Dopaminergic afferents arising from the ventral tegmental area (VTA) are crucial elements in the neural circuits that mediate arousal, motivation, and reinforcement. Two major targets of these afferents are the medial prefrontal cortex (mPFC) and the nucleus accumbens (NAc). Whereas dopamine (DA) in the mPFC has been implicated in working memory and attentional processes, DA in the NAc is required for responding to reward predictive cues. These distinct functions suggest a role for independent firing patterns of dopaminergic neurons projecting to these brain regions. In fact, DA release in mPFC and NAc can be differentially modulated. However, to date, electrophysiological studies have largely overlooked heterogeneity among VTA neurons. Here, we provide direct evidence for differential neurotransmitter control of DA neural activity and corresponding DA release based on projection target. κ opioid receptor agonists inhibit VTA DA neurons that project to the mPFC but not those that project to the NAc. Moreover, DA levels in the mPFC, but not the NAc, are reduced after local infusion of κ opioid receptor agonists into the VTA. These findings demonstrate that DA release in specific brain regions can be independently regulated by opioid targeting of a subpopulation of VTA DA neurons. Selective control of VTA DA neurons projecting to the mPFC has important implications for understanding addiction, attention disorders, and schizophrenia, all of which are associated with DA dysfunction in the mPFC.

GABA | reward | motivation | nucleus accumbens | ventral tegmental area

The dopaminergic neurons of the ventral tegmental area (VTA) play a critical role in motivation and reinforcement (1–3). Two major projection targets of VTA dopamine (DA) neurons are the medial prefrontal cortex (mPFC) and the nucleus accumbens (NAc). DA plays different roles in these two projection targets, contributing to working memory processes in the mPFC (4, 5) and motivated responding in the NAc (6). DA projection neurons contain an alternative neurotransmitter. To determine the neurotransmitter content of these two projections, brain slices containing retrogradely labeled cells were immunohistochemically analyzed by using antibodies for TH and the 67-kDa form of glutamic acid decarboxylase (GAD67) (13), for identification of DA and GABA neurons, respectively. The mPFC and NAc projections were colabeled. However, despite their distinct projections, the distributions of mPFC- and NAc-projecting neurons within the VTA were similar. These data support the hypothesis that largely separate populations of VTA neurons project to the mPFC and the NAc.

To compare the neurotransmitter content of these two projections, brain slices containing retrogradely labeled cells were immunohistochemically analyzed by using antibodies for TH and the 67-kDa form of glutamic acid decarboxylase (GAD67) (13), for identification of DA and GABA neurons, respectively. The mPFC and NAc projections each included TH(+) and GAD67(+) neurons (Fig. 2A). TH(+) neurons comprised 45% ± 10% (n = 6 rats) of the mPFC and 66% ± 10% (n = 3 rats) of NAc projections and were intermixed with TH(−) projections throughout the VTA (Fig. 2B). Similar percentages of GAD67(+) neurons were found among projections to the mPFC (20 ± 6%, n = 7 rats) and NAc (25 ± 5%, n = 4 rats) (Fig. 2B). That GAD67 and TH staining did not account for 100% of the retrogradely labeled neurons raises the possibility that some VTA projection neurons contain an alternative neurotransmitter, such as glutamate (14). However, it is also plausible that this these results to DA release in the projection targets in question, we measured DA levels by microdialysis in the NAc and mPFC of awake, unrestrained rats after local delivery of a KOP-R agonist into the VTA.

Results

DiI injections into the mPFC or NAc (Fig. 1A and B) labeled cell bodies in the VTA and substantia nigra pars compacta (Fig. 1C–F). To compare the density of these projections, retrogradely labeled neurons were counted in six regions of interest per slice (two fields in the lateral and one in the medial VTA, ipsilateral and contralateral to the unilateral injection site; ×250 magnification) in four nonadjacent horizontal 50-μm slices per animal throughout the dorsoventral extent of the VTA. Approximately twice as many retrogradely labeled neurons were observed in the VTA after unilateral injections into the NAc (210 ± 30 neurons per animal, n = 4) compared with unilateral mPFC injections (110 ± 10 neurons per animal, n = 6). Coinjections of DiI into the NAc and FluoroGold into the mPFC of the same animal showed that a relatively small proportion of neurons expressed both markers (14 ± 2 double-labeled neurons per animal, n = 3) (Fig. 1D): 19% of mPFC projections and 15% of NAc projections were colabeled. However, despite these findings, the distributions of mPFC- and NAc-projecting neurons within the VTA were similar. These data from young rats are consistent with previous observations of smaller injection volumes in adult rats (11, 12), and the data support the hypothesis that largely separate populations of VTA neurons project to the mPFC and the NAc.
difference in labeling represents an underestimation of GABAergic projections due to false negative results for GAD67 staining.

Stable whole-cell recordings were made in 50 retrogradely labeled neurons, of which both mPFC-projecting and NAc-projecting were similarly scattered throughout the medial–lateral, anterior–posterior, and dorsal–ventral aspects of the VTA (see Fig. 5, which is published as supporting information on the PNAS web site). Of these recordings, 26 cells were recovered and subsequently processed for TH immunoreactivity. Among recovered mPFC projections, 10 of 17 neurons (59%) were TH(+) (n = 5) (Fig. 3C). These neurons not only lacked an Ih but had other electrophysiological properties consistent with their classification as VTA secondary neurons (Table 1). Although secondary neurons have been proposed to be GABAergic interneurons (19), our data clearly demonstrate that many are projection neurons. Among Ih(−) neurons, those projecting to the mPFC expressed a significantly larger Ih than NAc projections (Fig. 3D). This difference was also present in confirmed TH(+) projections (Fig. 3E). Interestingly, the average action potential duration was the same for Ih(−) neurons and confirmed TH(+) mPFC-projecting neurons and was shorter.
neurons in the VTA are inhibited by KOP-R agonists (10). Consistent with our previous finding that only dopaminergic neurons (seven of seven) were inhibited by U69593 (Fig. 4A), mPFC-projecting neurons (Table 1). Action potential height were similar among mPFC- and NAc-projecting neurons (2 mV of hyperpolarization (n = 2940) than the action potential durations of NAc-projecting neurons (2290 pA and 50 ms) measured in mPFC-projecting (A) and NAc-projecting (B) VTA neurons. Almost half of the mPFC-projecting neurons recorded in this study were dopaminergic neurons (21), and none of the TH(−) mPFC-projecting neurons were pooled here.

Discussion

These data show that KOP-R agonists inhibit VTA DA neurons that project to the mPFC but not those projecting to the NAc. Despite the fact that separate subsets of midbrain DA neurons have distinct projection targets (11, 12, 20), the population as a whole is often considered to be physiologically and pharmacologically homogeneous. In addition to the pharmacological difference between these two subpopulations of DA projection neurons, we also show that the magnitude of one characteristic electrophysiological property of DA neurons, Ih, is correlated with projection target.

In this study, we identified two types of non-DA VTA projection neurons. Almost half of the mPFC-projecting neurons recorded in this study were Ih(−), a characteristic of VTA secondary neurons, which previously were hypothesized to be GABAergic interneurons (19). There were also Ih(+) but TH(−) neurons projecting to both mPFC and NAc. The electrophysiological characteristics of these Ih(+) neurons were overlapping with those of the TH(+) neurons. Furthermore, three of four of the Ih(+) neurons recorded in this study had action potentials with longer durations than the recently suggested 1.1-msec criterion for dopaminergic neurons (21), and these durations overlapped with those of the TH(+) neurons reported here. Therefore, immunohistochemistry was the only consistently reliable method for identifying the subset of Ih(+) neurons that were dopaminergic.

Table 1. Electrophysiological properties of VTA projection neurons

<table>
<thead>
<tr>
<th>Neurons</th>
<th>Ih</th>
<th>TH</th>
<th>Initial membrane potential, mV</th>
<th>Action potential height, mV</th>
<th>Action potential width, ms</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPFC-projecting</td>
<td>−</td>
<td>−</td>
<td>−47 ± 3</td>
<td>97 ± 7</td>
<td>1.1 ± 0.2</td>
<td>11*</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>−42 ± 3</td>
<td>89 ± 5</td>
<td>1.1 ± 0.1</td>
<td>10</td>
</tr>
<tr>
<td>NAc-projecting</td>
<td>+</td>
<td>+</td>
<td>−44 ± 1</td>
<td>80 ± 10</td>
<td>1.5 ± 0.3</td>
<td>7</td>
</tr>
</tbody>
</table>

*The five Ih(−), mPFC-projecting neurons processed for TH were confirmed to be TH(−). Therefore, all 11 Ih(−), mPFC-projecting neurons were pooled here.
contain the KOP-R-selective endogenous peptide dynorphin (DYN) (22, 23). DYN terminals synapse onto DA dendrites in the VTA (24) and are therefore optimally positioned to control DA neuron signaling by a direct postsynaptic action. The fact that DYN levels in the midbrain increase after amphetamine administration (25) suggests that KOP-R-mediated control of midbrain DA neurons projecting to the mPFC can have powerful behavioral effects. Furthermore, administration of drugs of abuse, such as alcohol and cocaine, produce long-lasting down-regulation of KOP-R mRNA levels in the VTA (26). The resulting changes in KOP-R expression may play an important role in the prolonged changes in mPFC DA signaling and behavior that are induced by exposure to these drugs (27–29). The dissociation between mPFC and NAc DA functioning is underscored by models of many cognitive disorders, including schizophrenia (30) and attention deficit hyperactivity disorder (31), characterized by DA dysfunction specifically in the mPFC. DA signaling in the mPFC, but not the NAc, has also been implicated in animal models of cocaine relapse (32). Alterations in DA release in the cortex may also contribute to the psychotomimetic effects of KOP-R activation in humans (33). Thus, mechanisms of differential modulation of dopaminergic afferents to the mPFC offer an important avenue for understanding and treating these common disorders.

Materials and Methods
Anatomy and Electrophysiology. Retrograde tracer injections. All retrograde-labeling experiments conformed to National Institutes of Health and Ernest Gallo Clinic and Research Center animal care policy standards. Male, 27-day-old Sprague–Dawley rats (Harlan Laboratories, San Diego) were anesthetized with isoflurane. A 1-μl Hamilton syringe was stereotactically placed in either the mPFC [anteroposterior (AP), +2.6 mm from bregma; mediolateral (ML), ±0.8 mm from bregma; ventral (V), −4.0 from skull surface] or the NAc (AP, +1.5; ML, ±0.8; V, −6.7). Neuro-Dil (1 μL, 7% in ethanol; Biotium, Hayward, CA) or hydroxystilamidine (FluoroGold 3% in H2O; Biotium) was slowly injected. Bilateral and unilateral injections were performed for electrophysiological and anatomical studies, respectively. Coronal sections containing mPFC or NAc were prepared with a sliding microtome and stained with 0.2% thionin to confirm all injection placements.

Standard methods were used for immunohistochemistry (see Supporting Materials and Methods, which is published as supporting information on the PNAS web site).

Slice preparation and electrophysiology. All recordings were made blind to injection site. Experiments were carried out 6–8 days after injection. Slice preparation, electrophysiological recordings, and data analysis were completed as described in ref. 10. Horizontal brain slices (150 μm thick) were prepared by using a vibratome (Leica Instruments, Solm, Germany) in Ringer solution (119 mM NaCl/2.5 mM KCl/1.3 mM MgSO4/1.0 mM NaH2PO4/2.5 mM CaCl2/26.2 mM NaHCO3/11 mM glucose saturated with 95% O2/5% CO2). Slices were visualized under a Zeiss Axioskop with differential interference contrast optics and infrared and epifluorescent illumination to visualize projecting neurons during recording.

Whole-cell recordings were made at 33°C with 2.5-4 MΩ pipettes containing 123 mM K-glucconate, 10 mM Hepes, 0.2 mM EGTA, 8 mM NaCl, 2 mM MgATP, 0.3 mM Na3GTP, and 0.1% biocytin (pH 7.2, osmolarity adjusted to 275). Pharmacological
experiments were carried out in current clamp mode (I = 0), measuring instantaneous firing rate and/or membrane potential.

U69593 was applied by bath superfusion. U69593 stock solution (1 mM diluted in 50% EtOH) was diluted in Ringer solution immediately before application. Drugs were obtained from Sigma.

**Data analysis.** I<sub>0</sub> was recorded by voltage clamping cells at −60 mV and stepping to −40, −50, −70, −80, −90, −100, and −120 mV. I<sub>0</sub> magnitude was measured as the difference between the initial capacitative response to the voltage step and the final step current.

Differences between NAc and mPFC projection populations were tested with a one-way ANOVA. The action potential width was taken as the width at half the height of the action potential. The action potential height was measured as the potential difference between the peak of the spike and the immediately following trough. P < 0.05 was required for significance in all analyses.

**Microdialysis. Animals.** Male, 250- to 300-g Sprague–Dawley rats (Charles River Laboratories) were housed in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care, and experiments were reviewed by the National Institute on Drug Abuse Intramural Research Program Institutional Care and Use Committee under National Institute on Drug Abuse (National Institutes of Health) guidelines.

**Surgical procedures.** Standard stereotaxic procedures were used to implant two unilateral microdialysis guide cannulae (CMA/11; CMA/Microdialysis, Acton, MA) in the NAc and VTA or mPFC and VTA (NAc: AP, +1.6 mm from bregma; ML, ±0.9 mm from bregma; V, −6.0 mm from dura surface; mPFC: AP, +3.2 mm from bregma; ML, ±0.6 mm from bregma; V, −2.0 mm from dura surface; VTA: AP, −5.6 mm from bregma; ML, ±0.6 mm from bregma; V, −7.5 mm from dura surface) of animals anesthetized i.p. with 3 ml/kg Equithesin.

**Microdialysis procedures.** Microdialysis experiments, as described in ref. 34, commenced 5 days after surgery. Before measurements, each probe was flushed overnight with 0.3 μl/min artificial cerebrospinal fluid (aCSF) containing 145 mM NaCl, 2.8 mM KCl, 1.2 mM MgCl<sub>2</sub>, 1.2 mM CaCl<sub>2</sub>, 0.25 mM ascorbic acid, and 5.4 mM D-glucose, adjusted to pH 7.2 with HPLC-grade NaOH or H2PO4. During the experiment, fresh aCSF was perfused at 0.6 μl/min. After 60 min of equilibration, 10-min dialysate sample collection commenced. All drugs were administered into the VTA by reverse dialysis. After baseline determination, the VTA aCSF was changed to that containing 5 μM U69593, and, after 30 min of equilibration, three consecutive samples were collected. To confirm receptor selectivity, experiments were conducted in a parallel group of rats that received the selective KOP-R antagonist nor-BNI at 1 μM perfused for 30 min before other drugs (n = 3) via the VTA dialysis probe (35).

**Probe location confirmation.** Upon completion of all experiments, the GABAR<sub>γ</sub> agonist baclofen (50 μM) was added to the VTA artificial cerebrospinal fluid to inactivate neurons and therefore pharmacologically confirm a functional connection between DA neurons targeted by the VTA probe and the projection target probe and to demonstrate that the dialysis method was sensitive to changes in DA concentration due to VTA neuron inhibition. Baclofen significantly reduced basal mPFC DA levels in saline-treated animals and nor-BNI-treated animals (decrease from baseline for saline: 0.52 ± 0.09 nM, n = 7; nor-BNI: 0.9 ± 0.6 nM, n = 3) and in the NAc (decrease from baseline: 0.6 ± 0.2 nM).

For anatomical confirmation, animals were euthanized by Equithesin, and probe placement was assessed on 25-μm serial coronal cryostat sections. Only data obtained from animals with histologically correct placements were used for subsequent analysis.

**DA determination.** DA was determined by HPLC coupled to electrochemical detection as described in ref. 34. The retention time for DA was 2.5 min, and the limit of detection was <0.25 nM.

**Data analysis.** Drug effects were analyzed by repeated-measures ANOVA, with one between-subjects factor (brain region) and two within-subjects factors (drug challenge and time). The data are presented as mean ± SEM. The accepted value of significance was P < 0.05.

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