Significance was defined at $P < 0.05$. In the case of postsynaptic drug effects, recovery during drug washout was also required.

All drugs were applied by bath perfusion. Stock solutions were made and diluted in Ringer immediately before application. (*trans*)-3,4-Dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl] benzeneacetamide methane-sulfonate hydrate (U69593) was diluted in 50% EtOH to a concentration of 1 mM; nor-binaltorphimine (nor-BNI; 1 mM), 5-CT (1 mM), CTAP (1 mM), and DAMGO (1 mM) were diluted in H_2O. Picrotoxin was diluted in DMSO (100 mM). All chemicals were obtained from Sigma Chemical (St. Louis, MO) or Tocris (Ballwin, MO).

RESULTS

Whole cell voltage-clamp recordings were made from neurons in the VTA. Pharmacologically isolated (100 μM picrotoxin) EPSCs were electrically evoked, and we confirmed that this evoked current was due to AMPA receptor activation by blocking the response with the non-NMDA glutamate receptor antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX; 10 μM, $n = 3$).

To address the question of whether opioids differentially alter glutamatergic transmission onto different cell types in the VTA, we classified neurons by their electrophysiological and pharmacological properties as principal, secondary, or tertiary (Margolis et al. 2003). In most neurons, the changes in stimulated EPSC amplitude following bath application of both the KOP-R selective agonist U69593 (1 μM) and the MOP-R selective agonist DAMGO (3 μM) were measured (Fig. 1). U69593 produced a modest reduction in EPSC amplitude in principal neurons (14 ± 4%), significantly smaller than the DAMGO effect in the same neurons (42 ± 8%, $n = 11$, ANOVA: $P < 0.01$). In contrast, in secondary cells, EPSCs

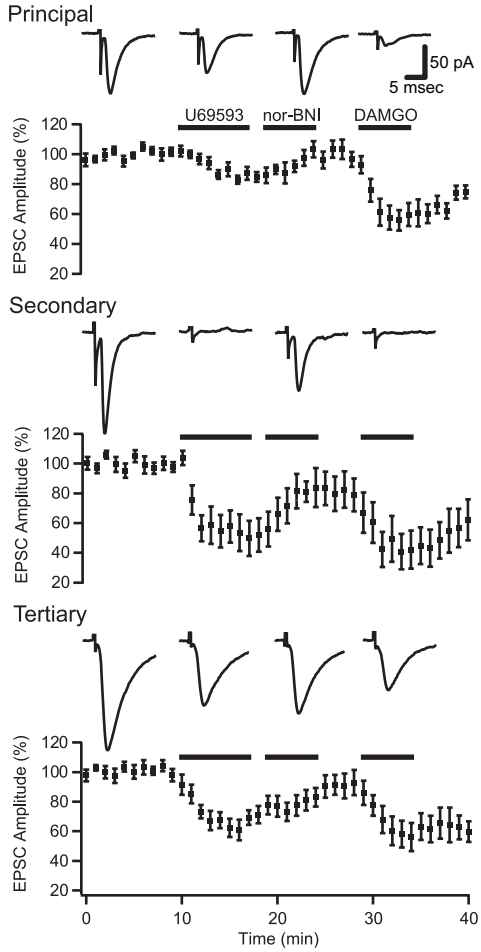


FIG. 1. Excitatory postsynaptic currents (EPSCs) in the ventral tegmental area (VTA) neurons are inhibited by both kappa opioid and mu opioid receptor (KOP-R and MOP-R) agonists. Evoked EPSCs in principal ($n = 11$), secondary ($n = 10$), and tertiary ($n = 9$) neurons are inhibited by the KOP-R agonist (*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl] benzeneacetamide methane-sulfonate hydrate (U69593; 1 μM). This inhibition is reversed by the KOP-R selective antagonist nor-binaltorphimine (nor-BNI; 100 nM). [D-Ala^2, N-Me-Phe^4, Gly-ol^5]-enkephalin (DAMGO; 3 μM), a MOP-R selective agonist, inhibits EPSC amplitude in the same cells of each cell type.

were inhibited to a similar degree by both U69593 (47 ± 10%) and DAMGO (45 ± 10%, $n = 10$). EPSCs in tertiary neurons were also inhibited by both U69593 (33 ± 6%) and DAMGO (35 ± 6%, $n = 9$). Because inhibition by the KOP-R agonist U69593 persisted for ≥15 min after washout commenced (1 μM, $n = 6$, data not shown), we used the KOP-R selective antagonist nor-BNI (100 nM) to reverse the KOP-mediated inhibition. Application of nor-BNI before U69593 completely blocked the KOP-R agonist effect (2 ± 2%, $n = 3$, 1 cell of each type). Because DAMGO was applied a second time to identify the cell type while recording in current-clamp mode, in most experiments no antagonist was used to reverse the prolonged presynaptic MOP-R activation response. However, in a separate set of cells, the application of the MOP-R selective antagonist D-Phe-Cys-Tyr-D-Tryp-Lys-Thr-Pen-Thr-NH2 (CTAP; 500 nM) reversed the DAMGO (3 μM) effect ($n = 4$, data not shown).

Comparing cell types, the observed U69593 effect was significantly larger in secondary and tertiary neurons than in principal neurons (SNK: principal vs. secondary, $P < 0.05$; principal vs. tertiary, $P < 0.05$). The DAMGO EPSC inhibition was not significantly different among cell types.

To provide evidence that the observed EPSC inhibitions were presynaptic, we examined changes in the PPR for each drug. A drug-induced decrease in the probability of release is typically correlated with an increase in the PPR (Manabe et al. 1993). We found no significant differences between the baseline PPRs of the different cell types (principal: 0.9 ± 0.1, $n = 12$; secondary: 1.2 ± 0.2, $n = 9$; tertiary: 0.93 ± 0.06 Hz, $n = 8$; ANOVA: $P > 0.05$).

Although opioid-induced changes in PPR for both U69593 and DAMGO varied greatly across cells and cell types, enhancement of PPR was observed for both receptor types. Figure 2*A* shows examples comparing baseline evoked EPSCs to those recorded in the presence of either U69593 or DAMGO and the time courses of the EPSC amplitude and PPR. This principal neuron exhibited a significant increase in PPR with both drugs. Overall, VTA neurons showed a significant facilitation of PPR in the presence of U69593 ($n = 29$, ANOVA: $P < 0.05$, Fig. 2*B*) and DAMGO ($n = 28$, ANOVA: $P < 0.05$, Fig. 2*C*). However, when broken down by cell type, the only significant effect observed was that produced by DAMGO in secondary neurons. Individually, less than one-third of all neurons (8/30) showed a significant increase in PPR with U69593 (Student's *t*-test, $P < 0.05$), and these neurons were distributed across all three cell types. A slightly higher proportion of neurons showed a significant increase in PPR with DAMGO (11/29), and these also included neurons of each type. There was overall a significant linear correlation between the magnitude of EPSC inhibition and the change in PPR for both U69593 (Fig. 2*D*; principal, $n = 12$; secondary, $n = 9$; tertiary, $n = 8$; $r^2 = 0.67$, $P < 0.05$) and DAMGO (Fig. 2*E*; principal, $n = 11$; secondary, $n = 8$; tertiary, $n = 8$; $r^2 = 0.22$, $P < 0.05$), but these relationships did not hold for individual neuron types.

The relative insensitivity of PPR in VTA neurons to U69593 and DAMGO leaves open the possibility that the observed KOP and MOP effects on EPSC amplitude may not be due to presynaptic mechanisms. Therefore, as an alternative method to test for a presynaptic site of action of the observed effects, we monitored spontaneous EPSCs (sEPSCs) simultaneously

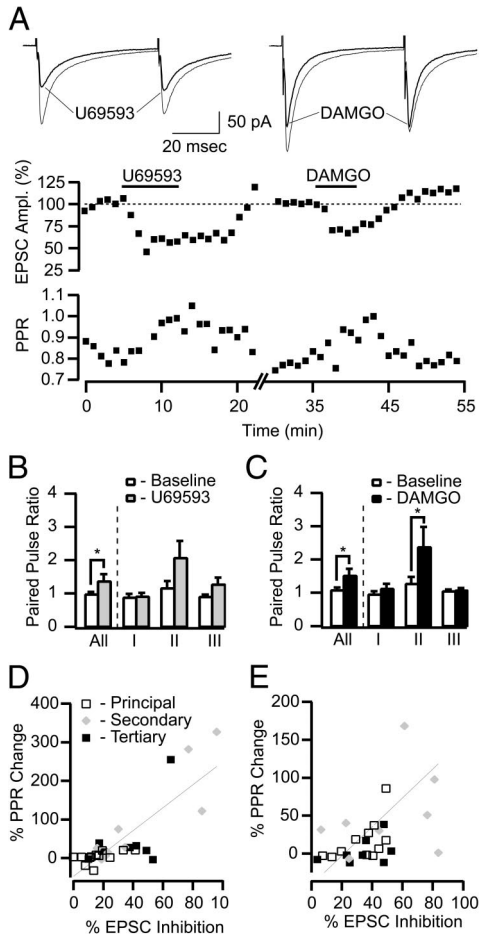
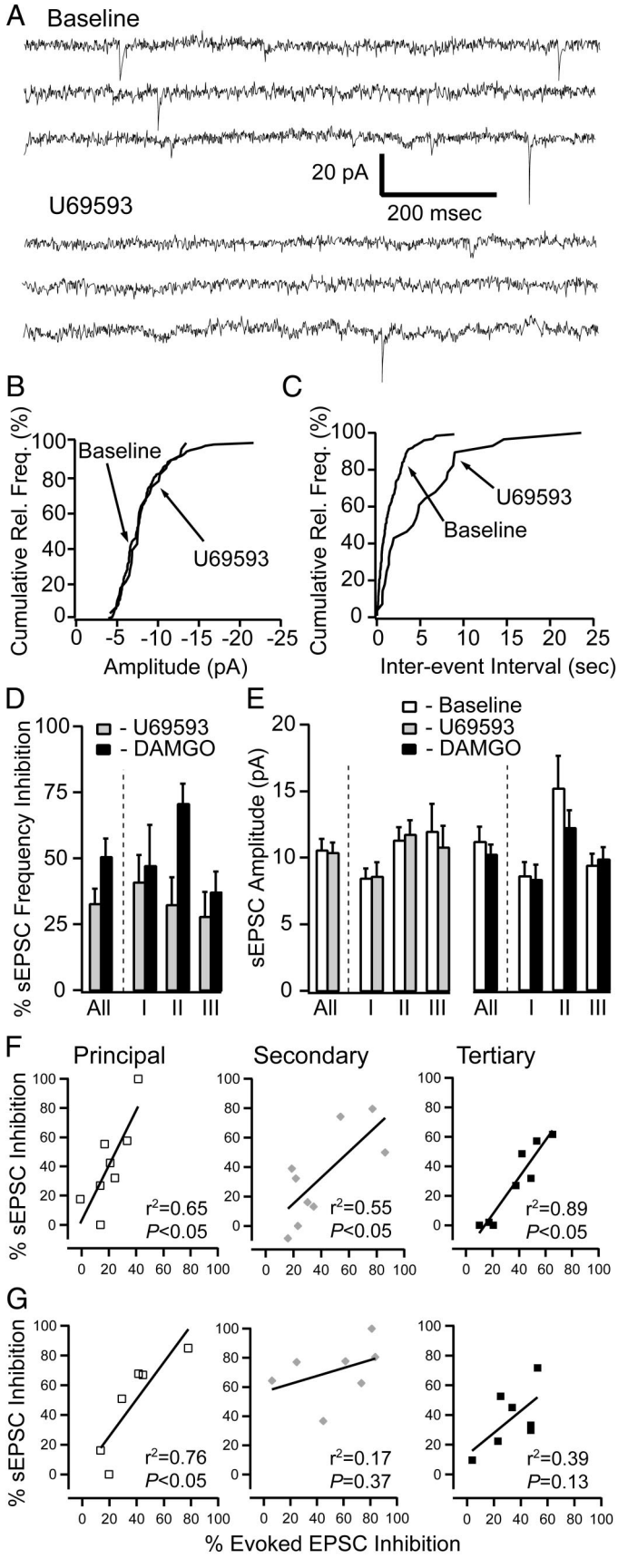


FIG. 2. Paired-pulse ratio (PPR) in VTA neurons increases due to KOP and MOP receptor agonists. *A*: significant paired-pulse facilitation (PPF) in response to both U69593 (1 μM) and DAMGO (3 μM). Recording traces show example baseline (light) and drug (dark) evoked EPSCs. Time courses show both inhibition of evoked EPSC amplitude and facilitation of PPR during drug applications. *B*: there is a significant PPF in pooled VTA neurons in the presence of U69593; however, no cell type alone exhibits a significant change in PPR (principal, $n = 12$; secondary, $n = 9$; tertiary, $n = 8$; ANOVA: $P < 0.05$, SNK: $P > 0.05$ principal, secondary, and tertiary). *C*: in addition to the overall PPF observed in the pooled VTA neurons during DAMGO application, there was also a significant increase among secondary neurons (principal, $n = 11$; secondary, $n = 8$; tertiary, $n = 8$; ANOVA: $P < 0.05$, SNK: $P < 0.05$ secondary, $P > 0.05$ principal and tertiary). *D*: there is a significant linear correlation ($P < 0.05$) between the magnitude of EPSC inhibition by U69593 and the change in the PPR caused by the drug in pooled VTA neurons. *E*: similarly, there is a significant linear correlation in pooled VTA neurons ($P < 0.05$) between DAMGO-induced EPSC inhibition and PPF. *$P < 0.05$.

with the evoked responses to test for correlations between changes in spontaneous and evoked EPSCs. Baseline sEPSC frequencies were variable across neurons, but there were no differences in baseline frequencies between cell types (principal: 1.3 ± 0.5 Hz, $n = 8$; secondary: 3.9 ± 1.4 Hz, $n = 9$; tertiary: 2.5 ± 0.5 Hz, $n = 8$, ANOVA: $P > 0.05$). U69593 was found to inhibit sEPSCs, but did not change their amplitude (Fig. 3). Pooled data show that the frequency of sEPSCs significantly decreased from baseline across all cell types in response to both U69593 (ANOVA: $P < 0.005$) and DAMGO ($P < 0.005$, Fig. 3*D*). These inhibitions were not accompanied by changes in sEPSC amplitude for either drug (ANOVA: U69593 $P > 0.05$, DAMGO $P > 0.05$, Fig. 3*E*). The presence of a decrease in sEPSC frequency and a lack of an effect on



sEPSC amplitude are consistent with an opioid-induced decrease in glutamate release probability. Furthermore, changes in sEPSC frequency were correlated with U69593-induced decreases in evoked EPSC amplitudes in each cell type (Fig. 3*F*). Although only principal neurons showed a significant correlation between evoked EPSC inhibition and sEPSC frequency in the presence of DAMGO, a trend toward a relationship is evident in secondary and tertiary neurons (Fig. 3*G*).

To ensure that the observed effects on sEPSC frequencies were not due to postsynaptic inhibitions of KOP- or MOP-sensitive spontaneously active glutamatergic neurons in the slice that synapse onto the neurons recorded in the preceding experiments, in separate experiments, we measured changes in miniature EPSCs (mEPSCs) in the presence of TTX (500 nM). The application of TTX did not change the frequency of observed spontaneous events in any cell type (principal: 1.8 ± 0.6 Hz baseline, 1.6 ± 0.6 Hz TTX, $n = 7$; secondary: 1.6 ± 0.4 Hz baseline, 1.5 ± 0.5 Hz TTX, $n = 10$; tertiary: 1.7 ± 0.3 Hz baseline, 1.4 ± 0.3 Hz TTX, $n = 8$, ANOVA: $P > 0.05$) or amplitude (principal: 14 ± 5 pA baseline, 13 ± 5 pA TTX, $n = 7$; secondary: 13 ± 1 pA baseline, 14 ± 1 pA TTX, $n = 10$; tertiary: 14 ± 2 pA baseline, 12.8 ± 0.6 pA TTX, $n = 8$, ANOVA: $P > 0.05$). The lack of an effect of TTX on either the frequency or the amplitude of spontaneous excitatory events in these neurons indicates that the previously observed sEPSCs are not action potential dependent.

In the presence of TTX, there was a significant inhibition of mEPSC frequency by U69593 (1 μM) among pooled VTA neurons ($n = 10$, ANOVA: $P < 0.001$), with the largest effect occurring in tertiary neurons (Fig. 4*A*). There was no change in mEPSC amplitude observed during U69593 application ($n = 10$, ANOVA: $P > 0.05$, Fig. 4*B*). In separate experiments, DAMGO (3 μM) also inhibited the frequency of mEPSCs in pooled VTA neurons ($n = 15$, ANOVA: $P < 0.005$, Fig. 4*A*). While as a group VTA neurons did show a significant decrease in mEPSC amplitude in the presence of DAMGO ($n = 15$, ANOVA: $P < 0.05$), no individual cell type showed a significant change (Fig. 4*B*). That the frequency of mEPSCs decreases with both U69593 and DAMGO further supports a presynaptic mechanism for the observed effects.

A subset of I_h-expressing neurons are postsynaptically inhibited by KOP-R agonists (Margolis et al. 2003). To confirm that this postsynaptic effect was not contributing to our evoked presynaptic observations, we compared the evoked presynaptic effects of U69593 in I_h expressing (principal and tertiary) neurons in which the KOP-R agonist induced a positive change in the holding current ($n = 6$) to those recorded in neurons that

FIG. 3. Frequency, but not amplitude, of sEPSCs is diminished by the KOP-R agonist U69593. *A*: sample traces of spontaneous activity recordings in a secondary neuron during baseline and U69593 application (1 μM). *B*: in the same neuron, the cumulative plot shows no difference between sEPSC amplitudes during baseline and in the presence of U69593. *C*: U69593 application shifts the cumulative plot of the inter-event interval to the right of baseline. *D*: sEPSC frequency was significantly decreased by both the KOP-R agonist U69593 (1 μM; principal, $n = 8$; secondary, $n = 9$; tertiary, $n = 8$) and the MOP-R agonist DAMGO (3 μM; principal, $n = 6$; secondary, $n = 7$; tertiary, $n = 7$). *E*: sEPSC amplitude was not affected by U69593 (1 μM) or DAMGO (3 μM). *F*: there is a significant correlation between the effect of U69593 on the amplitude of evoked EPSCs and the inhibition of sEPSC frequency in principal, secondary, and tertiary neurons. *G*: while there is a trend toward a relationship between the effect of DAMGO on the amplitude of evoked EPSCs and the inhibition of sEPSC frequency in secondary and tertiary neurons, only principal neurons show a significant relationship between these measures.

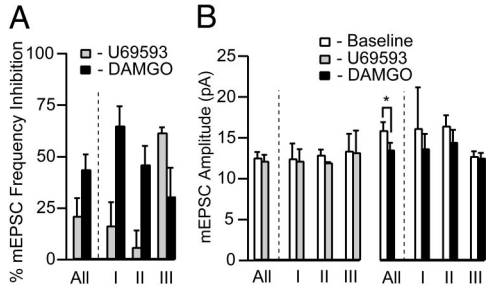


FIG. 4. Opioids decrease the frequency of miniature excitatory events. *A*: mEPSC frequency among pooled VTA neurons is significantly diminished by U69593 (1 μM, ANOVA: $P < 0.05$). Furthermore, tertiary neurons ($n = 2$), but not principal ($n = 4$) or secondary ($n = 4$) neurons, show a decrease in mEPSC frequency during U69593 application [Student-Newman-Keuls (SNK): $P < 0.05$ tertiary, $P > 0.05$ principal and secondary]. The MOP-R agonist DAMGO (3 μM) significantly decreased the frequency of mEPSCs in pooled (ANOVA: $P < 0.05$), principal ($n = 3$) and secondary ($n = 6$), but not tertiary ($n = 6$), neurons (SNK: $P < 0.05$ principal and secondary, $P > 0.05$ tertiary). *B*: U69593 did not significantly affect mEPSC amplitude in pooled data or any cell type. DAMGO significantly decreased mEPSC amplitude in the pooled data, but not in any individual neuron type. *$P < 0.05$.

exhibited no change in holding current with U69593 ($n = 13$, Fig. 5*A*). There was no difference between the KOP-R agonist-mediated presynaptic inhibitions of EPSCs for these two groups (ANOVA: $P > 0.05$). Interestingly, there was a significant difference in the MOP-R agonist DAMGO inhibition of evoked EPSCs in these two groups: I_h neurons that were postsynaptically hyperpolarized by the KOP-R agonist showed a significantly greater EPSC inhibition during a separate application of DAMGO (ANOVA: $P < 0.01$). Conversely, I_h neurons that were postsynaptically inhibited by the MOP-R agonist DAMGO (i.e., tertiary neurons, $n = 9$) showed a greater EPSC inhibition by U69593 than those that were not (i.e., principal neurons, $n = 12$, ANOVA: $P < 0.05$, Fig. 5*B*). There was no difference in the presynaptic DAMGO effect for neurons postsynaptically inhibited by MOP-R agonists compared with those that were not (Fig. 5*B*).

For both MOP and KOP receptor agonists, the largest presynaptic EPSC inhibitions were observed in secondary neurons, and only secondary cells showed a significant correlation between KOP and MOP receptor agonist EPSC inhibitions (Fig. 6). It is interesting to note that the EPSCs in a subset of secondary neurons (3/9) were inhibited >75% by both U69593 and DAMGO, a much larger effect than that observed in any principal or tertiary neuron. In the example traces in Fig. 1, the evoked EPSC signal appeared completely blocked, and this was typical of all three secondary neurons with these large evoked EPSC inhibitions. This effect would require that KOP and MOP receptors co-localize on glutamatergic terminals synapsing on to at least a subpopulation of secondary neurons.

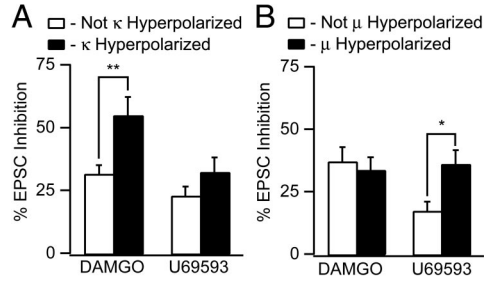


FIG. 5. Presynaptic KOP and MOP actions in I_h neurons partially segregate according to postsynaptic KOP and MOP actions. *A*: compared with I_h neurons not postsynaptically inhibited by KOP-R agonist U69593 (1 μM, $n = 13$), those that are exhibit a greater EPSC inhibition by DAMGO (3 μM, $n = 6$). There is no difference in EPSC inhibition by U69593 (1 μM) between neurons postsynaptically hyperpolarized by U69593 and those with no postsynaptic sensitivity to U69593. *B*: neurons that were postsynaptically hyperpolarized by the MOP-R agonist DAMGO (3 μM, tertiary neurons, $n = 9$) showed a greater EPSC inhibition by U69593 than those that were not (principal neurons, $n = 12$). There was no difference in EPSC inhibition by DAMGO between those neurons that were postsynaptically inhibited by DAMGO and those that were not. *$P < 0.05$, **$P < 0.01$.

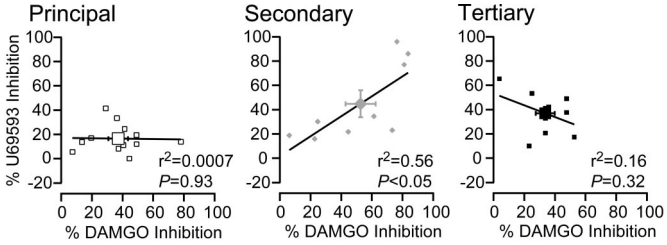


FIG. 6. EPSC inhibition by KOP and MOP in secondary neurons is correlated. There is no apparent relationship between the magnitude of EPSC inhibition by U69593 (1 μM) and DAMGO (3 μM) in principal ($n = 12$) or tertiary ($n = 8$) neurons. There is a significant linear correlation between EPSC inhibition by U69593 and DAMGO in secondary neurons ($n = 9$, $P < 0.05$). The greatest inhibitions by both U69593 and DAMGO were also observed in secondary neurons.

To address the question of whether MOP-Rs and KOP-Rs are segregated or are co-localized on the same glutamate terminals synapsing onto I_h-expressing neurons, we carried out occlusion experiments in principal and tertiary neurons. After a stable baseline was obtained, DAMGO was added to the bath, and the EPSC inhibition was allowed to stabilize. The addition of U69593 resulted in an additional inhibition of EPSC amplitude in all I_h neurons ($n = 8$, Fig. 7*A*). Furthermore, while there was no difference between the magnitude of the U69593 effect in the presence (8 ± 3%, $n = 4$) or absence of DAMGO among principal neurons, the U69593-mediated EPSC inhibition in tertiary neurons was significantly smaller in the presence of DAMGO (10 ± 3%, $n = 4$) than in ACSF alone (ANOVA: $P < 0.05$, Fig. 7*B*). These data are consistent with the hypothesis that MOP-Rs and KOP-Rs segregate to separate glutamatergic inputs to principal neurons, but require that at least some MOP-Rs and KOP-Rs are colocalized on glutamatergic terminals that synapse onto tertiary neurons.

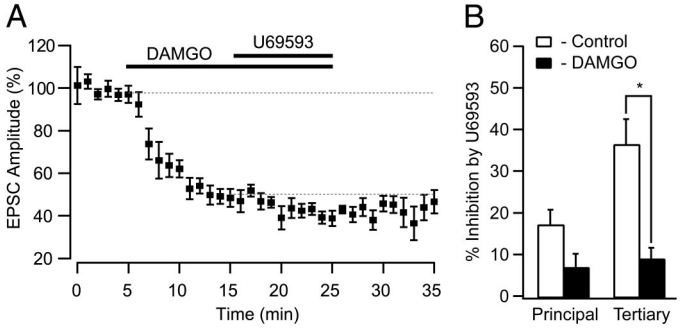


FIG. 7. MOP inhibition of glutamate release does not occlude the KOP-R mediated EPSC inhibition. *A*: I_h neurons exhibit further EPSC inhibition when U69593 (1 μM) is applied in the presence of DAMGO (3 μM, $n = 8$). *B*: among principal neurons, the magnitude of the additional EPSC inhibition due to U69593 in the presence ($n = 4$) or absence ($n = 11$) of DAMGO was not different. EPSC inhibition by U69593 in tertiary neurons was significantly smaller in the presence of DAMGO ($n = 4$) than in control artificial cerebrospinal fluid (ACSF; $n = 9$). *$P < 0.05$.

DISCUSSION

Our results show that glutamate terminals onto each class of VTA neuron are inhibited by both KOP and MOP receptor agonists. KOP EPSC inhibition is larger in secondary and tertiary cells than it is in principal neurons. Among neurons with an I_h (principal and tertiary), those that are postsynaptically hyperpolarized by KOP-R agonists show greater EPSC inhibition by MOP-R agonists. In those VTA neurons without an I_h (secondary), there is a significant correlation between the magnitudes of the KOP- and MOP-induced EPSC inhibitions.

Our results confirm and extend previous findings on opioid modulation of glutamate release in the VTA. The results presented here are in agreement with earlier observations of presynaptic inhibition of glutamate release by MOP-R agonists in I_h and non–I_h-expressing VTA neurons (Bonci and Malenka 1999; Manzoni and Williams 1999). However, the presynaptic KOP effect showed here was not observed in a previous investigation of glutamate release onto I_h-expressing neurons in the VTA (Manzoni and Williams 1999). Given the large variability reported for the seven neurons tested with U69593 in that study, an EPSC inhibition is likely to have occurred in a subset of those neurons. The variability of KOP inhibition across all I_h neurons reported here may have led to an average effect that was not significantly different from zero in their study when principal and tertiary neurons were not distinguished. The small but significant U69593 effect we observed in principal neurons was not only confirmed by sEPSC and mEPSC measurements, but was reversed by nor-BNI, confirming its KOP-R selectivity.

We observed a previously unreported and significant relationship between the pre- and postsynaptic effects of KOP and MOP receptor agonists in I_h neurons. The EPSC inhibition by KOP-R agonists was larger among neurons postsynaptically hyperpolarized by MOP-R agonists (i.e., tertiary neurons), and MOP-mediated EPSC inhibition was larger in neurons postsynaptically inhibited by KOP-R agonists. Interestingly, while about 70% of I_h-expressing neurons in the VTA are dopaminergic, all neurons hyperpolarized by KOP are dopaminergic (Margolis et al. 2003). Although there are also DA neurons that are not inhibited by KOP-R agonists, the data reported here suggest that glutamatergic inputs to DA neurons are more sensitive to MOP-R agonists than those onto non-DA, I_h-expressing neurons. This conclusion will need to be confirmed in future experiments.

The inhibition of excitatory input to DA neurons by MOP-R agonists seems counter to the observation that MOP-R agonists in the VTA excite DA neurons. However, it is possible that the function of MOP-R activation in the VTA is to increase firing rate without producing bursting in DA neurons. Glutamate input to DA neurons tends to shift the tonic, spontaneous activity of DA neurons to a bursting pattern, often without changing the overall firing rate of the neuron (Chergui et al. 1993; Connelly and Shepard 1997; Floresco et al. 2003; Johnson et al. 1992; Overton and Clark 1992). The combination of indirect disinhibition of DA neurons by MOP-R agonists (through the inhibition of local GABAergic neurons) with presynaptic EPSC inhibition could produce greater neuron activity without shifting the firing pattern to bursting. Such a change could increase a temporally broad DA signal, which is likely to carry very different information from the pulsed release associated with bursting of VTA DA neurons (Phillips et al. 2003).

Presynaptic inhibition of glutamate release in the VTA provides an important mechanism by which inputs to the VTA can be differentially controlled by KOP and MOP receptor agonists. The occlusion experiments reported here provide evidence that MOP-Rs and KOP-Rs differentially regulate glutamatergic inputs onto both principal and tertiary neurons. The lack of a difference among principal neurons in EPSC inhibition by U69593 applied in the presence of DAMGO compared with that observed in ACSF is consistent with KOP-Rs and MOP-Rs being segregated to separate terminals. However, secondary and tertiary neurons must have at least partial overlap of receptor expression on individual glutamatergic terminals. In tertiary neurons, this conclusion is supported by the finding that the KOP-R agonist mediated EPSC inhibition was diminished in the presence of the MOP-R agonist. In many secondary cells, U69593 and DAMGO each inhibited EPSC amplitude by >50%. Together with the correlation between U69593 and DAMGO EPSC inhibitions in these neurons, these data support the hypothesis that KOP-Rs and MOP-Rs are on the same glutamatergic terminals synapsing onto secondary neurons. Interestingly, glutamate excitation of secondary neurons in the VTA increases firing rates without causing bursting (Steffensen et al. 1998). Therefore not only does glutamate appear to have a very different postsynaptic function in secondary neurons compared with that in principal and tertiary cells, but the opioid regulation of these inputs seems to be fundamentally different.

Unlike the combinations of postsynaptic KOP-Rs and MOP-Rs that are differentially expressed in different VTA cell classes, presynaptic KOP and MOP receptor activation does inhibit glutamate release onto all VTA neurons. This provides a broad functional range of opioid modulation of VTA neuronal activity. Similar presynaptic KOP inhibition of glutamate release onto cell types having different postsynaptic opioid responses has also been observed in the nucleus raphe magnus (Bie and Pan 2003). Functional roles for these multiple opioid receptor sites in the VTA may be related to the synaptic location and timing of release of endogenous KOP and MOP receptor ligands. Projections to the VTA of neurons containing enkephalin, a MOP and δ opioid receptor agonist peptide, arise from the ventral pallidum, and those immunoreactive for endomorphin, a MOP-R selective agonist peptide, arise from the hypothalamus (Greenwell et al. 2002; Kalivas et al. 1993). It is possible then that endogenous ligands acting at the MOP-R in the VTA could be released by different events and at different times from those that lead to the release of the endogenous KOP-R selective ligand dynorphin, which is released from terminals of neurons located in the nucleus accumbens, lateral hypothalamus, and amygdala (Chou et al. 2001; Fallon et al. 1985).

Postsynaptic inhibitions by both KOP-R and MOP-R agonists in the VTA have previously been examined (Cameron et al. 1997; Johnson and North 1992; Margolis et al. 2003). Postsynaptically, only subsets of dopaminergic principal and tertiary neurons are inhibited by KOP-R agonists, while, by definition, all secondary and tertiary cells are directly inhibited by MOP-R agonists. Thus concurrent KOP- or MOP-induced presynaptic inhibition of glutamate release and postsynaptic hyperpolarization would be synergistic in secondary and ter-

tiary neurons. It is also important to point out that principal and tertiary neurons fire in the absence of synaptic input. Therefore opioids can modify the output of these VTA neurons in the absence of an excitatory input, and this modulation may have very different consequences from inhibiting excitatory inputs to the same neuron. There may also be an anatomical difference between the different actions of opioids on these signals. Endogenous opioids may have a limited radius of effect when released and therefore depending on their precise location, pre- and postsynaptic KOP-Rs and MOP-Rs could be activated independently in vivo. Such mechanisms could account for the seeming contradiction that MOP-R agonists both inhibit excitatory input and indirectly disinhibit principal neurons.

The sources of the differences in the responses to opioids reported here and their possible functional implications are unclear. Since in most cases, especially in the principal neurons, inhibition of glutamatergic inputs by KOP-R ligands was only partial, it is likely that not all glutamatergic terminals bear the KOP-R. Thus it is tempting to hypothesize that variation in EPSC modulation by opioids across cell types depends on the source of glutamatergic afferents. For instance, Carr and Sesack (2000) showed that glutamate afferents from the mPFC to the VTA synapse selectively on DA neurons that project back to the mPFC and GABAergic neurons that project to the NAc, but not DA neurons that project to the NAc or GABA neurons that project to the mPFC. Therefore some combination of PPN, STN, and hypothalamus afferents to the VTA likely provides major glutamate input to the nondopaminergic neurons that comprise 60% of the VTA projection to the mPFC, the dopaminergic neurons that comprise 80% of the VTA projection to the NAc, and possibly the VTA neurons that project to other targets such as the amygdala and hippocampus (Swanson 1982). However, since in other brain regions a single axon can give rise to multiple excitatory synapses with significantly different properties (Maccaferri et al. 1998; Markram et al. 1998; Scanziani et al. 1998), we cannot conclude that the observed differences are due to differential anatomical origins of the glutamatergic afferents. Further work needs to be done to discern if there is indeed an anatomical correlate to the effects observed here.

In conclusion, we show that KOP and MOP receptor agonists inhibit glutamatergic input onto all neuron types in the VTA. Presynaptic regulation of synaptic transmission by opioids in the VTA provides a mechanism for selective control of specific inputs to VTA neurons. How these presynaptic effects interact with the postsynaptic inhibitions through MOP and KOP receptor activation in the VTA not only depends on whether the glutamatergic afferents are active when opioid ligands are present, but may also depend on the site of origin of the neuron giving rise to the glutamatergic terminal or the projection target of the postsynaptic neuron. The modulation of glutamate release by KOP and MOP receptor agonists reported here provides important information for understanding signal modulation in the VTA and is an important key to elucidating the influence on motivation and reward of endogenous opioids acting in the VTA.

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REFERENCES

Arvidsson U, Riedl M, Chakrabarti S, Vulchanova L, Lee JH, Nakano AH, Lin X, Loh HH, Law PY, Wessendorf MW, and Elde R. The kappa-opioid receptor is primarily postsynaptic: combined immunohistochemical localization of the receptor and endogenous opioids. *Proc Natl Acad Sci USA* 92: 5062–5066, 1995.

Bals-Kubik R, Ableitner A, Herz A, and Shippenberg TS. Neuroanatomical sites mediating the motivational effects of opioids as mapped by the conditioned place preference paradigm in rats. *J Pharmacol Exp Ther* 264: 489–495, 1993.

Bie B and Pan ZZ. Presynaptic mechanism for anti-analgesic and anti-hyperalgesic actions of kappa-opioid receptors. *J Neurosci* 23: 7262–7268, 2003.

Bonci A and Malenka RC. Properties and plasticity of excitatory synapses on dopaminergic and GABAergic cells in the ventral tegmental area. *J Neurosci* 19: 3723–3730, 1999.

Cameron DL, Wessendorf MW, and Williams JT. A subset of ventral tegmental area neurons is inhibited by dopamine, 5-hydroxytryptamine and opioids. *Neuroscience* 77: 155–166, 1997.

Carr DB and Sesack SR. Projections from the rat prefrontal cortex to the ventral tegmental area: target specificity in the synaptic associations with mesoaccumbens and mesocortical neurons. *J Neurosci* 20: 3864–3873, 2000.

Charara A, Smith Y, and Parent A. Glutamatergic inputs from the pedunculopontine nucleus to midbrain dopaminergic neurons in primates: Phaseolus vulgaris-leucoagglutinin anterograde labeling combined with postembedding glutamate and GABA immunohistochemistry. *J Comp Neurol* 364: 254–266, 1996.

Chergui K, Charlety PJ, Akaoka H, Saunier CF, Brunet JL, Buda M, Svensson TH, and Chouvet G. Tonic activation of NMDA receptors causes spontaneous burst discharge of rat midbrain dopamine neurons in vivo. *Eur J Neurosci* 5: 137–144, 1993.

Chou TC, Lee CE, Lu J, Elmquist JK, Hara J, Willie JT, Beuckmann CT, Chemelli RM, Sakurai T, Yanagisawa M, Saper CB, and Scammell TE. Orexin (hypocretin) neurons contain dynorphin. *J Neurosci* 21: RC168, 2001.

Christie MJ, Bridge S, James LB, and Beart PM. Excitotoxin lesions suggest an aspartatergic projection from rat medial prefrontal cortex to ventral tegmental area. *Brain Res* 333: 169–172, 1985.

Connelly ST and Shepard PD. Competitive NMDA receptor antagonists differentially affect dopamine cell firing pattern. *Synapse* 25: 234–242, 1997.

Cornish JL, Nakamura M, and Kalivas PW. Dopamine-independent locomotion following blockade of N-methyl-D-aspartate receptors in the ventral tegmental area. *J Pharmacol Exp Ther* 298: 226–233, 2001.

Fallon JH, Leslie FM, and Cone RI. Dynorphin-containing pathways in the substantia nigra and ventral tegmentum: a double labeling study using combined immunofluorescence and retrograde tracing. *Neuropeptides* 5: 457–460, 1985.

Floresco SB, West AR, Ash B, Moore H, and Grace AA. Afferent modulation of dopamine neuron firing differentially regulates tonic and phasic dopamine transmission. *Nat Neurosci* 6: 968–973, 2003.

Garzon M and Pickel VM. Plasmalemmal μ-opioid receptor distribution mainly in nondopaminergic neurons in the rat ventral tegmental area. *Synapse* 41: 311–328, 2001.

Greenwell TN, Zangen A, Martin-Schild S, Wise RA, and Zadina JE. Endomorphin-1 and -2 immunoreactive cells in the hypothalamus are labeled by fluoro-gold injections to the ventral tegmental area. *J Comp Neurol* 454: 320–328, 2002.

Groenewegen HJ and Berendse HW. Connections of the subthalamic nucleus with ventral striatopallidal parts of the basal ganglia in the rat. *J Comp Neurol* 294: 607–622, 1990.

Harris GC and Aston-Jones G. Critical role for ventral tegmental glutamate in preference for a cocaine-conditioned environment. *Neuropsychopharmacology* 28: 73–76, 2003.

Johnson SW and North RA. Opioids excite dopamine neurons by hyperpolarization of local interneurons. *J Neurosci* 12: 483–488, 1992.

Johnson SW, Seutin V, and North RA. Burst firing in dopamine neurons induced by N-methyl-D-aspartate: role of electrogenic sodium pump. *Science* 258: 665–667, 1992.

Kalivas PW, Churchill L, and Klitenick MA. GABA and enkephalin projection from the nucleus accumbens and ventral pallidum to the ventral tegmental area. *Neuroscience* 57: 1047–1060, 1993.

Karreman M, Westerink BH, and Moghaddam B. Excitatory amino acid receptors in the ventral tegmental area regulate dopamine release in the ventral striatum. *J Neurochem* 67: 601–607, 1996.

Maccaferri G, Toth K, and McBain CJ. Target-specific expression of presynaptic mossy fiber plasticity. *Science* 279: 1368–1370, 1998.

Manabe T, Wyllie DJ, Perkel DJ, and Nicoll RA. Modulation of synaptic transmission and long-term potentiation: effects on paired pulse facilitation and EPSC variance in the CA1 region of the hippocampus. *J Neurophysiol* 70: 1451–1459, 1993.

Mansour A, Burke S, Pavlic RJ, Akil H, and Watson SJ. Immunohistochemical localization of the cloned kappa 1 receptor in the rat CNS and pituitary. *Neuroscience* 71: 671–690, 1996.

Manzoni OJ and Williams JT. Presynaptic regulation of glutamate release in the ventral tegmental area during morphine withdrawal. *J Neurosci* 19: 6629–6636, 1999.

Margolis EB, Hjelmstad GO, Bonci A, and Fields HL. Kappa-opioid agonists directly inhibit midbrain dopaminergic neurons. *J Neurosci* 23: 9981–9986, 2003.

Markram H, Wang Y, and Tsodyks M. Differential signaling via the same axon of neocortical pyramidal neurons. *Proc Natl Acad Sci USA* 95: 5323–5328, 1998.

Nader K and van der Kooy D. Deprivation state switches the neurobiological substrates mediating opiate reward in the ventral tegmental area. *J Neurosci* 17: 383–390, 1997.

Overton P and Clark D. Iontophoretically administered drugs acting at the N-methyl-D-aspartate receptor modulate burst firing in A9 dopamine neurons in the rat. *Synapse* 10: 131–140, 1992.

Overton PG and Clark D. Burst firing in midbrain dopaminergic neurons. *Brain Res Brain Res Rev* 25: 312–334, 1997.

Phillips AG and LePiane FG. Reinforcing effects of morphine microinjection into the ventral tegmental area. *Pharmacol Biochem Behav* 12: 965–968, 1980.

Phillips PE, Stuber GD, Heien ML, Wightman RM, and Carelli RM. Subsecond dopamine release promotes cocaine seeking. *Nature* 422: 614–618, 2003.

Rosin DL, Weston MC, Sevigny CP, Stornetta RL, and Guyenet PG. Hypothalamic orexin (hypocretin) neurons express vesicular glutamate transporters VGLUT1 or VGLUT2. *J Comp Neurol* 465: 593–603, 2003.

Scanziani M, Gahwiler BH, and Charpak S. Target cell-specific modulation of transmitter release at terminals from a single axon. *Proc Natl Acad Sci USA* 95: 12004–12009, 1998.

Schilstrom B, Nomikos GG, Nisell M, Hertel P, and Svensson TH. N-methyl-D-aspartate receptor antagonism in the ventral tegmental area diminishes the systemic nicotine-induced dopamine release in the nucleus accumbens. *Neuroscience* 82: 781–789, 1998.

Sesack SR and Pickel VM. Prefrontal cortical efferents in the rat synapse on unlabeled neuronal targets of catecholamine terminals in the nucleus accumbens septi and on dopamine neurons in the ventral tegmental area. *J Comp Neurol* 320: 145–160, 1992.

Speciale SG, Manaye KF, Sadeq M, and German DC. Opioid receptors in midbrain dopaminergic regions of the rat. II. Kappa and delta receptor autoradiography. *J Neural Transm Gen Sect* 91: 53–66, 1993.

Steffensen SC, Svingos AL, Pickel VM, and Henriksen SJ. Electrophysiological characterization of GABAergic neurons in the ventral tegmental area. *J Neurosci* 18: 8003–8015, 1998.

Svingos AL, Garzon M, Colago EE, and Pickel VM. μ-opioid receptors in the ventral tegmental area are targeted to presynaptically and directly modulate mesocortical projection neurons. *Synapse* 41: 221–229, 2001.

Swanson LW. The projections of the ventral tegmental area and adjacent regions: a combined fluorescent retrograde tracer and immunofluorescence study in the rat. *Brain Res Bull* 9: 321–353, 1982.

Xi ZX and Stein EA. Blockade of ionotropic glutamatergic transmission in the ventral tegmental area reduces heroin reinforcement in rat. *Psychopharmacology (Berl)* 164: 144–150, 2002.

Zheng F and Johnson SW. Group I metabotropic glutamate receptor-mediated enhancement of dopamine cell burst firing in rat ventral tegmental area in vitro. *Brain Res* 948: 171–174, 2002.