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Nociceptin/Orphanin FQ and Opioid Receptor-Like Receptor mRNA Expression in Dopamine Systems

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ABSTRACT

Although nociceptin/orphanin FQ (N/OFQ) influences dopamine (DA) neuronal activity, it is not known whether N/OFQ acts directly on DA neurons, indirectly by means of local circuitry, or both. We used two parallel approaches, dual in situ hybridization (ISH) and neurotoxic lesions of DA neurons by using 6-hydroxydopamine (6-OHDA), to ascertain whether N/OFQ and the N/OFQ receptor (NOP) mRNA are expressed in DA neurons in the ventral tegmental area (VTA) and substantia nigra compacta (SNc). In the VTA and SNc, small populations (~6–10%) of N/OFQ-containing neurons coexpressed mRNA for tyrosine hydroxylase (TH), the rate-limiting enzyme for DA synthesis. Similarly, very few (1–2%) TH-positive neurons contained N/OFQ mRNA signal. A majority of NOP-positive neurons (~75%) expressed TH mRNA and roughly half of the TH-containing neurons expressed NOP mRNA. Many N/OFQ neurons (~50–60%) expressed glutamic acid decarboxylase 65 and 67 mRNAs, markers for γ-aminobutyric acid (GABA) neurons. In the 6-OHDA lesion studies, NOP mRNA levels were nearly 80 and 85% lower in the VTA and SNc, respectively, on the lesioned side. These lesions appear to lead to compensatory changes, with N/OFQ mRNA levels approximately 60% and 300% higher in the VTA and SNc, respectively, after 6-OHDA lesions. Finally, N/OFQ-stimulated [^{35}S]guanylyl-5′-O-(γ-thio)-triphosphate levels were decreased in the VTA and SNc but not the prefrontal cortex after 6-OHDA lesions. Accordingly, it appears that N/OFQ mRNA was found largely on nondopaminergic (i.e., GABA) neurons, whereas NOP mRNA was located on DA neurons. N/OFQ is in a position to influence DA neuronal activity by means of the NOP located on DA neurons. J. Comp. Neurol. 444: 358–368, 2002.

Indexing terms: orphanin FQ; ORL1; substantia nigra compacta; ventral tegmental area; tyrosine hydroxylase; 6-hydroxydopamine

Three opioid receptors known as μ, κ, and δ have been cloned (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993; Meng et al., 1993; Thompson et al., 1993; Wang et al., 1993; Yasuda et al., 1993; Fukuda et al., 1994). With polymerase chain reaction amplification and low stringency hybridization techniques, several laboratories detected human, mouse (Mollereau et al., 1994), and rat (Bunzow et al., 1994; Chen et al., 1994; Fukuda et al., 1994; Wang et al., 1994; Wick et al., 1994) cDNAs encoding a novel receptor, which will be referred to as NOP (also known as ORL1). Approximately 65% of the residues within the membrane-spanning region of the NOP are similar to μ, κ, and δ opioid receptors (Mollereau et al.,

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1994; Lachowicz et al., 1995). Despite this similarity, classic opioid ligands do not specifically bind to the NOP with any significant affinity (Fukuda et al., 1994; Mollereau et al., 1994; Wang et al., 1994).

A 17 amino acid peptide termed orphanin FQ or nociceptin (N/OFQ) was discovered to be the high-affinity endogenous ligand for the NOP (Meunier et al., 1995; Reinscheid et al., 1995). There is a significant amino acid sequence similarity between N/OFQ and the endogenous opioid peptides. This resemblance is particularly striking in the case of dynorphin $A_{(1-17)}$. Orphanin FQ and dynorphin A have the same amino acid length, a similar amino acid motif ("Phe-Gly-Gly-Phe" in N/OFQ and "Tyr-Gly-Gly-Phe" in the opioid peptides) at the amino terminal and similar groups of positive charges in the midportion and C-terminal end (Meunier et al., 1995; Reinscheid et al., 1995). Like other opioid ligands, N/OFQ modulates Ca^{2+} channels (Knoflach et al., 1996; Connor and Christie, 1999), activates inward rectifying K^+ channels (Connor et al., 1996; Vaughan and Christie, 1996) and inhibits cAMP production (Meunier et al., 1995; Reinscheid et al., 1995). Opioid ligands exhibit varying degrees of affinity for each of the opioid receptors (Mansour et al., 1995). Despite the aforementioned similarities between opioid and the opioid-like orphanin system, N/OFQ does not bind with any appreciable affinity to the classic opioid receptors (Reinscheid et al., 1995). Accordingly, the orphanin system, although resembling the opioid system in many ways, has evolved into a distinct and independent system (Meng et al., 1996; Reinscheid et al., 1996; Danielson and Dores, 1999).

The widespread anatomic distribution of the orphanin system (Bunzow et al., 1994; Fukuda et al., 1994; Mollereau et al., 1994; Wick et al., 1994; Lachowicz et al., 1995; Neal et al., 1999a, b) suggests involvement in a broad array of neurologic functions. For example, N/OFQ administration (i.c.v.) decreases nociceptive threshold in the rodent tail flick test (Meunier et al., 1995; Reinscheid et al., 1995; Rossi et al., 1996), increases feeding (Pomonis et al., 1996; Stratford et al., 1997), activates neuroendocrine response to stress (Devine et al., 2001), decreases anxiety in behavioral tests (Jenck et al., 1997; Griebel et al., 1999), impairs motor coordination (Devine et al., 1996b), alters locomotion (Reinscheid et al., 1995; Devine et al., 1996b; Florin et al., 1996, 1997b), and influences learning and memory processes (Sandin et al., 1997; Manabe et al., 1998; Hiramatsu and Inoue, 1999).

Orphanin FQ and NOP are moderately to heavily expressed in dopamine (DA) -rich areas such as the ventral tegmental area (VTA), substantia nigra compacta (SNc), and prefrontal cortex (PFC; Florin et al., 1997a; Neal et al., 1999a, b). The orphanin system is, therefore, in a position to influence DA neuronal activity and several studies have shown a functional interaction between these two systems. Local N/OFQ application into the striatum increases DA levels as well as locomotor activity (Konya et al., 1998). Additionally, N/OFQ administration into the VTA or lateral ventricle decreases accumbal DA levels as measured with microdialysis (Murphy et al., 1996; Murphy and Maidment, 1999). Alterations in DA release are typically associated with motivational states; however, N/OFQ administration (i.c.v.) fails to produce conditioned place preference or aversion (Devine et al., 1996a). Despite these initial studies, the role of the N/OFQ system in mediating DA neuronal function is far from understood.

To better understand the anatomic relationships between the orphanin and DA systems, two parallel approaches were used to ascertain whether N/OFQ and NOP mRNA are expressed within DA-containing neurons in the VTA and SNc. In one series of experiments, we used dual in situ hybridization (ISH) techniques to examine potential coexpression of mRNA for tyrosine hydroxylase (TH), the rate-limiting enzyme for DA synthesis, with N/OFQ and NOP mRNA in tegmental and nigral neurons. Additionally, we determined whether N/OFQ mRNA was colocalized with mRNAs for glutamic acid decarboxylase 65 and 67 (GAD), markers for γ-aminobutyric acid (GABA) neurons. In another set of studies, N/OFQ and NOP mRNA expression levels were measured in the SNc and VTA after destruction of DA neurons with the neurotoxin 6-hydroxydopamine (6-OHDA). To determine whether the density, functional activity, or both, of this G-protein–coupled receptor (i.e., NOP) population was altered after 6-OHDA lesions, our final set of studies measured N/OFQ-stimulated [^{35}S]guanylyl-5′-O-(γ-thio)-triphosphate ([^{35}S]GTPγS) binding VTA, SNc, and PFC.

MATERIALS AND METHODS

Adult male Sprague-Dawley rats (275–300 g, Charles River, Wilmington, MA) were maintained and handled according to NIH guidelines. All protocols used in the present study were approved by the university unit for lab animal medicine at the University of Michigan. The rats were housed three per cage, maintained on a 12/12 hour light/dark schedule (lights on at 6 AM, off at 6 PM), and provided food and water ad libitum.

6-OHDA lesions

The rats were deprived of food 24 hours before surgery. On the day of surgery, each rat was first injected with atropine methyl nitrate (0.05 mg/kg, i.p., in 0.9% saline) followed by an injection of desmethylimipramine (15 mg/kg, i.p., in distilled water) and sodium pentobarbital (30–60 mg/kg, i.p.). Each rat was then placed in a stereotaxic frame, and by using neurosurgical techniques, a small hole was drilled through the skull. A 10-μl Hamilton syringe was lowered into the brain parenchyma and either 6-OHDA (2 mg/ml in 0.1 mg/ml ascorbic acid) or vehicle (0.1 mg/ml ascorbic acid) was injected into the right medial forebrain bundle at a rate of 0.5 μl/min over an 8.25-minute period. The injection coordinates were 3.0 mm anterior to bregma, 1.8 mm right of the midline, and 8.3 mm ventral with respect to the surface of the skull (Paxinos and Watson, 1986). Injections of 6-OHDA at these coordinates were used to lesion both nigral and tegmental dopamine neurons (Berridge and Robinson, 1998). After the 6-OHDA or vehicle injection, the microsyringe was left in place for an additional 2 minutes and then removed slowly. After surgical closure, each rat was injected with warm 0.9% saline (3–6 cc, s.c.) and placed in an incubator until it became ambulatory, after which it was returned to an individual cage with food and water supplied ad libitum. Fourteen days after the lesions, each rat was decapitated; the brain rapidly was removed, frozen in isopentane (−40°), and stored at −80°C. Brains were sectioned at 10 μm in the coronal plane, mounted onto polylysine-coated slides, and stored at −80°C.

Synthesis of labeled riboprobes

^{35}S-labeled riboprobes were used for single ISH experiments, whereas both digoxigenin (dig) and ^{35}S-labeled riboprobes were used for dual ISH experiments. Riboprobes were generated for the NOP (700 base pairs, Neal et al., 1999a), N/OFQ (580 base pairs, Neal et al., 1999b), TH (300 base pairs, courtesy of Tong H. Joh, Cornell University), GAD 65 (230 base pairs, courtesy of N.J.K. Tillakaratne, University of California), and GAD 67 (210 base pairs, courtesy of A. Tobin, University of California-Los Angeles). ^{35}S-labeled and dig-labeled riboprobes were generated with standard transcription reaction techniques. These reactions were terminated with DNAse I for 15 minutes at room temperature. Probes labeled with ^{35}S and dig were separated from free nucleotides by means of a Sephadex-G50 column. The dig-labeled fraction was determined by means of a color reaction (see dual ISH methods for details).

Single radioactive ISH histochemistry

A detailed description of the radioactive ISH technique has been described previously (see Watson et al., 1988). Briefly, slide-mounted sections were removed from −80°C and washed in the following solutions at room temperature: 4% formaldehyde solution for 1 hour, 3 times in 2× standard saline citrate (SSC), 0.1 M triethanolamine (pH 8.0) and acetic anhydride (0.25%) for 10 minutes, and water for 5 minutes. The slides were then dehydrated though graded alcohols. The ^{35}S-riboprobe was mixed with filtered hybridization buffer for a final concentration of $1–2 \times 10^6$ dpm/35 μl. Tissue sections were covered with the ^{35}S-riboprobe and hybridized in a 55°C oven overnight. On day 2, the coverslips were removed and sections were rinsed twice in 2× SSC. Tissue was then treated with RNase A solution followed by decreasing concentrations of salt solutions and soaked in 0.1× SSC for 1 hour at 70°C. The sections were then rinsed in water, dehydrated in graded alcohols, and air-dried. Slides were exposed to Biomax MR imaging film (Kodak) for the number of days required to obtain an adequate signal (approximately 10 days for N/OFQ and NOP probes, 2–3 days for TH probe). Brain images were captured with a CCD camera (TM-745, Pulnix, USA). The slides were dipped in NTB-2 emulsion, dried, and stored at 4°C for 1–3 weeks, then developed (Kodak D-19), dehydrated in graded alcohols, and cover-slipped with mounting fluid (Permount).

Routine in situ control studies were performed on sections by treating with either (1) RNase A solution before cRNA probe hybridization, or (2) ^{35}S-labeled mRNA (sense) strands. In all control studies, the radioactive signal did not differ from background.

Dual ISH histochemistry

Dig-labeled riboprobes were prepared as described above. During the first 2 days, the double ISH protocol was similar to the single ISH protocol. The only difference was during the hybridization step, i.e., the dig-labeled and ^{35}S-labeled probes were mixed together in hybridization fluid. In the GAD experiments, both dig-labeled GAD67 and GAD65 probes were added to the hybridization fluid. On day 2, after the 0.1× SSC wash in 70°C, the slides were cooled to room temperature in 0.1× SSC. The slides were then briefly placed in 0.1 M phosphate buffer (pH 7.4), and transferred to carrageenin blocking solution (0.1 M phosphate buffer, 0.25% carrageenan, and 0.5% Triton-X 100 [pH 7.4]) for 1 hour at room temperature on a shaker. The slides were then incubated overnight on a shaker in carrageenan blocking solution containing 1:20,000 alkaline phosphatase (AP) -conjugated anti-digoxigenin antiserum (Boehringer Mannheim). On day 3, sections were briefly washed in 0.1 M phosphate buffer (pH 7.4) followed by extensively washing in Tris-buffered saline (pH 7.5). The sections were then placed in alkaline substrate buffer (ASB; 100 mM Tris base, 150 mM NaCl, 50 mM $MgCl_2$, pH 9.5) and underwent a color reaction in ASB solution containing 5% polyvinyl [w/v], 0.45% 4-nitro blue tetrazolium chloride (NBT; Boehringer Mannheim), 0.35% 5-bromo-4-chloro-3-indoyl-phosphate (BCIP; Boehringer Mannheim), and 0.024% levamisole (Sigma). The color reaction was stopped when cells containing the dig label attained a bright purple color. The sections were then washed in water, placed in a 0.1 M glycine (pH 2.2)/0.5% Triton X-100 solution, and fixed in 2.5% glutaraldehyde. The slides were rinsed in water, dehydrated in graded alcohols, and exposed overnight to x-ray film to ensure the presence of ^{35}S-labeled probe. Sections were dipped in Ilford KD-5 emulsion (Polysciences), stored in the dark at 4°C and developed after appropriate time lapse (approximately 2–3 weeks). Brain sections were visualized by using a Leica microscope (Leitz DMR, Wetzler, Germany) connected to a camera (Sony DXC-970MD).

In vitro autoradiographic receptor-stimulated [^{35}S]GTPγS binding studies

For detailed description of this technique, see Sim et al. (1995). Briefly, slide-mounted sections were removed from −80°C and brought to room temperature 15–30 minutes before use. Slides were transferred to humidifying chambers with all incubations performed at room temperature. Sections were initially incubated in 300 μl of primary assay buffer (50 mM Tris-HCl, 0.1% bovine serum albumin, 5 mM $MgCl_2$, 1 mM EGTA, and 100 mM NaCl [pH 7.5]) for 10 minutes, followed by primary assay buffer containing 2 mM guanosine diphosphate (GDP; Sigma, St. Louis, MO) for 15 minutes. Sections were then incubated in primary assay buffer containing 1 μM cold GTPγS (Sigma) for 10 minutes and primary assay buffer for 10 minutes. The primary buffer was removed, and the sections were incubated for 2 hours in 300 μl of incubation medium (primary assay buffer containing 2 mM GDP, 0.04 nM ^{35}S-GTPγS [New England Nuclear, 1,250 Ci/mM], 1 μM DTT, protease inhibitor [0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM iodoacetamide] and the chosen agonist at varying concentrations). After incubation medium, sections were rinsed in ice-cold Tris buffer (50 mM Tris-HCl, pH 7.5), followed by ice-cold deionized water and then air-dried. Slide-mounted sections were exposed to Biomax MR imaging film (Kodak) for 20−48 hours, and brain images were captured with a CCD camera (TM-745, Pulnix, USA).

Agonist-stimulated GTPγS binding controls

Basal binding was measured on sections incubated with 2 mM GTP and 0.04 nM [^{35}S]GTPγS in the absence of agonist in the incubation medium. Agonist-stimulated binding was measured with respect to basal binding in adjacent sections. Basal binding was negligible in most sections analyzed. Nonspecific binding was assessed when 10 μM unlabeled GTPγS was added to the incubation medium containing 2 mM GTP and 0.04 nM [^{35}S]GTPγS

in the presence of agonist. Specific binding was completely eliminated when 10 μM unlabeled GTPγS added to the incubation medium in the presence of N/OFQ.

Data Analysis

Percentage colocalization in the dual ISH studies was calculated by dividing the number of dual-labeled neurons by either ^{35}S-labeled cells or dig-labeled cells × 100. The dig-labeled riboprobes appeared as a purple precipitate under brightfield conditions, whereas ^{35}S-labeled riboprobes appeared as silver grains under darkfield conditions. Labeled neurons were counted at 40× magnification by using a grid. The percentage colocalization is reported as the mean ± SEM. For N/OFQ/TH dual ISH studies, six brain sections (spanning from approximately bregma -5.2 to -6.04) were counted from each rat, with five to six rats per group. For N/OFQ/GAD dual ISH studies, four brain sections were counted from each rat with three rats per group.

N/OFQ, NOP, and TH mRNA expression, as well as N/OFQ-stimulated [^{35}S]GTPγS binding levels were determined with densitometric analysis. Each brain image (captured from film) was analyzed individually by using Scion Image (NIH image 1.61, Scion Corporation, National Institutes of Health, Frederick, MD). For single ISH studies, results are reported as the integrated density (area of pixels above background multiplied by the mean signal). Signal is a gray value at least 3.5 standard deviations above background. For N/OFQ-stimulated [^{35}S]GTPγS binding studies, results are reported as the integrated density values obtained for stimulated sections minus the integrated density values from basal sections (sections adjacent to stimulated sections).

Significance of differences in [^{35}S]GTPγS binding, N/OFQ and NOP mRNA values after 6-OHDA lesions was computed by using a one-way analysis of variance. All significant effects were further analyzed with Tukey-Kramer multiple comparisons test.

RESULTS

TH, N/OFQ, and NOP mRNA expression in the SNc and VTA

The distribution pattern of TH, GAD, N/OFQ, and NOP mRNA in the VTA and SNc was consistent with published studies (Chesselet et al., 1987; Esclapez et al., 1993, 1994; Sherman and Moody, 1995; Neal et al., 1999a, b). TH mRNA was expressed throughout the VTA and SNc. In contrast, GAD mRNA was found in a small population of neurons in these midbrain regions. NOP and N/OFQ mRNA was also found throughout the VTA and SNc, but these mRNAs exhibited varying degrees of expression within the subnuclei. Within the SNc, N/OFQ mRNA was present throughout medial and dorsal divisions, and slightly higher levels were found in the pars lateralis. Within the VTA, N/OFQ-containing cells were present throughout the region with a dense expression found in the paranigral subnuclei.

Orphanin receptor mRNA distribution was similar to that described for N/OFQ mRNA with a few notable exceptions. Within the SNc, NOP mRNA levels were high in ventral regions and lower levels were found in the dorsal compacta and pars lateralis. Expression of NOP mRNA was distributed throughout the VTA proper, with high levels noted in the parabrachial and paranigral nuclei and lower levels in the interfascicular nuclei. Within each region analyzed, NOP mRNA had a more diffuse distribution whereas N/OFQ was heavily concentrated in individual neurons throughout the VTA and SNc.

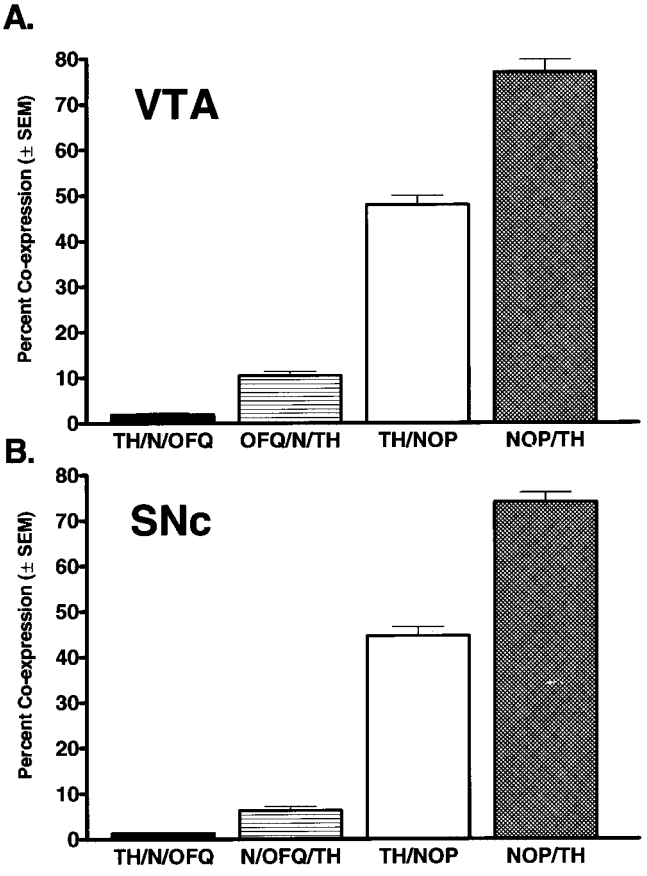

Fig. 1. Bar graphs representing the percentage coexpression of tyrosine hydroxylase (TH) mRNA with either nociceptin/orphanin FQ (N/OFQ) or N/OFQ receptor (NOP) mRNA in the ventral tegmental area (VTA) and substantia nigra compacta (SNc). **A:** Within the VTA, few TH-positive neurons exhibited N/OFQ mRNA signal, whereas a small population of N/OFQ neurons also expressed TH mRNA. Roughly 50% of TH-positive neurons coexpressed NOP mRNA, and approximately 80% of NOP-positive neurons also contained TH mRNA signal. **B:** Within the SNc, few neurons that expressed N/OFQ mRNA were positive for TH mRNA and vice versa. Almost half of the TH-containing neurons also contained NOP mRNA and nearly 75% of the neurons that expressed NOP mRNA also expressed TH mRNA. The bars represent the percentage of ^{35}S-labeled neurons containing digoxigenin label and vice versa. Values are the mean ± SEM.

Colocalization patterns between TH mRNA and either N/OFQ or NOP mRNA

Within the VTA (Figs. 1A, 2A–D), virtually none of the TH-positive neurons expressed N/OFQ mRNA (1.9 ± 0.3%, n = 6) and only a small portion of the N/OFQ-containing neurons expressed TH mRNA (10.5 ± 1.0%, n = 6). In contrast, approximately 50% of TH neurons contained NOP mRNA expression (47.9 ± 2.0%, n = 6) and nearly 80% of NOP-positive neurons exhibited TH mRNA expression (77.0 ± 2.7%, n = 6).

The pattern of TH colocalization with N/OFQ and NOP mRNAs in the SNc was similar to that observed in the

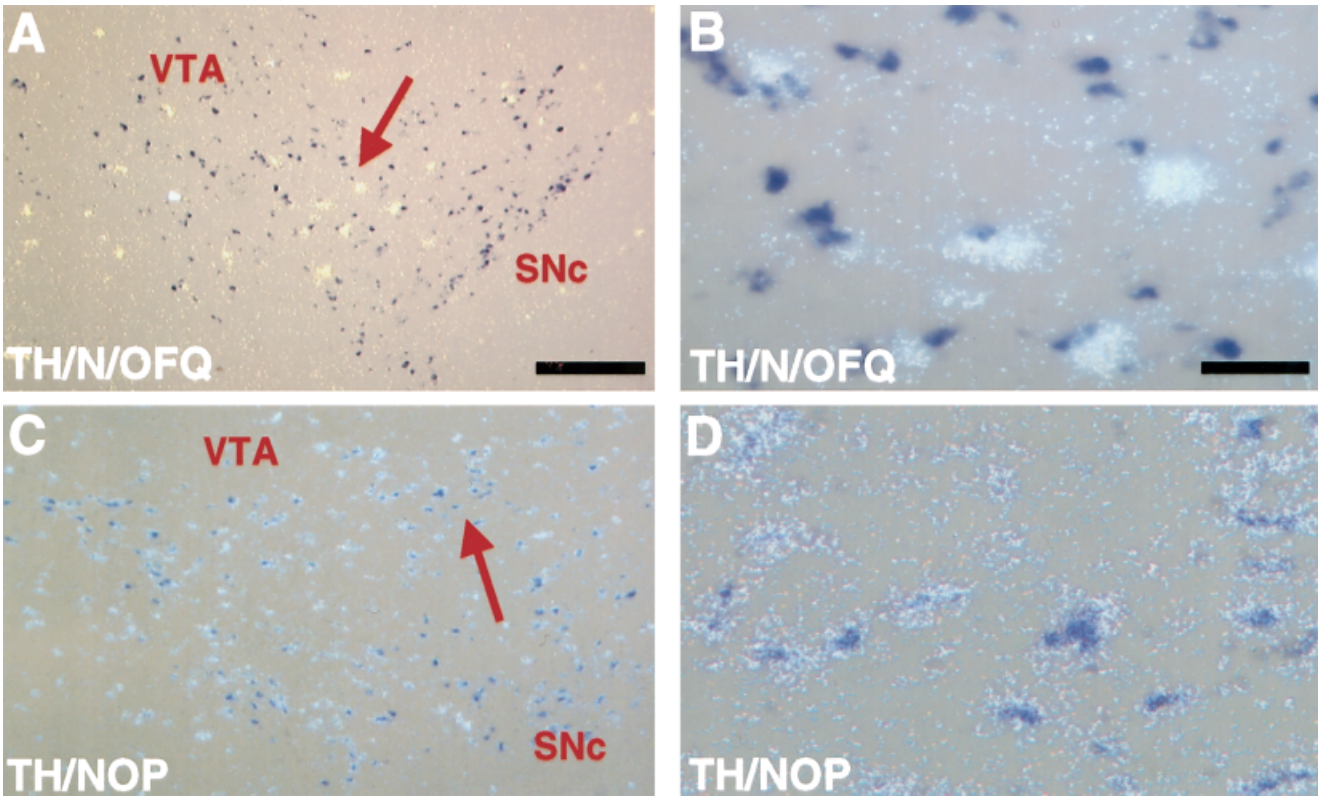

Fig. 2. Photomicrographs of dual in situ hybridization experiments comparing colocalization of either ^{35}S-labeled nociceptin/orphanin FQ (N/OFQ) or N/OFQ receptor (NOP) neurons (silver grains) with digoxigenin-labeled tyrosine hydroxylase (TH) neurons (purple precipitate) in the ventral tegmental area (VTA) and substantia nigra compacta (SNc). **A:** Most N/OFQ-positive neurons did not contain signal for TH mRNA. **B:** High-magnification photomicrograph of TH and N/OFQ mRNA expression patterns in the VTA. **C:** Many NOP-positive neurons contained signal for TH mRNA. **D:** High-magnification photomicrograph of TH and NOP mRNA expression pattern. Arrows in A and C indicate the area seen in B and D, respectively. Scale bar = 300 μm in A (applies to A,C), 60 μm in B (applies to B,D).

VTA (Figs. 1B, 2A–D). Few TH-containing neurons expressed N/OFQ mRNA (1.3 ± 0.2%, n = 6), and few N/OFQ-containing neurons were positive for TH mRNA (6.3 ± 0.9%, n = 6). Roughly half of the TH-containing neurons expressed NOP mRNA (44.6 ± 2.0%, n = 6), and approximately 75% of the neurons expressing NOP mRNA also expressed TH mRNA (73.9 ± 2.1%, n = 6).

N/OFQ and NOP mRNA expression in the VTA and SNc after 6-OHDA lesions

TH mRNA levels were assessed first to determine the extent of the lesion. Five of the 12 animals had an 85 and 95% loss of TH mRNA in the VTA and SNc, respectively, on the 6-OHDA–lesioned side compared with sham-lesioned controls. Tissue from these five rats was used for evaluation of [^{35}S]GTPγS binding, GAD, N/OFQ, and NOP mRNA values.

In the VTA, N/OFQ mRNA expression was roughly 60% higher on the 6-OHDA-lesioned side (3,411.3 ± 473.7) compared with sham-lesioned controls (2,105.4 ± 191.2, n = 5, $P < 0.05$) or the contralateral side (2,067.7 ± 352.0, n = 5, $P < 0.05$, Figs. 3B, 4A). In the SNc, N/OFQ mRNA levels were approximately three-fold higher in the 6-OHDA–lesioned side (12,415.9 ± 2,438.9) compared with sham-lesioned controls (4,285.6 ± 455.8, n = 5, $P < 0.01$) or the contralateral side (3,802.8 ± 578.9, n = 5, $P < 0.01$, Figs. 3B, 4A).

In contrast to the changes in N/OFQ mRNA expression in the VTA, NOP mRNA was almost 80% lower on the 6-OHDA–lesioned side (768.4 ± 186.4, n = 5) when compared with sham-lesioned controls (3,439.2 ± 431.4, n = 5, $P < 0.05$) or the contralateral control side (3,718.9 ± 849.6, n = 5, $P < 0.01$; Figs. 3C, 4B). The same holds true for the 6-OHDA lesion effect in the SNc (Figs. 3C, 4B) where NOP mRNA expression was nearly 85% lower on the 6-OHDA-lesioned side (722.8 ± 150.2, n = 5) when measured against sham-lesioned controls (4,365.3 ± 621.7, n = 5, $P < 0.01$) or the contralateral side (4,639.8 ± 675.8, n = 5, $P < 0.01$).

N/OFQ mRNA levels in the sham-lesioned controls did not differ significantly from the contralateral side (VTA, 1,920.6 ± 127.3; SNc, 4235.8 ± 494.0, n = 5). Similarly, NOP mRNA levels on the sham-lesioned control side did not differ significantly from the contralateral side (VTA, 4,205.3 ± 486.9; SNc, 4,861.7 ± 752.9, n = 5).

Colocalization patterns between GAD and N/OFQ mRNA

As stated above, the majority of N/OFQ-containing neurons did not contain TH mRNA signal; therefore, a follow-up set of studies determined whether N/OFQ mRNA was colocalized with GAD mRNA in VTA and SNc. Within the VTA (Fig. 5A), a large portion of N/OFQ-positive neurons also expressed GAD mRNA (57.8 ± 9.4%,

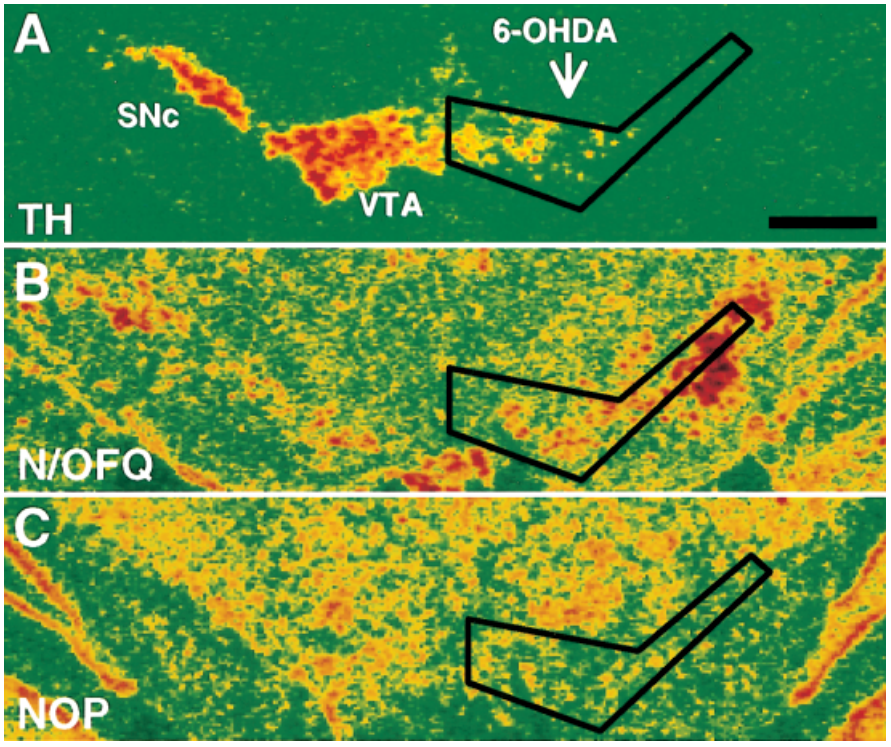

Fig. 3. Digitized photomicrographs of tyrosine hydroxylase (TH), nociceptin/orphanin FQ (N/OFQ), and N/OFQ receptor (NOP) mRNA in the ventral tegmental area (VTA) and substantia nigra compacta (SNc) after unilateral 6-hydroxydopamine (6-OHDA) lesions. The arrow points to the side that received the 6-OHDA injection. The SNc and VTA are outlined in black on the 6-OHDA–lesioned side. **A:** TH mRNA expression decreased on the side subjected to a 6-OHDA injection. **B:** Expression of N/OFQ mRNA increased on the 6-OHDA–lesioned side. **C:** Expression of NOP mRNA decreased on the 6-OHDA–lesioned side. Scale bar = 1 mm in A–C.

n = 3). A similar situation was noted in the SNc (Fig. 5A), where approximately half of the N/OFQ-positive neurons contained GAD mRNA (51.7 ± 8.3%, n = 3).

The percentage of OFQ-positive neurons that expressed GAD mRNA was analyzed in the SNc after 6-OHDA lesions (Fig. 6). This percentage was similar on the 6-OHDA–lesioned side (40.5 ± 1.6%, n = 3) compared with sham-lesioned controls (45.3 ± 8.3%, n = 3) or the contralateral sides (lesion, 38.6 ± 2.5%, n = 3; sham, 44.4 ± 9.7%, n = 3). Similar studies were performed in the VTA, and no significant differences were found (data not shown).

N/OFQ-stimulated [^{35}S]GTPγS binding studies

In the VTA, N/OFQ-stimulated [^{35}S]GTPγS binding was much lower on the 6-OHDA–lesioned side (767.4 ± 283.0, n = 5) compared with the sham-lesioned controls (6,871.8 ± 1,522.5, n = 5, $P < 0.004$; Fig. 7A). In this case, N/OFQ-stimulated [^{35}S]GTPγS binding was reduced on the contralateral side of the 6-OHDA lesion (3,151.6 ± 1,107.6, n = 5) when compared against the contralateral side of sham-lesioned controls (7,745.2 ± 1,716.7, n = 5, $P < 0.02$). In the SNc, N/OFQ-stimulated [^{35}S]GTPγS binding was lower on the 6-OHDA-lesioned side (2,014.2 ± 537.3, n = 6) when measured against the sham-lesioned controls (6,742.2 ± 1,155.3, n = 5, $P < 0.02$) or the contralateral side (5,177.2 ± 665.3, n = 6; Fig. 7A). In the SNc, N/OFQ-stimulated [^{35}S]GTPγS binding on the sham-lesioned side did not differ from the contralateral side (6,723.6 ± 1,412.9, n = 5).

In the PFC (Fig. 7B), N/OFQ-stimulated [^{35}S]GTPγS binding was similar on the 6-OHDA–lesioned side (45,947.1 ± 6,542.3, n = 4) when measured against sham-lesioned controls (54,095.8 ± 7,484.9, n = 4) or the contralateral side (47,064.6 ± 5,301.0, n = 4). Additionally, N/OFQ-stimulated [^{35}S]GTPγS binding in the sham-lesioned control did not differ significantly ($P < 0.05$) from the contralateral side (47,967.6 ± 7,323.2, n = 4).

DISCUSSION

In this study, we used two convergent approaches to characterize the anatomic relationship between the orphanin and DA systems in the brain: 6-OHDA lesions and dual ISH. Both approaches have led to consistent conclusions. In the VTA and SNc, many TH neurons expressed NOP mRNA and a majority of NOP-positive neurons also contained TH mRNA (Figs. 1A,B, 2C,D). In contrast, only a small percentage of TH neurons expressed N/OFQ mRNA and relatively few N/OFQ neurons expressed TH mRNA (Figs. 1A,B, 2A,B). Additional experiments demonstrated that N/OFQ mRNA was colocalized with GAD mRNA in these regions (Fig. 5). Accordingly, NOP mRNA was located on tegmental and nigral dopaminergic neurons, whereas N/OFQ mRNA was found on GABA neurons.

In agreement with the colocalization observations, N/OFQ mRNA increased while NOP mRNA decreased in the VTA and SNc after 6-OHDA lesions (Figs. 3, 4). Furthermore, N/OFQ-stimulated [^{35}S]GTPγS binding decreased in the VTA and SNc on the 6-OHDA-lesioned side

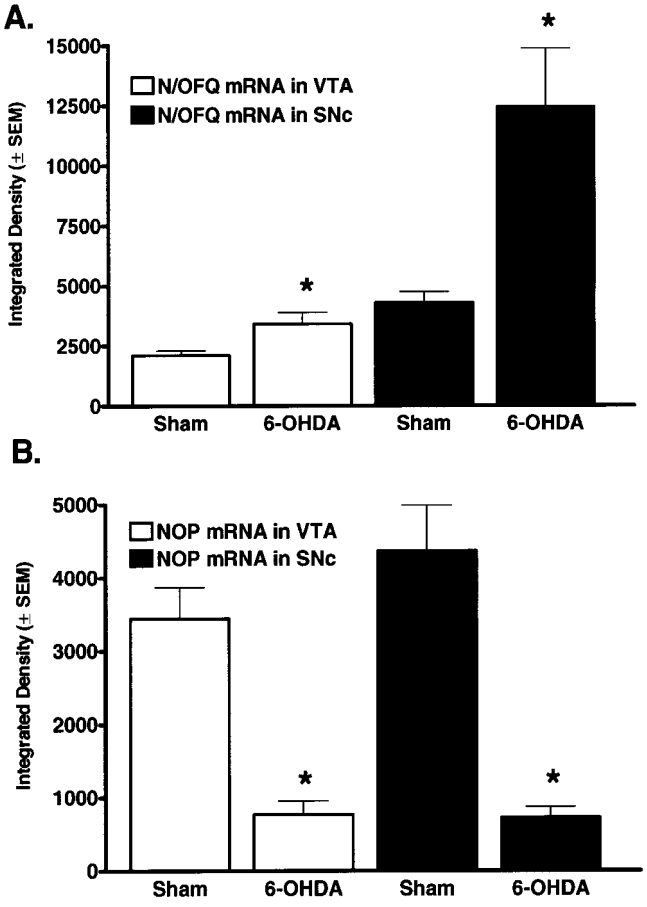

Fig. 4. Bar graphs illustrating the effects of a unilateral 6-hydroxydopamine (6-OHDA) lesion on nociceptin/orphanin FQ (N/OFQ) and N/OFQ receptor (NOP) mRNA in the ventral tegmental area (VTA) and substantia nigra compacta (SNc). **A:** N/OFQ mRNA was significantly increased in 6-OHDA–lesioned side compared with the sham-treated side in the VTA and SNc. **B:** NOP mRNA was significantly decreased in 6-OHDA–lesioned side compared with the sham-treated side in the VTA and SNc. Sham-treated sides do not differ from contralateral sides in either sham or 6-OHDA–lesioned rats. Rats that had received >85 and 95% lesion (as determined by tyrosine hydroxylase mRNA analysis) in the VTA and SNc, respectively, were used to quantify changes in N/OFQ and NOP mRNA. Values are the mean ± SEM.

(Fig. 7A). In contrast, N/OFQ-stimulated [^{35}S]GTPγS binding was unaltered in the PFC, a DA terminal region, after 6-OHDA lesions (Fig. 7B). The results from these experiments would suggest the NOP is found on the cell bodies of DA neurons in the midbrain.

A majority of the neurons originating in the VTA and SNc contain DA, whereas approximately 20–25% of the neurons are nondopaminergic and contain neurotransmitters such as GABA, CCK, or both (Swanson, 1982; Parent and Hazrati, 1995). As stated above, only a small population of N/OFQ mRNA was present on nigral and tegmental DA neurons (~10%), whereas a larger portion was found on GABAergic neurons (~50–60%). Although the number of GABAergic neurons in the SNc and VTA is small (Chesselet et al., 1987), these large neurons are known to exert local influence onto DA neurons as well as send projections to areas such as the thalamus and visual cortex (for review, see Fallon and Loughlin, 1995). The present study phenotyped the majority of the N/OFQ neurons in the VTA and SNc; however, a portion of cells containing N/OFQ mRNA may not have been identified. It is possible that neurons expressing low amounts of GAD mRNA may not have been detected with the nonradioactive ISH technique, thereby underrepresenting the total population of N/OFQ neurons that express GAD mRNA (see Day et al., 1999). Another feasible explanation is that N/OFQ mRNA is found in astrocytes and is regulated by ciliary neurotrophic factor (Buzas et al., 1998, 1999). As pointed out by Buzas et al. (1999), N/OFQ may play a role in neurotrophic responses to neural injury and the N/OFQ mRNA increase observed in the present study may be in response to the loss of cells in the VTA and SNc after the 6-OHDA lesion. Future studies will need to determine whether N/OFQ mRNA is also colocalized with markers for astrocytes (i.e., glial fibrillary acidic protein).

Roughly half of the identified DA neurons also expressed NOP mRNA, and, as would be expected, lesions specific to DA neurons diminished this mRNA pool (Figs. 3, 4). The expression of NOP mRNA changed markedly in both midbrain regions, but there is a slightly greater reduction of NOP mRNA in the SNc as opposed to the VTA (Fig. 4B). Dopamine neurons in the VTA are known to be somewhat resistant, compared with DA neurons in the SNc, to the neurotoxin 6-OHDA. Specific coordinates were chosen to optimize the destruction of both tegmental and nigral DA-containing neurons (Berridge and Robinson, 1998), resulting in greater than 85 and 95% lesion in the VTA and SNc, respectively. Although the extent of the 6-OHDA lesion would be considered massive (Schwarting and Huston, 1996), the greater reduction of NOP mRNA in the SNc may be the result of a more pronounced loss of DA neurons. One cannot discount the possibility that the surviving DA neurons, the small population (approximately 20%) of nondopaminergic neurons, or both, had altered NOP mRNA production subsequent to the neurotoxic insult (all alterations in NOP mRNA, increases or decreases, would have contributed to our overall results). However, in light of our finding that nearly 80% of NOP mRNA was colocalized with TH mRNA and alterations in NOP mRNA after DA lesions correlated closely with the extent of DA neuronal loss, it seems reasonable to conclude that the NOP mRNA decreases after 6-OHDA lesions was due to the specific loss of NOP mRNA-containing dopaminergic neurons.

The complex neuroanatomic organization of DA projections systems has been worked out in detail (for review, see Swanson, 1982; Oades and Halliday, 1987; Joel and Weiner, 2000). In brief, the mesostriatal DA system originates in the SNc and projects primarily to the dorsal striatum (STR). The mesostriatal system also includes the projections from the VTA to the anteromedial STR. The mesolimbic DA system originates in the VTA and projects mainly to the nucleus accumbens (NAc) but also innervates areas such as the olfactory tubercles (Tu), bed nucleus of the stria terminalis (BNST), amygdala (AM), and lateral septum (LS). The mesocortical DA system originates in the VTA and extends largely to the prefrontal, cingulate, and entorhinal cortices.

As presented herein, only a small percentage of DA neurons within the mesostriatal, mesolimbic, and mesocortical systems contain signal for N/OFQ mRNA. Hence, it was not surprising that lesions targeting DA neurons did not decrease N/OFQ mRNA expression levels. In fact,

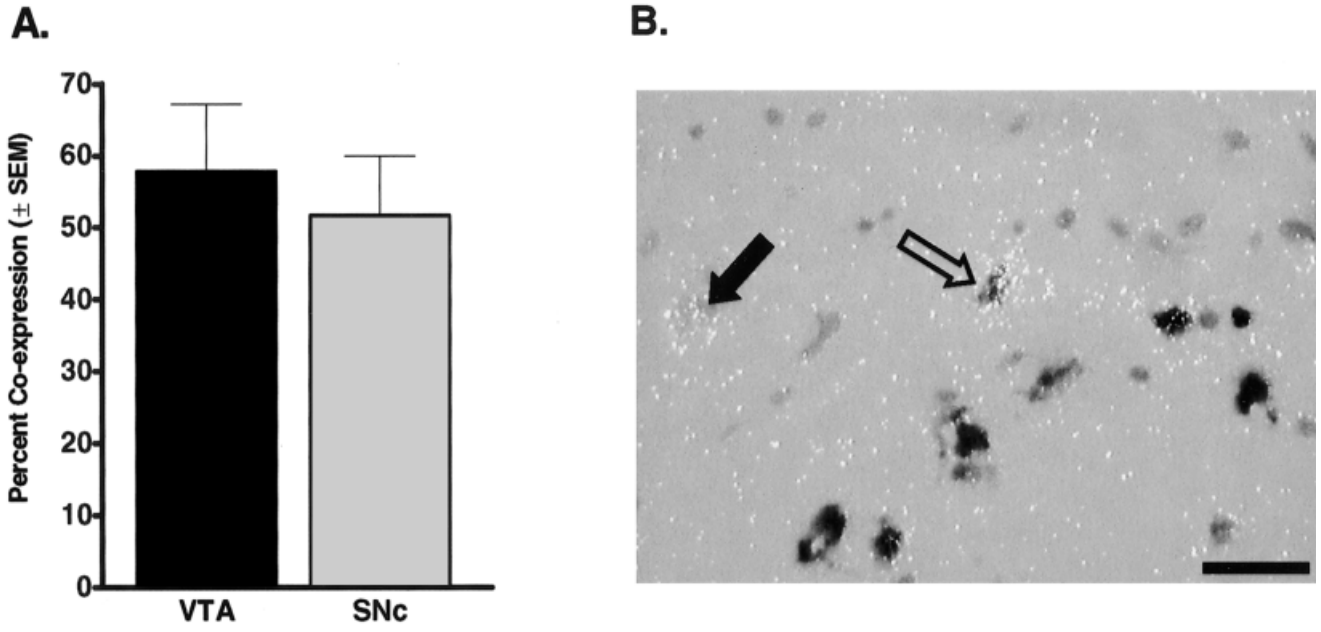

Fig. 5. Bar graph representing the percentage coexpression of nociceptin/orphanin FQ (N/OFQ) with glutamic acid decarboxylase (GAD) mRNA in the ventral tegmental area (VTA) and substantia nigra compacta (SNc). **A:** Approximately 50–60% of N/OFQ-positive neurons contained GAD mRNA signal. Values are the mean ± SEM. **B:** A photomicrograph of a dual in situ hybridization experiment comparing the colocalization of ^{35}S-labeled N/OFQ neurons (silver grains) with digoxigenin-labeled GAD neurons (purple precipitate) in the SNc. The black arrow points to a ^{35}S-labeled neuron only, whereas the open arrow points to a neuron containing both labels. Scale bar = 40 μm in B.

N/OFQ mRNA expression levels increased after the lesion and this increase was much greater in the SNc compared with the VTA (Fig. 4A). Furthermore, the percentage of N/OFQ neurons expressing GAD mRNA (Fig. 6) and the number of N/OFQ-positive neurons (data not shown) did not change significantly after the lesion, suggesting that the increase in N/OFQ mRNA expression was largely due to an increase in the amount of mRNA in cells already expressing N/OFQ mRNA rather than a recruitment of a new population of cells.

The greater response of N/OFQ mRNA in the SNc compared with the VTA may be due to a more pronounced lesion in the SNc (similar to the NOP ISH studies). As pointed out by Schwarting and Huston (1996), most changes postsynaptic to DA neurons occur only when there is a substantial lesion of DA neurons and the near total loss of striatal DA innervation disrupts feedback pathways that project to the nigral DA neurons. These feedback pathways include projections from the STR, NAc core, and dorsolateral ventral pallidum (Heimer et al., 1991; Zahm, 1989). Additionally, the subthalamic nucleus projects primarily to the SNreticulata (some minor projections may extend to the SNc), and these SNreticulata neurons are known to have extensive anatomic interactions with the cell bodies and long dendrites of DA neurons (for review see Joel and Weiner, 2000). The 6-OHDA lesions observed in the present study may have led to altered feedback control which, in turn, could have resulted in the large increase in N/OFQ mRNA expression levels.

Although NOP mRNA was found in DA neurons within the mesolimbic and mesocortical systems, the site of receptor protein expression could be in the DA cell body or terminal region (or both). Indeed, binding studies support

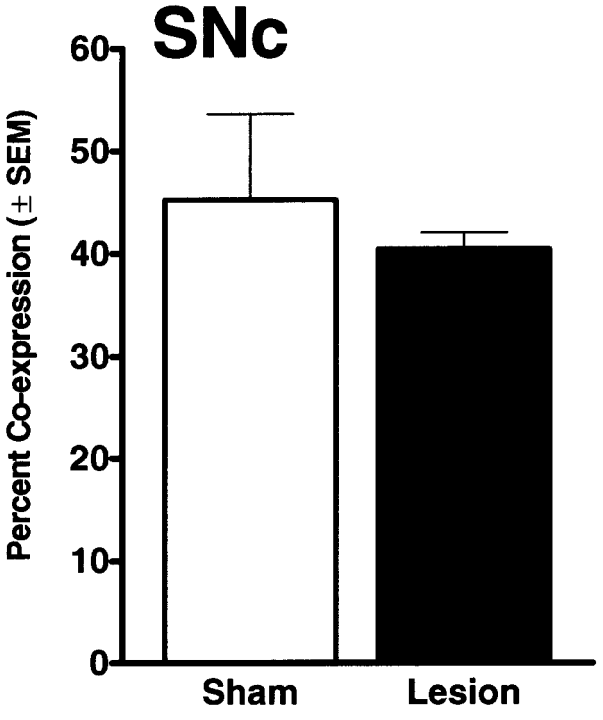

Fig. 6. Bar graph illustrating the effects of unilateral 6-hydroxydopamine (6-OHDA) lesions on the percentage of nociceptin/orphanin FQ (N/OFQ) neurons that expressed glutamic acid decarboxylase mRNA in the substantia nigra compacta (SNc). The levels of colocalization were not altered on the side that received the 6-OHDA lesion compared with the sham-lesioned side. Values are the mean ± SEM.

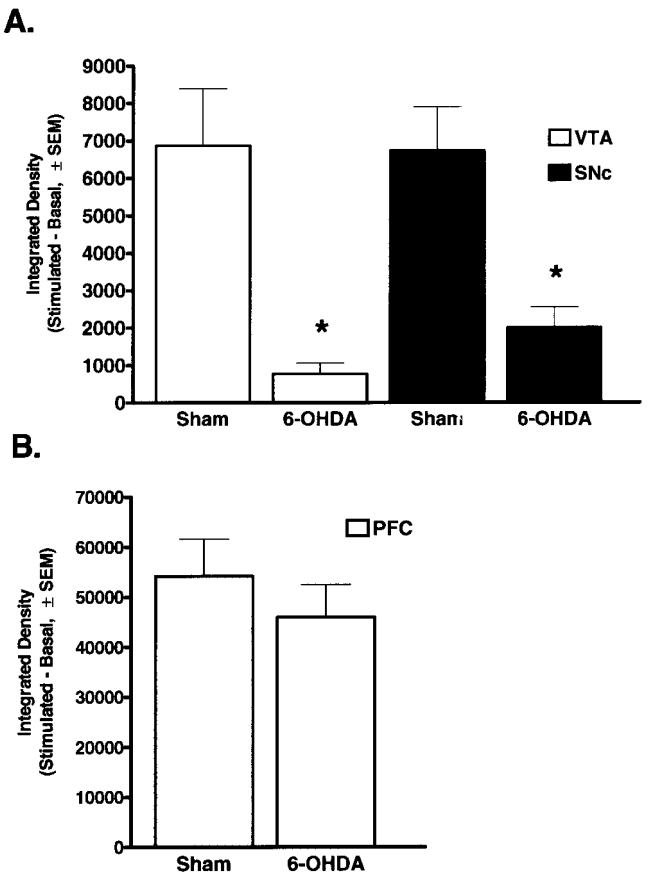

Fig. 7. Bar graphs illustrating the effects of unilateral 6-hydroxydopamine (6-OHDA) lesions on nociceptin/orphanin FQ (N/OFQ) -stimulated [^{35}S]GTPγS binding in the ventral tegmental area (VTA), substantia nigra compacta (SNc), and prefrontal cortex (PFC). **A:** N/OFQ-stimulated [^{35}S]GTPγS binding in the VTA and SNc was reduced on the side subjected to a 6-OHDA lesion. **B:** N/OFQ-stimulated [^{35}S]GTPγS binding in the PFC was not altered on the 6-OHDA–lesioned side compared with the sham-lesioned side. Values are the mean ± SEM.

the existence of the NOP in brain regions associated with both DA cell bodies and terminals (Neal et al., 1999a). It should be noted that the presence of binding in the DA regions does not indicate whether the receptor is located presynaptically, on the DA cell body or terminal itself, postsynaptically on DA receptive neurons, or possibly even on adjacent but non–DA-related cell bodies or terminals. Nonetheless, N/OFQ binding levels are low in the VTA and low to moderate in several DA projection areas, including the NAc, Tu, LS, and AM (Neal et al., 1999a). In contrast, these binding levels are moderate to high in the BNST and entorhinal cortex and dense in the prefrontal, cingulate, and frontal cortices (Neal et al., 1999a). Similarly, N/OFQ-stimulated [^{35}S]GTPγS binding is low in the VTA, NAc, LS, BNST, and all areas of the AM. The highest levels of N/OFQ-stimulated [^{35}S]GTPγS binding are found in cingulate and frontal cortices (Shimohira et al., 1997; Sim and Childers, 1997). These studies suggest that the NOP protein is present and highly activatable in the cortex as opposed to the other projection areas within the mesolimbic and mesocortical DA systems.

Another conclusion may be drawn when considering the mesostriatal DA system. NOP binding with ^{125}I-[^{14}Tyr]-N/OFQ is situated throughout the SNc while absent in the STR (Neal et al., 1999a), but N/OFQ stimulated [^{35}S]GTPγS binding is low in both the SNc and STR (Shimohira et al., 1997; Sim and Childers, 1997). Thus, NOP may be expressed on mesostriatal DA cell bodies, as opposed to terminals, but it is uncertain to what extent these receptors are functional.

Although the current study did not systematically compare N/OFQ-stimulated [^{35}S]GTPγS binding across all brain regions associated with DA circuitry, it is clear that after a 6-OHDA lesion, binding was dramatically reduced in the VTA and SNc but not in the PFC, the main terminal projection region of the DA mesocortical system. Levels were not evaluated in the NAc or STR because low to nearly nonexistent (in the case of the STR) levels of N/OFQ-stimulated [^{35}S]GTPγS binding were found in these areas. Current studies in our laboratory (MH Miquel Kabbaj, personal communication) show alterations in N/OFQ-stimulated [^{35}S]GTPγS binding could be due to changes in the number of receptors available, the coupling efficiency of the receptor, and/or number of G-proteins present. The large reduction in NOP mRNA combined with the specific loss in binding in the cell body vs. the projection areas after a 6-OHDA lesion would suggest a differential expression of NOP protein in the soma as opposed to terminals.

The high levels of ^{125}I-[^{14}Tyr]-N/OFQ binding and N/OFQ-stimulated [^{35}S]GTPγS binding in the cortical regions (Neal et al., 1999a) is most likely due to the NOP located on nondopaminergic neurons within these regions. Alternatively, NOP proteins originating in the DA neurons may be transported to DA terminals in the PFC; however, this may represent a small subpopulation of the total pool of NOP in the cortex. In this scenario, the loss of such a subpopulation after 6-OHDA lesions may not be detected by this technique. Taken together, the orphanin system may interact with mesencephalic DA systems directly by means of its receptor on DA cell bodies and dendrites and indirectly through its receptor on adjoining circuitry at the level of the terminal.

Although changes in mRNA levels correlated with the extent of the 6-OHDA lesion, alterations in N/OFQ-stimulated [^{35}S]GTPγS binding did not (Fig. 7A). In other words, the decrease in N/OFQ-stimulated [^{35}S]GTPγS binding was not as great in the SNc compared with the VTA. The receptors remaining in the SNc may have an increased coupling capacity to compensate for the dramatic loss of receptors, leading to slightly higher binding levels. Clearly, the ISH studies demonstrated that N/OFQ mRNA increased to a much greater extent in the SNc compared with the VTA. Assuming the mRNA was translated into protein, the higher levels of peptide in the SNc could have influenced the functional activity of the residual receptors.

Our observations suggest that the orphanin system is in an anatomic position to influence DA neuronal activity, supporting recent studies demonstrating N/OFQ influence on DA release. For example, 3 and 30 μg of N/OFQ (i.c.v.) decreases accumbal DA levels (measured with microdialysis) by 25 and 50% of baseline values, respectively (Murphy et al., 1996). Placement of N/OFQ (1 mM) into the VTA increases tegmental GABA, glutamate, and aspartate while decreasing basal DA levels by 40% in the NAc (Murphy et al., 1999). Local N/OFQ (0.1–10 μM) administration of 1- and 10-μM increases striatal DA levels by 383 and 398%, respectively (Konya et al., 1998). Interest-

ingly, N/OFQ (10 nmol, i.c.v.) reduces morphine-induced (5 and 10 mg/kg, i.p.) DA release in the NAc as measured by in vivo voltametry (Di Giannuario et al., 1999). Our current findings suggest a mechanism for this modulation: activation of NOP may directly inhibit DA neuronal activity at the level of the cell body and indirectly excite DA activity at the level of the terminal by means of disinhibition of adjoining circuitry (e.g., GABA).

The presence of the orphanin peptide and receptor in the mesostriatal DA system might suggest some involvement with sensorimotor functions (Graybiel et al., 1994). Indeed at low and high doses, N/OFQ augments and inhibits locomotion, respectively (Reinscheid et al., 1995; Devine et al., 1996b; Florin et al., 1996). Moreover, a high dose of N/OFQ (10 nmol, i.c.v.) interferes with fine and gross motor coordination (Devine et al., 1996b). Orphanin FQ may be acting on the mesostriatal DA system to influence locomotion (Konya et al., 1998), but, as pointed out by Devine and colleagues (1996b), N/OFQ may be acting at several key regions involved in motor function (i.e., brainstem and basal ganglia).

The mesolimbic and mesocortical DA systems are implicated in motivation and reward (Le Moal and Simon, 1991; Bardo, 1998; Schultz, 1998), and, although N/OFQ alters DA release, N/OFQ (0.1–100 nmol, i.c.v.) does not appear to produce conditioned place preference or aversion (Devine et al., 1996a). However, N/OFQ (3 and 10 nmol, i.c.v.) impairs acquisition of morphine and alcohol conditioned place preference (Ciccocioppo et al., 1999; Murphy and Maidment, 1999b). In these studies, N/OFQ can influence several brain regions, due to the route of N/OFQ administration (i.c.v.), possibly confounding the findings. Although the role of the orphanin system in mediating mesocorticolimbic DA systems and, therefore, possibly interfering with motivational states remains to be fully determined, our findings lay some of the anatomic groundwork for understanding this complex interplay.

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