Chronic morphine induces visible changes in the morphology of mesolimbic dopamine neurons


ABSTRACT The mesolimbic dopamine system, which arises in the ventral tegmental area (VTA), is an important neural substrate for opiate reinforcement and addiction. Chronic exposure to opiates is known to produce biochemical adaptations in this brain region. We now show that these adaptations are associated with structural changes in VTA dopamine neurons. Individual VTA neurons in paraformaldehyde-fixed brain sections from control or morphine-treated rats were injected with the fluorescent dye Lucifer yellow. The identity of the injected cells as dopaminergic or nondopaminergic was determined by immunohistochemical labeling of the sections for tyrosine hydroxylase. Chronic morphine treatment resulted in a mean ~25% reduction in the area and perimeter of VTA dopamine neurons. This reduction in cell size was prevented by concomitant treatment of rats with naltrexone, an opioid receptor antagonist, as well as by intra-VTA infusion of brain-derived neurotrophic factor. In contrast, chronic morphine treatment did not alter the size of nondopaminergic neurons in the VTA, nor did it affect the total number of dopaminergic neurons in this brain region. The results of these studies provide direct evidence for structural alterations in VTA dopamine neurons as a consequence of chronic opiate exposure, which could contribute to changes in mesolimbic dopamine function associated with addiction.

Dopaminergic neurons in the ventral tegmental area (VTA) and their projections to the limbic forebrain are important neural substrates for the acute reinforcing properties of opiates and other drugs of abuse (1–4). It has been proposed that chronic exposure to a drug of abuse elicits long-lasting adaptations within these same brain regions that underlie changes in drug reinforcement mechanisms, as well as drug craving, associated with drug addiction.

Among the most consistent drug-induced adaptations in the VTA is induction of tyrosine hydroxylase (TH; refs. 5–8), the rate-limiting enzyme in the biosynthesis of dopamine. In addition, chronic morphine administration has been shown to decrease levels of the three major neurofilament proteins, NF200, NF160, and NF68 (9), and to increase levels of glial fibrillar acidic protein (10) in this brain region. Each of these biochemical adaptations occurs specifically in the VTA and is not elicited by chronic exposure to other psychotropic drugs that lack reinforcing properties. In addition, similar adaptations in the VTA are observed in rats after chronic heroin self-administration, indicating that they are induced in response to self-regulated drug intake (11).

The lower levels of neurofilament proteins and the reciprocal increase in glial fibrillar acidic protein present in the VTA after chronic opiate administration raises the possibility that chronic exposure to opiates may result in prominent structural changes within this brain region (12, 13). Indirect support for this possibility is provided by the observation that chronic morphine treatment results in a 50% impairment in apomorphine-induced locomotion in the VTA to a major forebrain target, with no change in transport observed in other neural pathways studied (14). Moreover, intra-VTA infusion of brain-derived neurotrophic factor (BDNF) or related neurotrophins, known to support the survival of dopaminergic neurons in cell culture (see Discussion), has been shown recently to prevent the morphine-induced biochemical adaptations in this brain region (15).

The goal of the present study was to assess directly whether the various biochemical adaptations elicited in the VTA by chronic morphine administration are associated with morphological changes in this brain region. We show here that chronic exposure to morphine elicits a selective reduction in the size of VTA dopamine neurons, with no change observed in nondopaminergic neurons in this brain region. Moreover, the reduction in size of VTA dopamine neurons is prevented by intra-VTA infusion of BDNF.

MATERIALS AND METHODS

In Vivo Drug Treatments. Sprague–Dawley rats (initial weight 100–120 g; Camm Research Institute, Wayne, NJ) were used in these studies. Morphine was administered chronically, as described (5), by daily subcutaneous implantation of morphine pellets (containing 75 mg of morphine base; National Institute on Drug Abuse) for 5 days under light halothane anesthesia. Rats were used 24 hr after the last pellet implantation. This treatment paradigm produces morphine tolerance and dependence based on behavioral, electrophysiological, and biochemical criteria (see ref. 5). Control rats received equivalent implantation of placebo pellets.

Naltrexone (Sigma) was administered daily [50 mg/ml i.p. in saline and 50 mg/ml in an emulsion of saline and lipid] immediately before morphine or placebo pellet implantation as described (10). This treatment paradigm has been shown to completely block the development of morphine tolerance and dependence based on behavioral, electrophysiological, and biochemical criteria (see ref. 10).

BDNF was provided by Ronald Lindsay (Regeneron Pharmaceuticals, Tarrytown, NY). It was infused at a dose of 2 µg/day (in 10 mM sodium phosphate, pH 7.4/0.9% NaCl/1% bovine serum albumin) directly into the VTA by osmotic minipumps via midline canulae exactly as described (15). This

Abbreviations: VTA, ventral tegmental area; TH, tyrosine hydroxylase; BDNF, brain-derived neurotrophic factor.

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BDNF dose has been shown to prevent many of the biochemical effects elicited in the VTA by chronic morphine administration (15). Control rats received intra-VTA infusions of vehicle.

Measurements of VTA Neuronal Morphology. The morphology of VTA neurons was assessed by filling individual cells with Lucifer yellow by use of a procedure based on previous studies (16-19). Rats were deeply anesthetized with chloral hydrate (400 mg/kg i.p.) and then perfused transcardially with 50 ml of ice-cold phosphate-buffered saline (PBS), followed by 200 ml of 4% paraformaldehyde in PBS at a rate of 15 ml/min. Brains were removed and 100- to 150-μm thick oblique slices (described below) were obtained through the VTA by use of a vibratome. Neurons in the fixed slices were injected with Lucifer yellow under an upright fluorescence microscope. Individual neurons were visualized with Nomarski optics, impaled with a glass microelectrode filled with 0.2% Lucifer yellow in PBS, and injected by applying negative current pulses (2 Hz, 400 msec, 1 nA) for 5-10 min. After filling 4-8 cells per slice, the slices were fixed in 4% paraformaldehyde in PBS overnight at 4°C.

Oblique brain slices, illustrated in Fig. 1, were used in these studies according to published procedures (21). In these oblique slices, VTA dopamine neurons can be readily distinguished from substantia nigra dopamine neurons as being located medial to the medial terminal nucleus of the accessory optic tract (Fig. 1). The VTA cells injected with Lucifer yellow were located at ±5.3 ± 0.2 mm Bregma.

The dopaminergic phenotype of the Lucifer yellow-injected neurons was determined by TH immunohistochemistry. The fixed slices were washed with PBS and permeabilized by 2-3 cycles of freezing (at −20°C) and thawing. The slices were incubated for 18 hr with a mouse monoclonal anti-TH antibody (1:1500; Incstar, Stillwater, MN) in PBS plus 0.3% Triton X-100 at 4°C. The slices were washed three times in PBS at room temperature, after which time they were incubated for 2.5 hr with Texas Red-conjugated anti-mouse IgG made in horse (1:200; Vector Laboratories) in PBS plus 0.3% Triton X-100 and 10% normal horse serum at room temperature. Slices were washed three times in PBS, mounted on gelatin-coated slides, dried, dehydrated, cleared in methyl salicylate, and coverslipped with DPX (Merck).

Slices were examined under fluorescence microscopy (Nikon) to visualize Lucifer yellow-filled neurons and to determine whether the filled neurons were TH+ or TH−. The filled neurons were recorded with a Xabion charged coupled device-gated video camera. The morphological parameters of the neurons were measured by National Institutes of Health image version 1.57 software; this was done with the experimenter blinded to the identity of the neurons as TH+ or TH− and of the slices as control or morphine-treated. Cell perimeter and area were then calculated.

In pilot experiments, 400-μm thick oblique midbrain slices containing the VTA from control and morphine-treated rats were prepared as described (21). Dopamine neurons, identified by well-established electrophysiological criteria (for example, see refs. 16 and 21), were recorded in whole cell mode with electrodes filled with an intracellular solution containing 1% Lucifer yellow. The electrodes were withdrawn after 15 min in cell-attached mode. One to two neurons were injected per slice. The slices were then fixed in 4% paraformaldehyde, cut into 50- to 100-μm thick sections, and mounted for analysis of cell morphology as described above.

Counting VTA Dopamine Neurons. Control and morphine-treated rats were perfused with paraformaldehyde, as described above. TH immunohistochemistry was then conducted on fixed 40-μm thick coronal brain sections, and the number of TH+ neurons was counted at ×400 magnification, exactly as described (22). Briefly, TH+ neurons were counted in every 40-μm section throughout the rostral-caudal extent of the ventral midbrain. An immunoreactive area was counted as a cell if a region of central pallor, consistent with the absence of staining of a nucleus, could be visualized. The slides were counted in a blind fashion. Regional boundaries of the VTA were determined by use of neuroanatomical landmarks according to published criteria (20, 23). The major landmark defining the lateral boundary of the VTA was the medial lemniscus. Other major landmarks used to establish level and orientation included the interpeduncular nucleus, the red nucleus, and the fasciculus retroflexus. Cross regions of TH immunoreactivity were compared and aligned with previously published studies of the protein in the rat (23, 24).

RESULTS
Regulation of VTA Neuronal Morphology by Chronic Morphine Administration. In the present study, we assessed directly the changes in cellular morphology in the VTA produced by chronic morphine administration. We used an approach based on previous work (see refs. 16-19), which involved filling individual neurons with the fluorescent dye Lucifer yellow in paraformaldehyde-fixed brain slices. The injected cells were then identified as dopaminergic or nondopaminergic by immunohistochemically labeling the slices for TH.

This technique resulted in the reliable labeling of individual neurons and the proximal extents of their processes as visualized under fluorescence microscopy. In control rats (Fig. 24), Lucifer yellow-filled TH+ neurons exhibited considerable variability in their shape, although most cells were elliptical with several large caliber proximal processes, consistent with previous descriptions of the morphology of VTA dopaminergic neurons (see refs. 16, 17, and 25). In contrast, in morphine-treated rats (Fig. 2B), Lucifer yellow-filled TH+ neurons exhibited smaller cell bodies and tended to show a more spherical shape with narrowed proximal processes. Analysis of 41 neurons from seven control and from seven morphine-treated rats revealed a 20-25% reduction in mean cell body area and perimeter elicited by chronic morphine exposure (Table 1). An equivalent (≈25%) reduction in the area and perimeter of VTA dopamine neurons was observed after chronic morphine administration when the neurons were injected with Lucifer yellow via patch-recording electrodes in living brain slices (data not shown).

In addition to a reduction in mean cell body size, there was a dramatic shift in the distribution of cell body sizes between the control and chronic morphine-treated conditions. This is
illustrated in Fig. 3A, which shows that the perimeter of all but one of the VTA dopamine neurons of morphine-treated rats was below the median value exhibited by the neurons from control rats. A similar shift in distribution was observed for cell body area (data not shown).

Chronic morphine exposure also was found to result in a significant (~30%) reduction in the mean length of dopaminergic neuronal processes. The average length of Lucifer yellow-filled processes in TH+ neurons from control rats was 80 ± 10 μm (n = 25) compared with 56 ± 6 μm (n = 20) in TH+ neurons from morphine-treated rats (P < 0.03 by Student's t test).

The reduction in cell body area and perimeter elicited by chronic morphine administration was blocked by concomitant treatment of rats with the opioid receptor antagonist naltrexone, which by itself had no effect on the size of the neurons.

Table 1. Effect of drug treatments on the size of VTA dopamine neurons

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Perimeter, μm</th>
<th>Area, μm²</th>
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<tbody>
<tr>
<td>Control</td>
<td>27.5 ± 0.6 (41)</td>
<td>43.5 ± 1.7 (41)</td>
</tr>
<tr>
<td>Morphine</td>
<td>22.1 ± 0.5 (41)†</td>
<td>33.2 ± 1.4 (41)†</td>
</tr>
<tr>
<td>Naltrexone</td>
<td>26.8 ± 2.8 (9)</td>
<td>40.2 ± 8.0 (9)</td>
</tr>
<tr>
<td>Morphine plus naltrexone</td>
<td>26.4 ± 1.5 (15)</td>
<td>41.6 ± 4.6 (15)</td>
</tr>
<tr>
<td>Vehicle‡</td>
<td>26.2 ± 2.1 (9)</td>
<td>43.3 ± 2.9 (9)</td>
</tr>
<tr>
<td>BDNF</td>
<td>27.9 ± 0.8 (28)</td>
<td>49.2 ± 2.4 (28)</td>
</tr>
<tr>
<td>Morphine plus BDNF</td>
<td>37.4 ± 1.1 (19)</td>
<td>48.1 ± 3.5 (19)</td>
</tr>
</tbody>
</table>

Data are expressed in means ± SEM (n).

*All animals that were not treated with morphine received subcutaneous implantation of placebo pellets. Number of animals used were as follows: control, seven; morphine, seven; naltrexone, three; morphine plus naltrexone, four; vehicle, three; BDNF, five; and morphine plus BDNF, four.

†P < 0.01 by Student's t test.
‡Animals received intra-VTA infusions of vehicle solution (see ref. 15) as well as subcutaneous implantation of placebo pellets.
VTA dopamine neuronal counts in various rodent strains (see refs. 22 and 24).

At the higher magnification, there did appear to be a small decrease in the size of dopamine neurons under morphine-treated conditions (Fig. 4 C and F). However, this was difficult to quantify due to the high density of TH+ immunoreactivity in cell bodies and processes in the tissue sections and to the difficulty in identifying complete (i.e., unsectioned) neurons. Indeed, this limitation in the quantitative capability of immunohistochemistry necessitated the use of Lucifer yellow injections originally.

DISCUSSION

The results of the present study provide the first direct demonstration that dopaminergic neurons in the VTA exhibit structural changes after chronic morphine administration. The observed reduction in cell size is consistent with earlier speculation (12, 13), which was based on adaptations in cytoskeletal proteins (9, 10) and on the impairment in axoplasmic transport (14) in this brain region associated with chronic morphine exposure. In the present study, we show that this reduction in cell size is specific to dopaminergic neurons within this brain region and requires the specific action of morphine at opioid receptors. At the same time, this reduction in cell size was not associated with gross abnormalities in the VTA, nor was it associated with death of VTA dopamine neurons. Since other drugs of abuse are known to produce some similar biochemical adaptations in the VTA compared with morphine (see Introduction), it would be interesting in future studies to determine whether the drugs also produce similar effects on the morphology of VTA dopamine neurons.

We also show in the present study that the morphine-induced changes in the size of VTA dopamine neurons can be prevented by intra-VTA infusion of BDNF. VTA dopamine neurons are known to express TrkB, the protein tyrosine kinase receptor that exhibits high affinity for BDNF (26).

Moreover, BDNF has been shown to enhance the survival of midbrain dopamine and other neurons in cell culture in vitro and to protect the neurons from neurotoxins (e.g., MPTP or 6-hydroxydopamine) administered in vivo (e.g., see refs. 27–35). The present study shows that BDNF can in addition exert a more subtle effect on these neurons: it cannot only rescue the cells and prevent cell death, it can also prevent neuroplastic changes that are not associated with cell death in response to chronic drug exposure.

The results of the present study are also of interest in light of recent work, which suggests that some of the morphine-induced biochemical adaptations in the VTA may be achieved via morphine perturbation of BDNF signaling cascades. For example, chronic morphine administration has been shown to increase the phosphorylation state and activity of extracellular signal regulated kinase (ERK), a major effector of BDNF and related neurotrophins, selectively in this brain region (36). Direct evidence has been obtained to implicate such regulation of ERK in chronic morphine induction of TH in the VTA. In this sense, the ability of BDNF to prevent some of morphine’s effects may represent not only a pharmacological intervention, but also an intervention at or near the pathophysiological mechanisms that mediate some of the long-term actions of morphine in this brain region. In addition, these studies suggest possible approaches to the development of novel pharmacological treatments of opiate addiction; they raise the possibility that agents that activate the BDNF signaling cascade may be useful in treating certain sequelae of long-term opiate exposure.

The morphine-induced reduction in the size of VTA dopamine neurons did not appear to be due to a different rate of diffusion of the Lucifer yellow in the neurons between control and morphine-treated conditions. This is because the dye was injected into individual neurons visualized under fluorescent microscopy, and was observed to completely fill all of the neurons sampled during the injection process. However, we cannot exclude the possibility that a different rate of diffusion influenced the morphine-induced reduction in the mean length of VTA neuronal processes observed in these studies.

A major question raised by the present findings is: What are the functional consequences of the morphine-induced structural changes observed in VTA dopamine neurons? We know that opiates acutely activate VTA dopamine neurons, an effect mediated, at least in brain slices, via inhibition of inhibitory
GABAergic interneurons (37). However, the electrophysiological effects of chronic opiate exposure on these neurons have received virtually no attention to date. In addition, we do not know whether the structural changes in VTA dopamine neurons seen after chronic morphine administration are mediated via transsynaptic effects of morphine through the GABAergic neurons or via direct effects of morphine on the dopaminergic neurons. Evidence for such direct effects is the apparent expression of opioid receptors by VTA dopamine neurons (38) as well as the synaptic specializations between these neurons and enkephalin-containing nerve terminals observed by electron microscopy (39).

One possible model of opiate regulation of the VTA is that opiates acutely activate VTA dopamine neurons with an intensity and persistence not seen under normal conditions. After chronic exposure, compensatory adaptations occur in the VTA to oppose this activation. In the absence of drug, these changes would contribute to deficient VTA neuronal function and possibly to an aversive state postulated to occur during drug withdrawal (3, 4, 40–42). According to this model, the structural alterations in VTA neurons could reflect an impairment in the functional capacity of these cells, which would thereby contribute to motivational dependence.

It is also possible that the changes observed in the morphology of VTA dopamine neurons after chronic morphine exposure represent a form of neural injury. Indirect support for this possibility comes from prior observations that chronic morphine administration, as well as heroin self-administration, results in reduced levels of neurofilaments and elevated levels of glial fibrillary acidic protein selectively within the VTA (see Introduction). Glial fibrillary acidic protein is an astrocyte-enriched protein often induced in concert with astrocyte activation or proliferation. While this would represent the first evidence for neural injury in response to chronic opiate administration, damage to midbrain dopamine neurons and concomitant induction of glial fibrillary acidic protein in select brain regions have been well-documented in response to chronic exposure to stimulants, particularly amphetamine and its derivatives (for example, see refs. 43 and 44). However, it is important to emphasize that the altered levels of neurofilaments and glial fibrillary acidic protein and the changes in VTA neuronal morphology seen after morphine administration, while perhaps suggestive of neural injury, could instead reflect neuroplastic changes associated with increased or decreased functioning of these cells. Indeed, one must be very cautious in interpreting the observed changes as reflecting neural injury, given the current and successful practice of treating opiate addiction with opiate agonists, such as methadone (see ref. 45).

While more work is clearly needed to study the validity of these and alternative interpretations, this study highlights the complex types of adaptations that can be elicited in the brain following a chronic drug treatment. It also adds to the growing evidence that many examples of plasticity in the functioning of neurons in the fully differentiated adult brain are associated with plasticity that can be visualized at the anatomical level (for example, see refs. 46–51). As the array of molecular and cellular adaptations induced in the VTA and other brain regions by chronic opiate exposure is delineated, these changes can be related to specific behavioral features of addiction and may direct the development of fundamentally new approaches to the treatment of addictive disorders.

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