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Inputs to Serotonergic Neurons Revealed by Conditional Viral Transneuronal Tracing

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ABSTRACT

Descending projections arising from brainstem serotonergic (5HT) neurons contribute to both facilitatory and inhibitory controls of spinal cord "pain" transmission neurons. Unclear, however, are the brainstem networks that influence the output of these 5HT neurons. To address this question, here we used a novel neuroanatomical tracing method in a transgenic line of mice in which Cre recombinase is selectively expressed in 5HT neurons (ePet-Cre mice). Specifically, we injected the conditional pseudorabies virus recombinant (BA2001) that can replicate only in Cre-expressing neurons. Because BA2001 transports exclusively in a retrograde manner, we were able to reveal a subset of the neurons and circuits that are located upstream of the Cre-expressing 5HT neurons. We show that diverse brainstem regions differentially target the 5HT neurons of the dorsal raphe (DR) and the nucleus raphe magnus of the rostroventral medulla (RVM). Among these are several catecholaminergic and cholinergic cell groups, the periaqueductal gray, several brainstem reticular nuclei, and the nucleus of the solitary tract. We conclude that a brainstem 5HT network integrates somatic and visceral inputs arising from various areas of the body. We also identified a circuit that arises from projection neurons of deep spinal cord laminae V–VIII and targets the 5HT neurons of the NRM, but not of the DR. This spinoreticular pathway constitutes an anatomical substrate through which a noxious stimulus can activate 5HT neurons of the NRM and in turn could trigger descending serotonergic antinociceptive controls. J. Comp. Neurol. 514:145–160, 2009.

Indexing terms: PRV; pain; serotonin; brainstem; analgesia; RVM

Although there is considerable evidence that serotonergic neurons of the medullary raphe nuclei regulate the transmission of nociceptive messages at the level of the spinal cord and trigeminal nucleus, recent studies have deemphasized this contribution. For example, activity of serotonergic cells appears not to be required for the analgesia evoked by opioids or by electrical stimulation of the nucleus raphe magnus (NRM) or periaqueductal gray (PAG; Proudfit and Anderson, 1975; Yaksh et al., 1977; Barbaro et al., 1985; Porreca et al., 2002; Zeitz et al., 2002; Zhao et al., 2007). In fact, two major populations of spinally projecting neurons in the rostroventral medulla (RVM) that regulate nociceptive processing are not serotonergic (Potrebic et al., 1994; Mason, 1997; Gao and Mason, 2000). The "on" cell population is activated by noxious stimuli and facilitates the transmission of nociceptive messages as well as nocifensive reflexes; the "off" cell population contributes to the inhibition of nociceptive processing and, not surprisingly, is activated by morphine as well as by analgesia-producing electrical stimulation of the PAG. The 5HT neurons, by contrast, constitute a heterogeneous population, with slow, regular discharge patterns and variable responses to noxious stimuli and to opioid agonists (Auerbach et al., 1985; Chiang and Pan, 1985; Gao et al., 1998; Gao and Mason, 2001; Zhang et al., 2006). Thus, despite considerable evidence that 5HT neurons are activated by noxious stimulation (Dong et al., 1997; Suzuki et al., 2002; Chen et al., 2003; Imbe et al., 2007), whether that activation leads to a feedback antinociceptive control is not clear.

To date, there is no anatomical evidence for direct connections between spinal cord and brainstem 5HT neurons. Anterograde studies demonstrated projections from the cord to the RVM (Gallager and Pert, 1978; Abols and Basbaum, 1981; Cervero and Wolstencroft, 1984; Willis and Westlund, 1997) and the PAG (Beitz, 1982; Mantyh, 1982; Keay and Bandler,

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1993; Bernard et al., 1995; Vanderhorst et al., 1996; Mouton and Holstedge, 2000), but none has reported synaptic contacts between ascending terminals and 5HT neurons. Although spinal cord neurons can be retrogradely labeled after tracer injections into the RVM, these studies cannot distinguish between inputs to 5HT and non-5HT neurons.

Here we reexamined the question in the mouse using a technique that can specifically determine whether or not 5HT neurons receive direct or indirect inputs from spinal cord neurons. We used a modified pseudorabies virus (PRV) retrograde tracer (Bartha 2001; BA2001) developed by DeFalco et al. (2001). Infection by BA2001 spreads transneuronally in the retrograde direction, but the virus replicates only in Cre-expressing neurons and thus will spread only to neurons that are located upstream of these Cre-expressing neurons. To identify selectively the CNS networks that regulate serotonergic neurons, we injected BA2001 in the RVM and/or dorsal raphe (DR) of ePet-Cre mice (Scott et al., 2005), in which Cre recombinase is expressed exclusively in 5HT neurons. With these animals, we could follow the retrograde transneuronal transport of Cre-activated PRV from 5HT to non-5HT neurons by expression of viral-encoded green fluorescent protein (GFP). To validate some of the findings, we also report anterograde transport of biotinylated dextran amine (BDA) from BA2001-labeled areas that were not previously reported to project directly to the DR. We report that diverse brainstem regions differentially target the 5HT neurons of the DR and the NRM. Among these are several catecholaminergic and cholinergic cell groups. We found evidence for a circuit that inputs the 5HT neurons of the NRM (but not of the DR). This circuit originates in presumed nociresponsive neurons of spinal cord laminae V–VIII and defines a route through which 5HT neurons at the origin of descending antinociceptive controls can be activated by noxious stimuli.

MATERIALS AND METHODS

Animals

All experiments were reviewed and approved by the Institutional Care and Animal Use Committee at the University of California San Francisco. ePet-Cre mice express the Cre recombinase under the control of the ePet-1 promoter (Scott et al., 2005), which is selective for 5HT neurons.

Virus and infections

We used injections of the BA2001 virus, which is a thymidine kinase-deficient pseudorabies virus (PRV) recombinant (DeFalco et al., 2001). This recombinant virus is dependent on a Cre-mediated recombination event to coexpress thymidine kinase (which is required for replication) and tau-GFP (a GFP reporter).

For infection of mouse brainstem, ePet-Cre animals were anesthetized by an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (8.0 mg/kg) mixture and placed in a stereotaxic apparatus. After incision of the skin overlying the brainstem, a small burr hole was made directly over the midline of the skull. To target the dorsal raphe or NRM, we inserted a micropipette attached to a manual microinjector (Sutter Instruments, Novato, CA) to 3 or 6 mm depth, respectively, below the skull. After a pause of 2 minutes for pressure equalization, we made a single injection of 1.0 μl (approximately 10^6 total plaque forming units) of the concentrated BA2001 suspension. To minimize spread of the injection, it was made over a 1-minute period, and the micropipette was kept in place for an additional 2 minutes, then withdrawn. Once injections were complete, the scalp was sutured, and the mouse was kept under a warming lamp until it recovered from the anesthesia then returned to standard housing. In this study, mice were followed up to 5 days postinfection. We did not observe any morbidity or mortality among the mice infected with BA2001.

Fluorogold and BDA injections

To inject Fluorogold (FG) or BDA in the brain of BA2001-infected ePet-Cre mice, we followed the procedure described above for virus injections. We made a single injection of FG (1.0 μl of a 2.0% solution) in the spinal cord or BDA (0.5 μl of a 10% solution) in the cochlear nucleus. Animals injected with FG or BDA were killed 3 days or 2 weeks postinjection, respectively.

Immunohistochemistry

Antibodies included mouse antityrosine hydroxylase (1:5,000; RBI, Nattick, MA; No. T-186), rat anti-5HT (1:500; Protos Biotech Corporation; No. NT 101), and rabbit anti-GFP (1:1,000; Molecular Probes, Eugene, OR). Our studies have established that there is no GFP immunoreactivity in wild-type mice (i.e., in mice that were not infected with the BA2001). Anti-TH antibodies were raised with rat TH as the immunogen. This antibody recognizes an epitope present in the N-terminal region (between amino acids 9 and 16) of both rodent (~60 kD) and human (62–68 kD) TH. In Western blots of PC-12 rat pheochromocytoma cells, the anti-TH antibody detects a single band at 60 kD. Anti-5HT antibodies were raised in rats with serotonin conjugated to hemocyanin as immunogen. The patterns of 5HT and TH immunoreactivity that we observed with these antisera are very comparable to those reported in many other studies of the distribution of 5HT and TH in the mouse and rat brain (Dahlstrom and Fuxe, 1965; Beitz, 1982; Vander-Horst and Ulfhake, 2006).

One, five, or seven days after injection of BA2001, infected mice were anesthetized (Nembutal; 100 mg/kg) and then perfused transcardially with 10 ml saline (0.9% NaCl) followed by 30 ml of 3.7% formalin in PB 0.1 M, pH 7.4, at room temperature (RT). Tissues were dissected out, postfixed in the same solution for 3 hours, and cryoprotected in 30% sucrose phosphate-buffered saline (PBS) overnight at 4°C. Twenty (spinal cord)- or forty (brain)-micrometer cryostat sections were preincubated for 30 minutes at RT in PBS, pH 7.4, containing 0.5% Triton X-100 and 10% normal goat serum (NPBST) and then immunostained overnight at RT in the same buffer containing the primary antibodies. After being washed in NPBST, sections were incubated for 1 hour with Alexa-conjugated anti-IgG secondary antibodies (1:700), rinsed in NPBST, mounted in fluoromount-G (Southern Biotechnology, Birmingham, AL), and coverslipped. To detect BDA-positive fibers, brainstem sections were preincubated for 30 minutes at RT in NPBST and then incubated for 3 hours at RT in NPBST containing Alexa 546-conjugated streptavidin (1:1,000). After final rinses (3 × 10 minutes) in PBS, sections were mounted in fluoromount-G and coverslipped.

Sections were viewed with a Nikon Eclipse fluorescence microscope, and images were collected with a Spot camera.

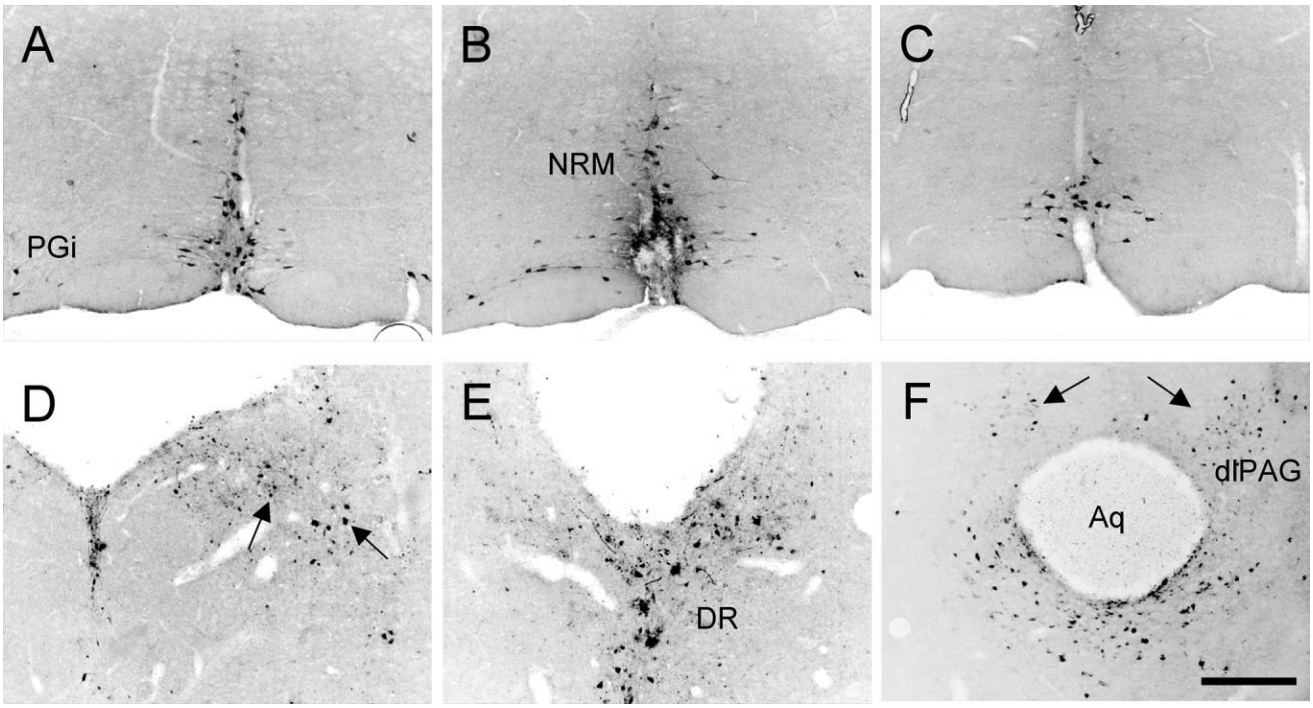

Figure 1.
Replication of BA2001 in brainstem 5HT neurons. We injected BA2001 into the rostroventral medulla (**A–C**), which includes the 5HT-containing neurons of the nucleus raphe magnus, or dorsal raphe (**D–F**) of ePet-Cre mice, in which the Cre recombinase is expressed exclusively in 5HT neurons. Three days after the injection, we observed GFP in large number of neurons at the injection site (black). This demonstrates that replication of BA2001 was restored in Cre-expressing neurons. There are also many GFP-positive neurons in areas that do not contain 5HT neurons (arrows in D,F), which is indicative of retrograde, transneuronal transfer of BA2001 from 5HT neurons to non-5HT neurons. Aq, aqueduct; dlPAG, dorsolateral periaqueductal gray; DR, dorsal raphe; NRM, nucleus raphe magnus; PGi, paragigantocellularis. Scale bar = 100 μm.

Brightness and contrast were adjusted in Adobe Photoshop, version 6.0. Magenta-green copies of Figures 2, 4–6, and 11 are available online as Supporting Information. Brain structures were determined according to the atlas of Paxinos and Franklin (2001).

RESULTS

In this study, we used a recombinant pseudorabies virus that lacks the thymidine kinase (TK) gene. The virus is normally replication incompetent, but it can be rendered competent because the virus encodes a transcriptional cassette (containing tau-GFP-IRES-TK) that is transcribed after Cre-mediated excision of a lox-STOP-lox sequence between the CMV promoter and the transcriptional cassette (DeFalco et al., 2001). BA2001 cannot replicate in neurons of wild-type animals; however, when BA2001 infects a Cre-expressing neuron, the lox-STOP-lox sequence is excised. This initiates expression of TK, which not only restores replication competence to the recombinant but also allows the spread of this recombinant to any other neuron with which the infected neuron is in synaptic contact. As a result, all neurons that are upstream of the Cre-expressing neuron will replicate the recombinant virus. Because GFP is concurrently expressed when the lox-STOP-lox sequence is excised, the transneuronal spread of PRV can be followed by immunostaining for GFP.

Previous studies with BA2001 reported low to undetectable Cre-independent replication in their systems (DeFalco et al., 2001; Yoon et al., 2005; Wintermantel et al, 2006; Campbell and Herbison, 2007). It was critical to confirm this feature in our studies. Thus, to examine whether BA2001 replicates only in neurons that express the Cre recombinase, we injected BA2001 in wild-type animals (i.e, mice that do not express Cre recombinase). Consistently with previous reports, we found no evidence of BA2001 replication (no expression of GFP; data not shown).

Figure 1 illustrates an example of a BA2001 injection that targeted the midline RVM, i.e., in the region of the NRM (upper row), and another that targeted the DR (lower row) of ePet-Cre mice. In both cases, we detected intense GFP staining in the region of the injection site. The GFP signal identifies cells infected by the recombinant of BA2001 that had undergone Cre-dependent recombination (black in Fig. 1). With a view to determining whether the labeling arose from direct infection of 5HT cells by BA2001 or whether it resulted from retrograde transneuronal transport from primary infected cells, we performed double-labeling experiments with antisera directed against 5HT. This analysis revealed that both 5HT and non-5HT neurons within the injected raphe contained the virus (Fig. 2). The presence of GFP in non-5HT neurons must have arisen from the transneuronal transport of Cre-recombined BA2001 from 5HT (first-order/Cre-expressing neuron) to non-5HT (second-order cell that is upstream of the first-order Cre-expressing cell) neu-

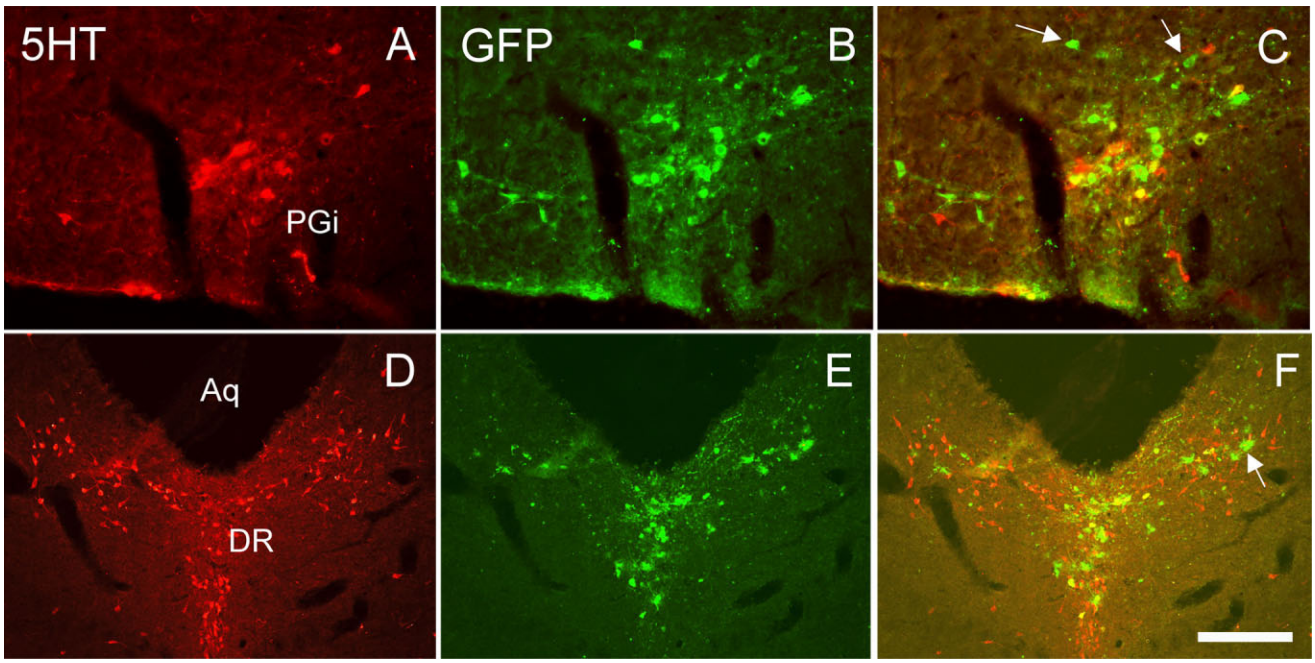

Figure 2.
Cre-dependent GFP expression from BA2001 is present in both 5HT and non-5HT neurons. Double-labeling experiments with antibodies against 5HT revealed that GFP was present in both 5HT and non-5HT neurons. This was the case for injections that targeted the NRM (**A–C**) or the dorsal raphe (**D–F**) and demonstrates that the Cre-recombinant of BA2001 was retrogradely transported from 5HT neurons (first-order neurons that express Cre) to non-5HT neurons (second-order and higher order neurons, upstream of the 5HT neurons), both within and outside of the raphe nuclei (see also Fig. 1). Arrows point to non-5HT neurons that contain GFP. Aq, aqueduct; DR, dorsal raphe; PGi, paragigantocellularis. A magenta-green version of this figure is available online as Supporting Information. Scale bar = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

rons. This observation indicates that non-5HT neurons send inputs to 5HT neurons, within the raphe nucleus.

Time-dependent retrograde transneuronal transport of BA2001: 24 hours

We also detected PRV infection as marked by GFP expression outside of the injected raphe nuclei, throughout the brainstem (see also Supp. Info. Table 1). Twenty-four hours after PRV injection in the DR, we detected high numbers (>20 cells per section) of GFP-positive neurons in the locus ceruleus (LC), Barrington's nucleus, lateral and dorsolateral periaqueductal gray (PAG), laterodorsal tegmental nucleus, area postrema, vestibular nucleus, dorsal cochlear nucleus, and paraventricular nucleus of the thalamus. Moderate to low numbers of GFP cells (five to 20 cells per section) were detected in the subceruleus area, nucleus of the solitary tract (NTS), dorsomedial PAG, prepositus hypoglossal nucleus, peduculopontine tegmental nucleus (PPTg), and A1, A5, and A7 cell groups. Scattered labeled cells (one to five per sections) were found in the NRM, paraventricular and lateral hypothalamus, striatum, septum, and cerebellum (Purkinje cells).

Twenty-four hours after injection of PRV in the RVM, we found the highest number of labeled neurons (>20 per section) in the PPTg, ventral LC/subceruleus area, and paragigantocellular nucleus. Moderate to low numbers of labeled neurons (five to 20 cells per section) were found in the lateral and ventrolateral PAG, B9 cell group, DR, vestibular nucleus, prepositus hypoglossal nucleus, A7 cell group, paraventricular and lateral hypothalamus, and septum. Scattered labeled neurons (fewer than five cells per section) were observed in the dorsomedial PAG, NTS, and A1 and A5 cell groups.

Time-dependent retrograde transneuronal transport of BA2001: 48 hours

Two days after the injection, we recorded a pattern of labeling similar to that seen at 24 hours, with a slight overall increase in the number of GFP-positive cells. We also detected GFP-positive neurons in areas that were not labeled at 24 hours. For example, injections in either the DR or the RVM resulted in labeling of neurons in the nucleus reticularis magnocellularis and gigantocellularlis. Injections of PRV in the DR, but not in the RVM, also resulted in labeling of neurons in the medial parabrachial nucleus at 48 hours.

Time-dependent retrograde transneuronal transport of BA2001: 5 days (Fig. 3)

Five days after PRV injections, we observed a significant decrease in the number of labeled neurons at the injection sites (DR and RVM). We presume that this resulted from a lytic effect of the virus. However, at the 5-day time point, we also recorded labeling in regions not seen at the earlier time points, including the deeper laminae (V–VIII) of the spinal cord (five to 10 cells per section; Fig. 4). Lower numbers (one to five cells per section) were recorded in superficial laminae of the trigeminal nucleus caudalis (TNC). In con-

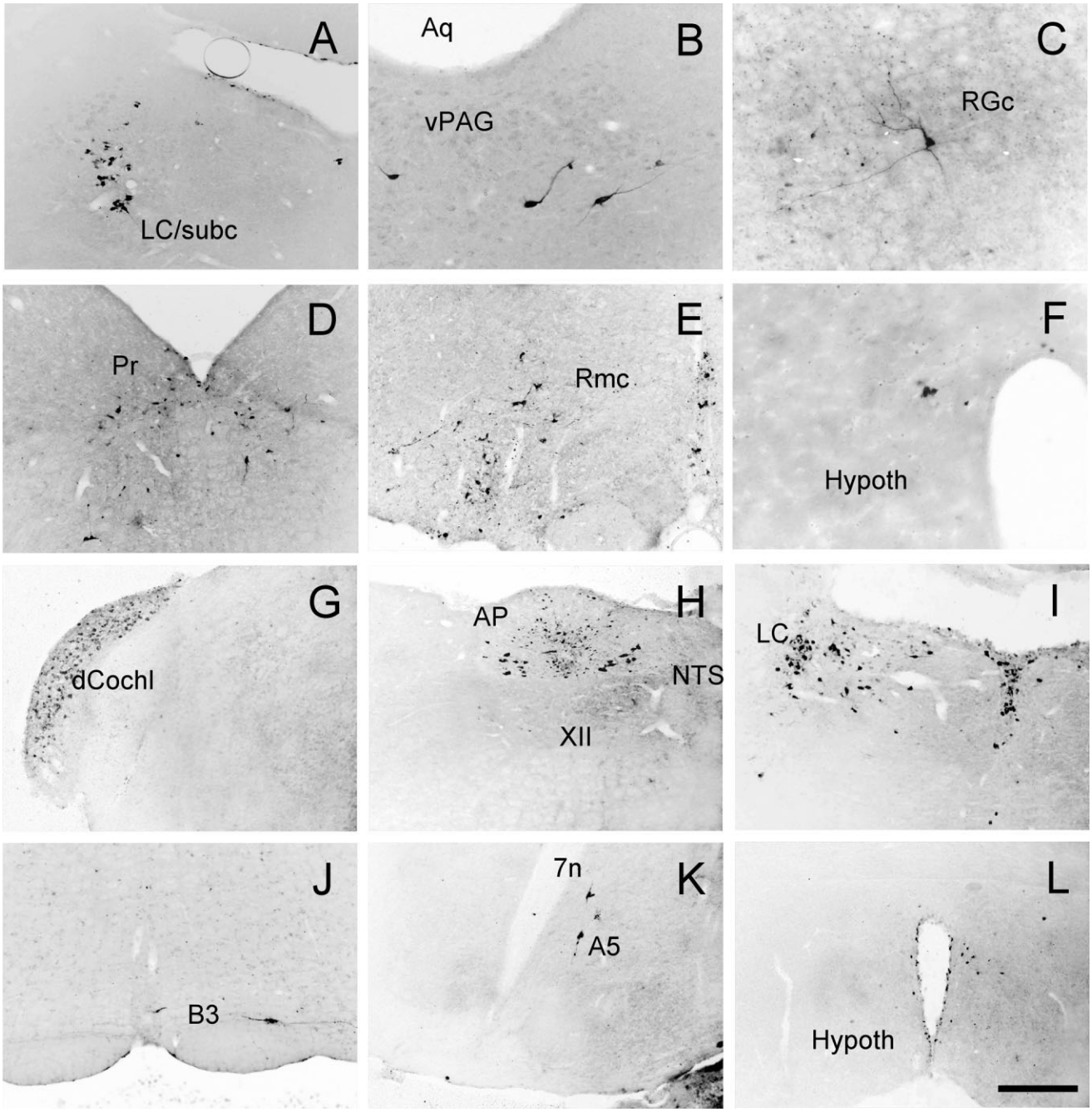

Figure 3.
Retrograde transneuronal transport of BA2001 in the brain after infection of Cre-expressing neurons. Five days after injections into the rostroventral medulla (**A–F**) or dorsal raphe (**G–L**), GFP was detected in diverse areas of the brain (black neurons), including the locus ceruleus/subceruleus region (LC/subc), ventral PAG (vPAG), nucleus reticularis gigantocellularis (RGc), nucleus reticularis magnocellularis (Rmc), prepositus hypoglossi nucleus (Pr), hypothalamus (Hypoth), dorsal cochlear nucleus (dCochl), area postrema (AP), nucleus of the solitary tract (NTS), area B3, and the A5 noradrenergic cell group. XII, motor XII, 7n, seventh nerve; Aq, aqueduct. Scale bar = 50 μm in L (applies to C,F,L); 100 μm for A,B,D,E,G–K.

trast, DR injections never resulted in labeling of spinal cord or TNC neurons, even at the later time points. The fact that GFP-positive neurons appeared in spinal cord and TNC only at longer survival times suggests that the connection between spinal cord/TNC projection neurons and 5HT neurons of the RVM is multineuronal.

Neurochemistry of BA2001-infected neurons

We found , based on double-labeling experiments with tyrosine hydroxylase (TH) antisera, that the greatest number of non-5HT/virus-infected neurons is catecholaminergic. Figure 5 illustrates examples of neurons that were infected (green)

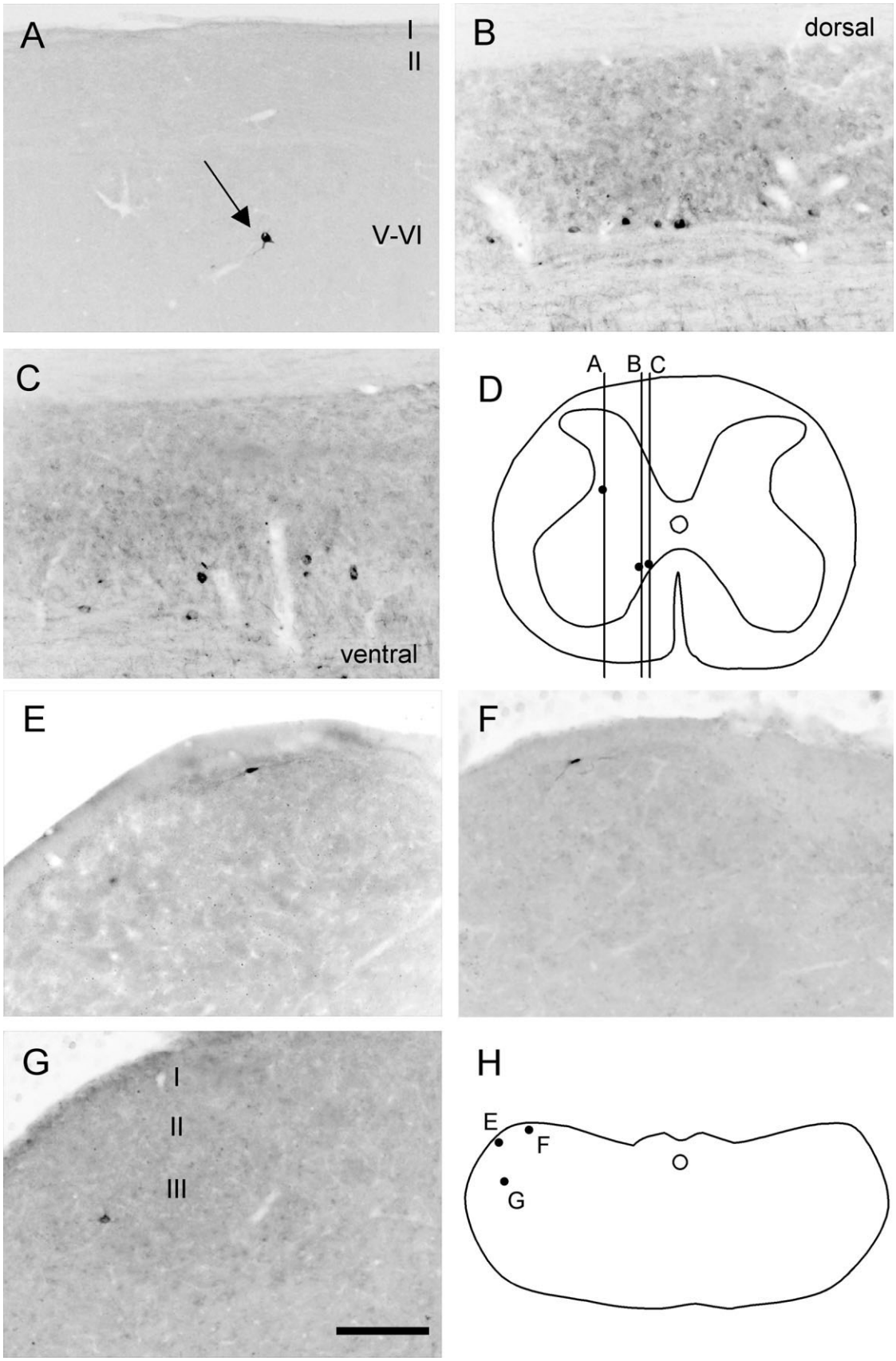

Figure 4.
Ascending spinal and trigeminal nucleus pathways target 5HT neurons of the nucleus raphe magnus, but not those of the dorsal raphe. Injections of BA2001 directed at the NRM resulted in retrograde infections of neurons in deep laminae (V–VIII) of the spinal cord (**A–C**) and of neurons in superficial laminae (I–III) of the trigeminal nucleus caudalis (**E–G**). Lines A–C in **D** show the location of the labeled neurons in the photomicrographs of A–C, respectively, from sagittal sections of the spinal cord. The black dots in **H** show the location of neurons in the photomicrographs of E–G, which are taken from the trigeminal nucleus caudalis. In contrast to RVM, injections of BA2001 in the DR did not result in retrograde infection of spinal cord or of trigeminal nucleus caudalis neurons. Scale bar = 100 μm.

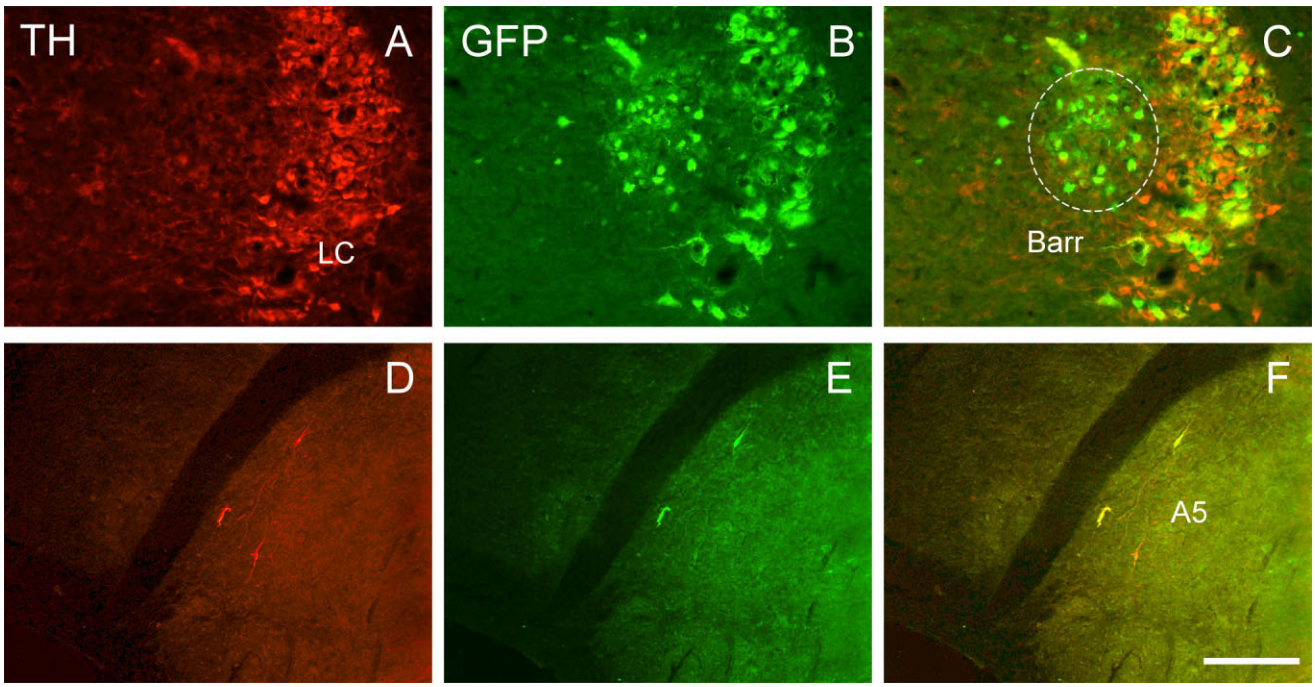

Figure 5.
Brainstem catecholaminergic cell groups target 5HT neurons of the dorsal raphe. Injections of BA2001 in the dorsal raphe resulted in its retrograde transport to all catecholaminergic cell groups of the brainstem as determined by expression of GFP. **A–C:** GFP-positive noradrenergic neurons of the locus ceruleus. **D–F:** GFP-positive noradrenergic neurons of the A5 cell group. The same results were obtained after injections of BA2001 in the RVM (see Supp. Info. Table 1). Note that in the LC GFP was present in both NA (yellow) and non-NA (green) neurons. Barrington's nucleus (Barr) also contained large numbers of virus-infected neurons. These results indicate that both the catecholaminergic cell groups and the Barr neurons are presynaptic to the 5HT neurons of the DR. A magenta-green version of this figure is available online as Supporting Information. Scale bar = 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

and expressed TH (red) in the A5 (lower row) and A6 cell groups (upper row) after injection of BA2001 in the DR. As described above, double-labeled neurons were recorded in several noradrenergic (NA) cell groups (A1, A2, A5, A6, and A7), indicating that the brainstem NA system lies upstream of the 5HT system. NA-positive neurons were detectable within 24 hours of the PRV injection, which suggests that there is a monosynaptic connection between the TH- and 5HT-positive neurons.

The laterodorsal tegmental (LDTg) and pedunculopontine tegmental (PPTg) nuclei also labeled intensely for GFP (i.e, were virus infected). Although we found that the PPTg collateralizes to both DR and NRM 5HT neurons, we found only GFP-positive LDTg neurons after PRV injection into the DR (Fig. 6).

We found strong evidence for reciprocal connections between the DR and the NRM. Thus, BA2001 injections in DR and RVM resulted in retrograde labeling of 5HT neurons in the NRM and DR, respectively. This is illustrated in Figure 7A–C, which shows neurons located in the ventral PAG that contain BA2001 after it was injected in the RVM. Almost 50% of the PAG neurons labeled after RVM injection were concentrated in the DR, and all of these were 5HT immunoreactive. These results illustrate that large numbers of 5HT neurons in the DR target the 5HT neurons of the NRM. By contrast, we found many fewer RVM cells labeled after injection of BA2001 into the DR. Together these results indicate that there is limited reciprocity in the circuits between the 5HT neurons of the NRM and the DR. As discussed below, we suggest that the DR to NRM 5HT projection is an indirect one.

To determine whether PRV-infected brainstem neurons that were retrogradely labeled after RVM injection also project to the spinal cord, we injected the retrograde tracer FG in the spinal cord of three BA2001-infected ePet-Cre mice. Three days later, we studied the distribution of FG^+ and GFP^+ neurons. Figure 8 illustrates that many brainstem neurons that target the 5HT neurons of the RVM (i.e., are GFP-positive) send a collateral to the spinal cord (i.e., are FG-positive). These include non-5HT neurons of the NRM, nucleus reticularis magnocellularis (Rmc), LC/subceruleus, and A5. By contrast and consistent with reports of there being minimal spinal projections from the PAG and DR, we did not find double labeling of BA2001-positive neurons in these regions.

Differential inputs to the dorsal raphe and nucleus raphe magnus

A more detailed analysis of viral labeling after DR or RVM injections revealed that there are both common inputs to these serotonergic brainstem nuclei and regions that differentially target the two sites. For example, we uncovered a strong projection from neurons of the dorsolateral PAG (dlPAG) to 5HT neurons of the DR (Fig. 1F). The same region did not label after BA2001 injection into the RVM (data not shown). Connections between the dlPAG and DR and between the dlPAG and NRM have, in fact, been reported previously (Gallager and Pert, 1978; Beitz et al., 1983; Hermann et

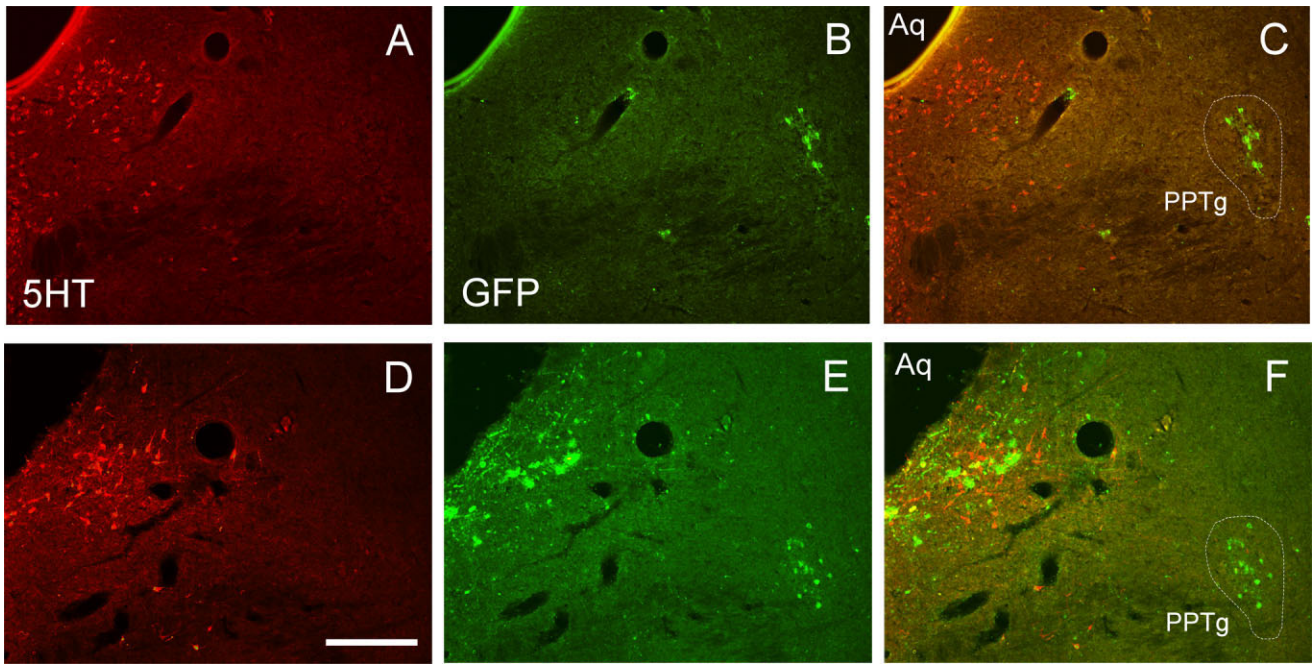

Figure 6.
Brainstem cholinergic cell groups target 5HT neurons of the dorsal raphe and NRM. Injections of BA2001 into either the NRM (**A–C**) or the dorsal raphe (**D–F**) resulted in retrograde infection of neurons in the peduculopontine tegmentum nucleus (PPTg), indicating that these neurons, presumably cholinergic, send inputs to 5HT neurons of the brainstem. In contrast to the PPTg, we detected GFP in cholinergic neurons of the laterodorsal tegmentum nucleus only when it was injected in the dorsal raphe (see Supp. Info. Table 1). Aq, aqueduct. A magenta-green version of this figure is available online as Supporting Information. Scale bar = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

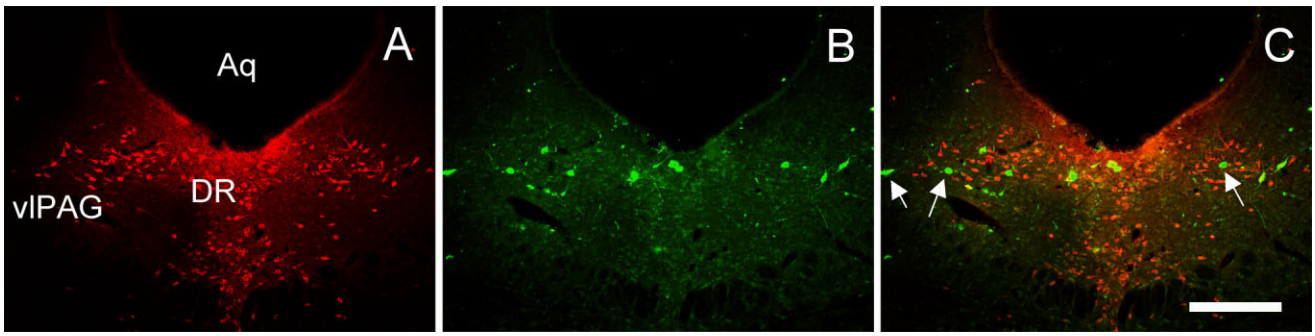

Figure 7.
Connections among neurons of the DR, PAG, and RVM. After injection of BA2001 into the RVM, we observed retrograde labeling in 5HT neurons of the dorsal raphe as well as in non-5HT neurons (arrows) in the immediate vicinity. Many of the latter are located in the ventrolateral PAG. Aq, aqueduct; DR, dorsal raphe; vlPAG, ventrolateral periaqueductal gray. A magenta-green version of this figure is available online as Supporting Information. Scale bar = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

al., 1997; Peyron et al., 1998; Lee et al., 2005). Our results thus indicate that, whereas dlPAG neurons target the 5HT population of DR, they target the non-5HT population of the RVM. Similarly, we found significant labeling of neurons of the dorsal cochlear and Barrington's nuclei after injection of BA2001 into the DR (Figs. 3G, 5, 9) but not after injection into the RVM (data not shown). In contrast, neurons located in lateral paragigantocellularis (LPGi) and Rmc nuclei target preferentially the 5HT neurons of NRM (Fig. 3C,E) but not those of the DR (data not shown).

Finally, Figure 9 illustrates that subdivisions of a given brainstem nucleus can differentially target the 5HT neurons of the DR and NRM. Here, for example, we observed that neurons located in the dorsal aspect of the LC (A6) project to 5HT neurons of the DR. In contrast, the RVM is targeted by ventrally located LC neurons, some of which appear to extend ventrally into the nucleus subceruleus. Double labeling of these BA2001-infected neurons with antisera against TH confirmed that these differential inputs were both noradrenergic.

Anterograde studies

We were surprised to find significant labeling of neurons in the dorsal cochlear nucleus following injections in DR, in that

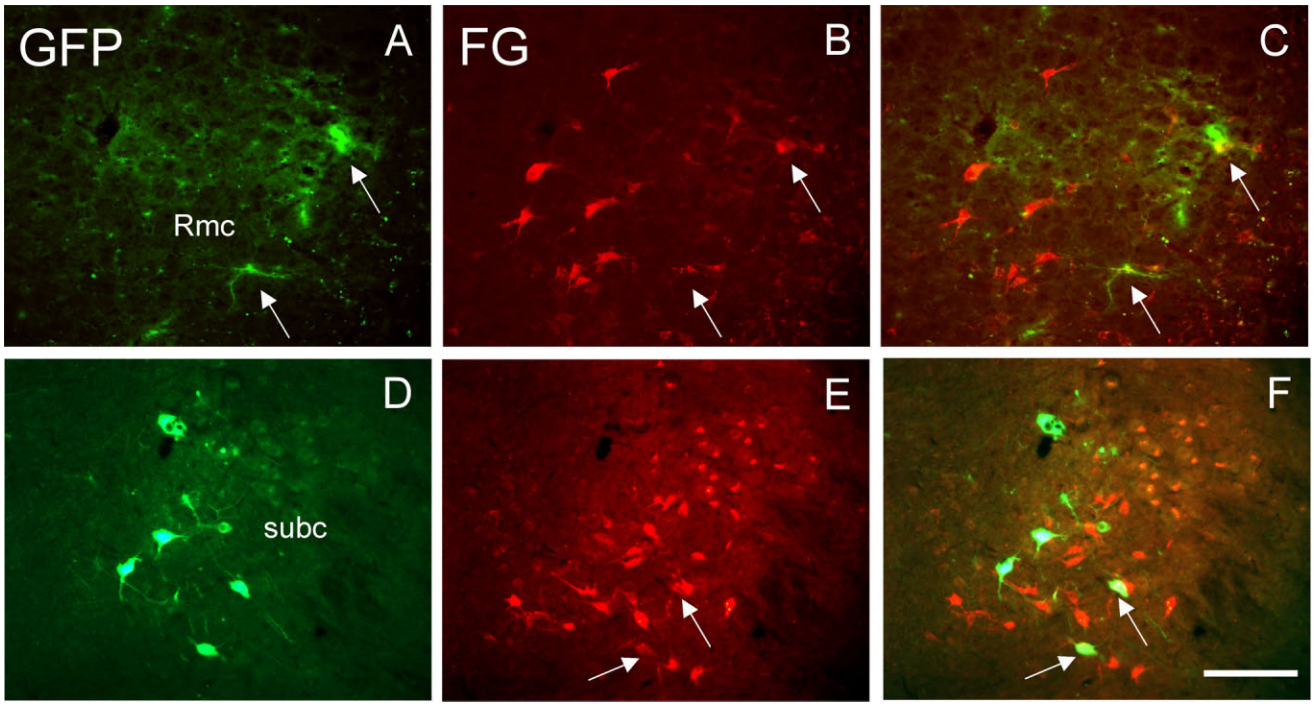

Figure 8.
Some spinally projecting neurons of the brainstem have collaterals that innervate 5HT neurons of the NRM. Coinjections of BA2001 that targeted the NRM and of Fluorogold in the spinal cord of ePet-Cre mice resulted in double labeling of neurons in diverse areas of the brainstem, including the nucleus reticularis magnocellularis (Rmc, **A–C**) and the subceruleus (subc, **D–F**). A magenta-green version of this figure is available online as Supporting Information. Scale bar = 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

no previous study had reported that dorsal cochlear neurons project to the DR. Clearly this pattern of labeling could have arisen from an indirect projection, but the numbers of neurons that we recorded were nevertheless unexpected. To provide an independent, reciprocal approach to determining whether dorsal cochlear neurons input 5HT neurons of the DR, we injected the anterograde tracer BDA in the cochlear nucleus and recorded all regions in the brainstem that contained BDA-positive terminals. Figure 10 shows that the injection site was restricted to the cochlear nucleus, with little or no spread to the more dorsally situated cerebellum. As expected, we found that cochlear fibers send a massive projection via the trapezoid body (Figs. 10C, 11I) and terminate densely in the superior olive (Fig. 10C) and inferior colliculus (Fig. 10B). However, of particular relevance to the question at hand, anterograde labeling was also detected in the dorsolateral PAG (dlPAG; Fig. 11A,B), ventrolateral PAG (data not shown), and A1 and A7 noradrenergic cell groups (Fig. 11G,H), with a limited projection to the LC (Fig. 11D,E). We never observed labeled terminals in the raphe nuclei, including the dorsal raphe (Fig. 10B). We also studied the pattern of projections arising from injections of BDA into the region of the cerebellum that lies just dorsal to the dorsal cochlear nucleus. These injections spared the dorsal cochlear nucleus. The cerebellar injections produced a very different labeling pattern. In these animals, we never detected BDA-positive terminals in the dlPAG or in the A1 and A7 noradrenergic cell groups. We did, however, observe some anterograde labeling in the LC and vlPAG (data not shown).

We conclude, based on the results of the anterograde labeling studies, that the retrograde labeling of the dorsal cochlear nucleus after injections of the virus into the DR must have resulted from labeling of a circuit that indirectly inputs the DR. Specifically, because injections of BA2001 into the DR resulted in retrograde labeling of all noradrenergic group neurons, including the LC (Fig. 11F) as well neurons in the dlPAG (Fig. 11C), we suggest that infection of neurons of the dorsal cochlear nucleus was indirect. In other words, BA2001 injections into the 5HT neurons of the DR could readily have resulted in labeling of neurons of the dorsal cochlear nucleus after retrograde transneuronal passage of the virus, via connections with neurons of the dlPAG, vlPAG, A1, A7, and LC, all of which project to the 5HT neurons of the dorsal raphe. Figure 10 further illustrates that unilateral injections of BDA into the cochlear nucleus resulted in extensive contralateral labeling of the dorsal cochlear nucleus. The presence of these dense reciprocal connections would, if anything, enhance any transneuronal retrograde labeling of dorsal cochlear neurons, a feature that conceivably contributes to our detection of labeling of these neurons after injections of BA2001 into the DR.

In contrast to the labeling of dorsal cochlear neurons after BA2001 injections that targeted the DR, we found no labeling after injections directed at the NRM. This result not only distinguishes the circuits that engage the DR and NRM but provides a critical control for the possibility that labeling of the dorsal cochlear nucleus resulted from uptake of BA2001 by fibers of passage in the region of the 5HT neurons of the DR,

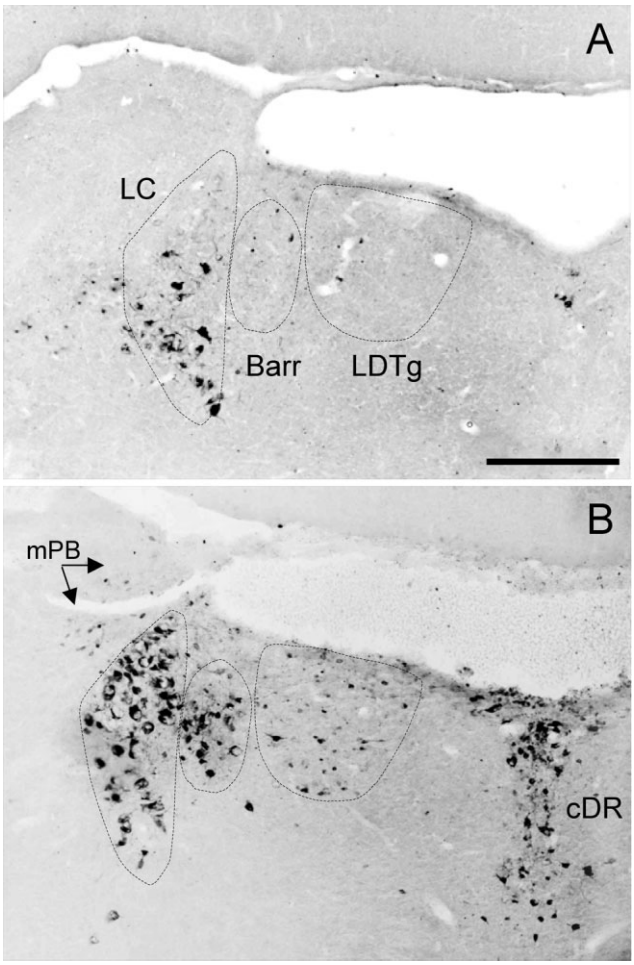

Figure 9.
Differential inputs to the 5HT neurons of the dorsal raphe and the nucleus raphe magnus. Injections of BA2001 into the NRM (**A**) or the dorsal raphe (**B**) resulted in differential labeling of catecholaminergic and cholinergic neurons of the brainstem. With injection into the NRM, we observed retrograde infection of neurons in the ventral part of the locus ceruleus (LC) and the more ventrally located subceruleus. In contrast, with injections in the DR, we found retrograde infection of neurons that were concentrated in the more dorsal part of the LC. Dorsal raphe injections of BA2001 also resulted in retrograde infection of Barrington nucleus (Barr), medial parabrachial nucleus (mPB), and cholinergic neurons of the laterodorsal tegmental nucleus (LDTg). cDR, caudal dorsal raphe. Scale bar = 50 μm.

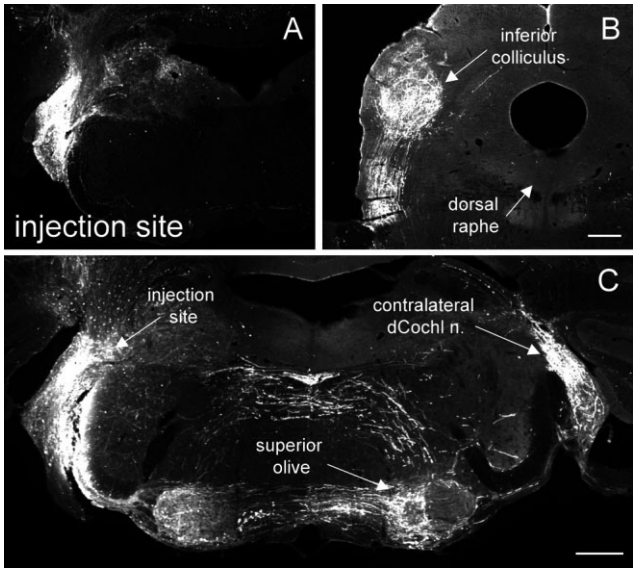

Figure 10.
Anterograde labeling of brainstem auditory nuclei after injection of BDA into the cochlear nucleus. **A:** In this case, the injection site was restricted to the cochlear nucleus and did not spread dorsally to the cerebellum. Cochlear nucleus axons course through the trapezoid body and terminate in major brainstem auditory nuclei, including the superior olive and inferior colliculus (**B**). In addition, there is a dense projection to the contralateral dorsal cochlear nucleus (**C**). Note the lack of labeling in the dorsal raphe (B). Scale bars = 100 μm in A (applies to A,B); 100 μm in C.

namely, in the neurons that rendered the virus replication competent. Figure 11 illustrates this important point. Thus, despite the presence of bundles of trapezoid body axons in the immediate vicinity of the 5HT neurons of the NRM (Fig. 11I), we never found labeling of the dorsal cochlear nucleus after BA2001 injections into this region. This clearly indicates that fiber of passage uptake of replication-competent BA2001, after its release from neighboring 5HT neurons, is not a significant concern in the use of BA2001 in our studies.

DISCUSSION

This Cre-dependent viral transneuronal retrograde tracing approach permitted a comprehensive analysis of the brainstem and spinal cord networks that modulate the 5HT population of raphe neurons in the RVM and midbrain PAG of the mouse. We conclude that, within the brainstem, most catecholaminergic and cholinergic neurons send strong inputs to 5HT neurons of both the NRM and the DR. Furthermore, the detection of GFP in the periaqueductal gray, NTS, Barrington's nucleus, and dorsal cochlear nucleus illustrates that the 5HT neuronal network contributes to the integration of somatic, visceral, and auditory inputs that arise from various areas of the body. Finally, we have uncovered a polysynaptic pathway that links deep spinal cord projection neurons with 5HT neurons of the medullary but not of the midbrain raphe. We suggest that this pathway is a route through which 5HT neurons are activated by noxious stimuli. The latter, in turn, are likely part of the descending controls that regulate the transmission of nociceptive messages at the level of the spinal cord.

Technical considerations

Unlike traditional transneuronal tracers that are rapidly diluted after they cross synapses, viral tracers such as PRV replicate in each neuron of the circuit (self-amplification). This provides an intense signal that does not dampen as the virus progresses along the circuit. Electron microscopic studies have shown that PRV is preferentially released at sites of synaptic contact and that the virus is taken up by the terminals of input neurons (Card et al., 1993; Carr et al., 1999). Because of these unique properties, PRV is a powerful tool for retrograde transsynaptic tracing (Jasmin et al., 1997; O'Donnell et al., 1997; Leak et al., 1999). We recognize, how-

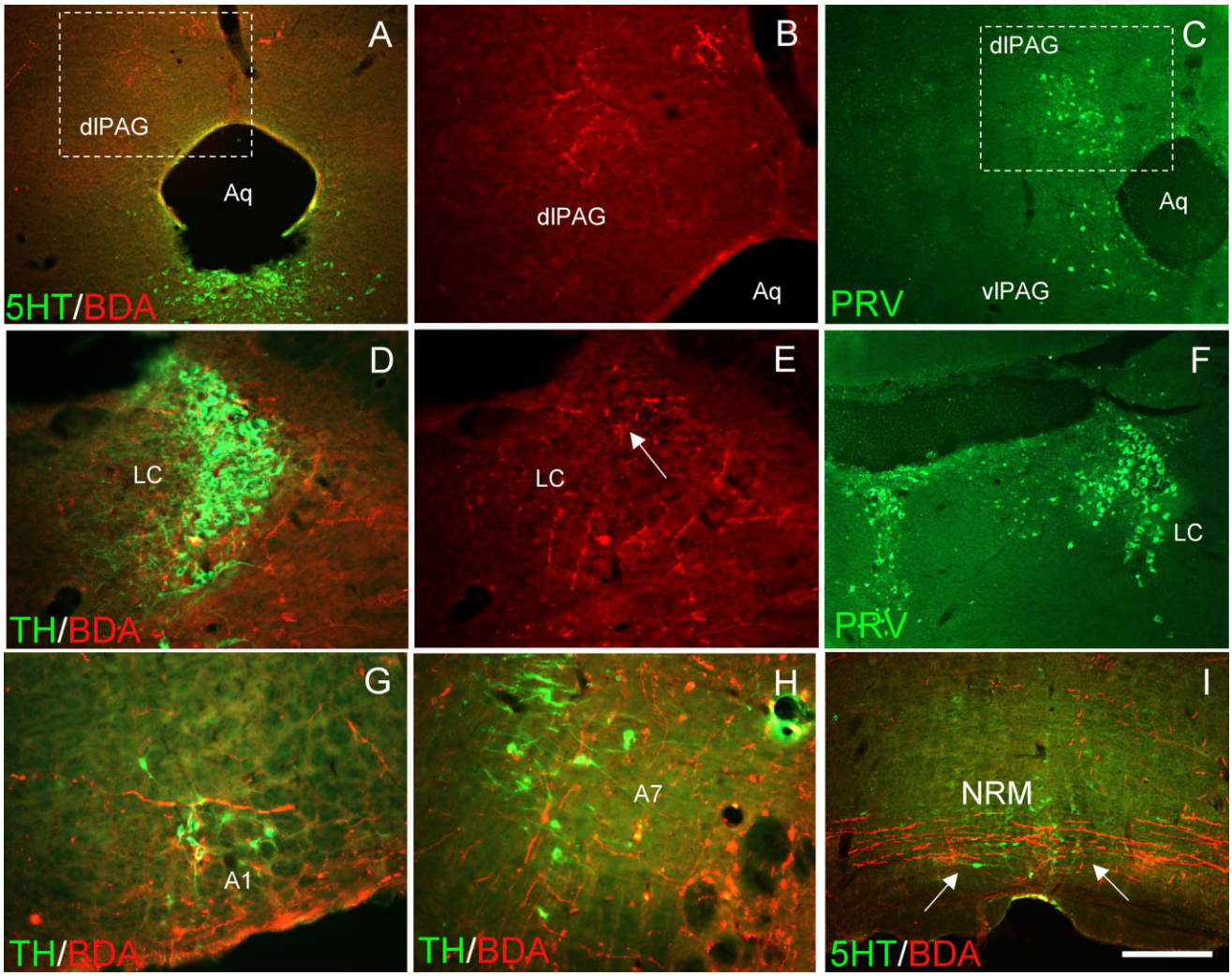

Figure 11.
Anterograde labeling of nonauditory brainstem regions after injection of BDA into the cochlear nucleus. We recorded considerable labeling in nonauditory loci, in addition to the brainstem auditory regions noted in Figure 10. Among these were the dorsolateral periaqueductal gray (**A**; see B for higher magnification of boxed area in A), the locus ceruleus (**D,E**), and the catecholaminergic cell groups A1 (**H**) and A7 (**G**). Note that the location of BDA-positive terminals in the dlPAG (**A,B**) overlaps with the location of retrogradely labeled neurons in dlPAG (green in **C**) after injection of BA2001 in the DR. Compare also the location of BDA-positive terminals in the locus ceruleus (LC; D,E) with the location of BA2001-infected LC neurons (green in **F**). BDA-positive cochlear nucleus axons course through the nucleus raphe magnus, via the trapezoid body (**I**). We never detected BA2001 in dorsal cochlear nucleus neurons after injection of BA2001 in the NRM. This argues against the possibility of uptake of BA21001 by fibers of passage in the trapezoid body. Aq, aqueduct; A1 and A7, noradrenergic cell groups A1 and A7, respectively; dlPAG, dorsolateral periaqueductal gray; LC, locus ceruleus; NRM, nucleus raphe magnus, vlPAG, ventrolateral periaqueductal gray. A magenta-green version of this figure is available online as Supporting Information. Scale bar = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ever, that we cannot rule out the possibility that nonsynaptic transfer of the virus occurs (see below). For this reason, we refer to transneuronal rather than transynaptic labeling of neurons and to multineuronal rather than polysynaptic circuits.

As with traditional retrograde tracers, PRV is not selective for a particular type of neuron. In contrast, the Cre-dependent BA2001 strain allows for the identification of networks that regulate a neurochemically distinct subpopulation of neurons (DeFalco et al., 2001; Yoon et al., 2005; Wintermantel et al., 2006; Campbell and Herbison, 2007). Although the system is powerful, some limitations should be noted. For example, in our study it is unlikely that BA2001 infected all Cre-expressing 5HT neurons. Thus, we probably underestimated the number of neurons that influence the 5HT populations of the medullary and midbrain raphe. Furthermore, the fact that many brain regions previously reported to project to the NRM or DR did not contain labeled neurons indicates that BA2001 was not taken up by all circuits that input the 5HT neurons. Clearly, only a subset of the brainstem networks that regulate directly or indirectly the 5HT neurons were revealed in the present study. We appreciate that false negatives are also possible if a specific group of neurons is resistant to infection, which could occur if their terminals lack the viral docking protein

receptors required for uptake (Martin and Dolivo, 1983; Sik et al., 2006). In other words, it is possible that differential virus uptake in specific neurotransmitter systems contributes to the pattern of viral spread. In fact, it is apparent from this and other studies that pseudorabies readily infects monoaminergic neurons. If such selective viral spread occurs, it could account for our failure to detect GFP-positive neurons in brain regions previously reported to project to the raphe nuclei. We are also cognizant of the fact that viral infection of neurons can alter their phenotypic properties (Ray and Enquist, 2004), which may also produce false negatives. We believe, however, that this was not a major concern in the present study, because we were able to identify many neurons that colabeled for neurotransmitter markers appropriate for particular brainstem subgroups (e.g., TH and 5HT). Thus, although the approach that we have taken revealed only a subset of the regions that differentially target the midbrain and medullary raphe, we are confident that the retrogradely labeled neurons are part of a circuit that either directly or indirectly involves the 5HT neurons of those raphe nuclei. In contrast, more traditional tracing studies can define inputs to only a mixed population of neurons. Without electron microscopic confirmation, these studies cannot unequivocally conclude that there are inputs to a defined population within the projection zone (e.g., 5HT neurons).

A major limitation of traditional retrograde tracing techniques is the often unavoidable uptake of the tracer by fibers of passage in the region of the injection site. On the other hand, despite reports of viral uptake by fibers of passage (Chen et al., 1999), this appears to be less of a concern with PRV compared with other tracers. This issue should be even less problematic with BA2001, insofar as this virus is not competent in neurons that do not express the Cre recombinase. Thus, if the virus were taken up and transported by fibers of passage that course in the region of 5HT neurons, it would not be detected. Because only 5HT neurons express Cre in the ePet-Cre mice (Scott et al., 2005; Braz and Basbaum, 2007), the GFP would never be expressed in the cell bodies that give rise to the fibers of passage in question. Of course, this feature defines one of the great strengths of this technique over traditional tracing methods, where spread of the injection always introduces questions regarding the circuit that generated the retrograde labeling pattern.

On the other hand, because BA2001 reverts to a wild-type virus after Cre recombination, it is possible that wild-type virus that is released from the Cre-expressing neurons is taken up by fibers of passage or by nearby, unrelated neurons (Jansen et al., 1993; Vizzard et al., 1995). Furthermore, because the injection site is adjacent to the cerebral ventricular system, we cannot rule out the possibility that there is release of competent virus into the cerebrospinal fluid, which could lead to nonsynaptic transfer of BA2001 over long distances. Although this caveat must be recognized, we do not believe it is a major concern in our study. Thus, for example, whereas injections of BA2001 in the DR resulted in retrograde labeling of dorsal cochlear nucleus neurons, injections of BA2001 in the NRM never did. Thus, if BA2001, after reverting to a competent state, were released extrasynaptically, we would have detected the virus in the dorsal cochlear nucleus after injections in the nucleus raphe magnus, which is traversed by trapezoid body axons.

Finally, and perhaps most importantly, it has been suggested that, by studying animals at different times after viral injection, it is possible to construct a circuit through which the different populations of neuron are labeled. Based on the replication time of the virus (Chen et al., 1999), labeling of neurons within 24 hours of injection is presumed to represent a direct (i.e., monosynaptic) connection with the target neurons (in this case, the 5HT neurons). By contrast, those neurons labeled at longer time points presumably represent a multineuronal circuit that is connected with the 5HT neurons. In the present study, we found that catecholaminergic brainstem nuclei were labeled 24 hours postinfection, whereas the spinal cord contained labeled neurons only 5 days after the injection. Keeping the caveat concerning transynaptic vs. transneuronal labeling in mind, we suggest that the catecholaminergic neurons are directly connected with the 5HT neurons but that the spinal cord input is part of a multineuronal circuit. However, we also report very early labeling (within 24 hours) of several structures that we strongly believe are at least two synapses away from the DR (see below). Based on these results, we conclude that it is inappropriate to make conclusions regarding mono- vs. polyneuronal connectivity based purely on the temporal pattern of retrograde transneuronal labeling.

Comparison with previous studies

In general, our results are consistent with previous studies that used traditional retrograde tracers (see Supp. Info. Table 1). However, our results allow for the more precise conclusion that the pattern of retrograde labeling arose from either direct or indirect connections with the 5HT neurons of the DR and NRM. The pattern did not merely reflect inputs to the region that included 5HT neurons. The areas that contained the largest numbers of GFP-positive neurons, notably, the monoaminergic and presumed cholinergic cell groups (see below), were previously demonstrated to project directly to the midbrain and medullary raphe nuclei (Sakai et al, 1977; Gallager and Pert, 1978; Abols and Basbaum, 1981; Beitz, 1982; Yezierski et al., 1982; Beitz et al., 1983; Marchand and Hagino, 1983; Li et al, 1990; Peyron et al., 1996; Hermann et al., 1997). In contrast, several regions not reported in previous studies were also retrogradely labeled with BA2001. We suggest that these regions do not project directly to the 5HT neurons but rather were labeled indirectly after retrograde transneuronal transfer of BA2001, via multineuronal circuits. Among these areas are the area postrema (AP), the dorsal cochlear nucleus, and the paraventricular thalamus. Because there is no evidence for direct projections from the AP to any of the raphe nuclei, it was surprising to find significant AP labeling. We suggest that BA2001-labeled neurons of the AP regulate 5HT neurons of DR indirectly, via a multineuronal circuit that involves the NTS, an area heavily labeled in our study. In fact, it has been shown that AP neurons, through their projection to NTS, modulate ascending interoceptive information and influence autonomic outflow (Shapiro and Miselis, 1985).

As for the AP, our ability to detect GFP expression in the dorsal cochlear and the paraventricular nuclei of the thalamus occurred at early time points and was somewhat unexpected. On the other hand, Ye and Kim (2001) did report that, in the cat, there is a direct projection (albeit a limited one) from the cochlear nucleus and adjacent structures to the DR. Furthermore, Kandler and Herbert (1991) reported labeled fibers in

the LC, a region that contained large numbers of GFP-positive neurons, after injections of PHA-L centered in the cochlear nucleus. Finally, and of functional relevance, several studies reported that DR neurons can be activated by auditory stimuli (see, e.g., Trulson and Trulson, 1982). Whether those responses are driven by a direct projection from dorsal cochlear neurons or by a multineuronal circuit is unclear, but the functional connection exists, so there must be an anatomical correlate. Here, we showed that the connection between dorsal cochlear and 5HT neurons of the DR is unquestionably indirect (i.e., multineuronal), and likely involves neurons of the dorsolateral PAG as well as the A1 and A7 noradrenergic cell groups. With regard to the thalamic input, Moga and colleagues (1995) reported a small number of labeled fibers in the "central gray" after injection of PHA-L into the paraventricular thalamus. Because we detected BA2001 in all regions of the PAG after injections into the DR, it is possible that BA2001 was transneuronally transferred from 5HT neurons of the DR to neurons of the PAG and from the PAG to neurons in the paraventricular nucleus of the thalamus. Of course, some structures might have been labeled both through a direct projection to the raphe nuclei and via multineuronal connections. This could be the case for the pontine micturition center (Barrington's nucleus). Thus, virus labeling in Barrington's nucleus could have resulted from its direct projection to the ventral PAG (Valentino et al., 1995) or indirectly via its projection to the LC (Lee et al., 2005) or the NTS (Loewy et al., 1979; Valentino et al., 1995). Given the difficulty in discriminating direct from indirect projection when using BA2001, it is advisable to incorporate some correlative anterograde tracing analysis, especially when one obtains results that are very different from the prevailing view.

Finally, it is of interest that, after BA2001 injection in the RVM, we observed labeling of 5HT neurons in the DR. This result provides evidence for connections between 5HT neurons of the DR and the NRM. Retrograde studies have, in fact, shown that large inputs to the NRM originate in the PAG (Hermann et al., 1997). However, very few of these inputs are serotonergic (Beitz, 1982). This fact, taken together with our own study showing that 5HT neurons of the NRM are not postsynaptic to 5HT neurons of the DR (Braz and Basbaum, 2007), suggests that BA2001 injections in the NRM resulted in labeling of 5HT neurons of the DR through a multineuronal pathway, possibly involving reciprocal connections between the DR and the LC (Kim et al., 2004) and between the LC and the RVM (Hermann et al., 1997).

Ascending pathways that target brainstem serotonergic neurons

Most spinal cord neurons that project to the reticular formation, including the RVM, originate in deep laminae of the spinal cord, laminae V–VIII and X (Fields et al., 1975; Gallager and Pert, 1978; Abols and Basbaum, 1981; Andrezik et al., 1981; Chaouch et al., 1983; Menétrey et al., 1983; Shokunbi et al., 1985; Villanueva et al., 1991; Wang et al., 1999). Electrophysiological studies have demonstrated that neurons in laminae VII–VIII of the spinal cord have very large receptive fields and are responsive to noxious stimulation (Fields et al., 1975, 1977). These are precisely the regions where we found GFP-containing neurons after injections into the RVM. The fact that we never detected GFP in primary sensory neurons presumably reflects the rather limited labeling of spinal cord neurons that we observed and the likelihood that there are multiple synapses between the primary afferent nociceptor and the projection neurons of laminae VII and VIII.

It is of interest, however, that reticular nuclei that receive spinal cord inputs do not include the medullary raphe nuclei (Gallager and Pert, 1978; Abols and Basbaum, 1981). For this reason, we believe that the neuronal pathway that links laminae V–VIII spinoreticular neurons with the 5HT neurons of the NRM is multineuronal, probably involving neurons of the RGc and Rmc. The fact that we found GFP-containing neurons in the RGc and Rmc is consistent with this conclusion, as is our finding that transneuronal viral labeling of spinal cord neurons was always detected at longer times (5 days) postinoculation compared with the labeling in the medullary reticular formation (48 hours).

The nucleus raphe magnus is the major source of serotonergic axons that target the superficial laminae of the spinal cord (Oliveras et al., 1977; Basbaum and Fields, 1979; Skagerberg and Björklund, 1985) and is generally considered to be the origin of descending 5HT-mediated antinociceptive controls. In fact, Cervero and Wolstencroft (1984) suggested, based on studies in the cat, that the NRM and the adjacent reticular formation are engaged in a positive feedback loop between the brainstem and the spinal cord. Our present analysis indicates that, at least in the mouse, 5HT neurons are in fact part of a spinobulbospinal circuit through which noxious stimulation engages descending serotonergic antinociceptive controls. This spinoreticulo-5HT-spinal loop corresponds to a pathway that parallels the spinomesencephalic-spinal loop (including the PAG and RVM), which also participates in the descending modulation of nociceptive messages at the level of the spinal cord.

In contrast to the case in the medullary raphe, we never detected GFP in the spinal cord after BA2001 injection in the DR. Thus, 5HT neurons of the DR appear to be neither directly nor indirectly influenced by ascending pathways that originate in the spinal cord. This was surprising insofar as, after injections of BA2001 in DR, the brainstem structures that were retrogradely labeled include many of those known to receive spinal cord and trigeminal nucleus caudalis ascending inputs. Among these brainstem regions are the A6 and A7 noradrenergic cell groups, PAG, NTS, and cholinergic neurons of the laterodorsal tegmental and pedunculopontine tegmental nuclei (Menétrey et al., 1982; Mantyh, 1982; Menétrey and Basbaum, 1987; Esteves et al., 1993; Carlson et al, 2004). Our results suggest that the spinal cord projection to these latter regions targets neurons that differ from those that target the 5HT neurons of the dorsal raphe.

Catecholaminergic modulation of 5HT systems

The existence of catecholaminergic inputs to DR and RVM is well established (Baraban and Aghajanian, 1981; Hammond et al., 1980; Beitz, 1982; Sagen and Proudfit, 1986). These inputs arise from all catecholaminergic cell groups of the lower brainstem (Kwiat and Basbaum, 1990; Herbert and Saper, 1992). It was not surprising, therefore, to find GFP in all NA cell groups of the brainstem. Although Peyron et al. (1996) reported that the A7 input to the DR derives from non-NA cells, we found retrograde labeling of both TH-positive and TH-negative cells in the A5, A6, and A7 cell groups after DR or RVM injection. These results suggest that that both NA- and non-NA neurons in these regions regulate the 5HT neurons.

Traditional retrograde tracing cannot unequivocally establish inputs to neurochemically defined cells (such as the 5HT neurons of the NRM and DR). Although EM analysis showed synaptic contacts between NA fibers and 5HT neurons in the DR (Baraban and Aghajanian, 1981) and NRM (Tanaka et al., 1994), these studies could not determine the origin of the NA contacts. By contrast, with this new approach, we not only found that the 5HT populations of the DR and NRM are directly targeted by NA cell groups but also have provided a much broader perspective on the populations of catecholamine neurons that directly or indirectly influence the 5HT neurons.

Whether the NA neurons that target the 5HT neurons of the DR also send a collateral to the medullary 5HT neurons remains to be determined. We suggest, based on the pattern of labeling in the LC, that this is not the case. Thus neurons located more dorsally in the LC were labeled after injections of BA2001 in the DR; the ventrally located neurons of the LC and the subceruleus were labeled after injections into the RVM. These observations agree with results from a more traditional retrograde tracing study that found a predominant input from the subceruleus nucleus to the NRM (Hermann et al., 1997). Whether there is a differential functional consequence of the NA regulation of 5HT neurons in the NRM and DR is not known. It is significant, however, that LC neurons contribute to a tonic control of sleep-waking cycles and that LC activity is altered by behavioral state (Foote et al., 1983; Berridge and Waterhouse, 2003). The fact that 5HT neurons also discharge slowly and steadily, in a state-dependent manner (Jacobs and Azmitia, 1992), suggests that LC activity indeed contributes to a tonic modulation of 5HT neurons in various stages of sleep and arousal (Mason, 2001). Finally, it is of interest that noradrenergic neurons of the A5, A6, and A7 cell groups contribute to both antinociceptive and cardiovascular controls, through their projections to the spinal cord (Clark and Proudfit, 1993; Bajic and Proudfit, 1999). The patterns of labeling that we recorded after spinal cord coinjection of FG indicate that the spinally projecting A5, A6, and A7 NA cell groups also regulate 5HT neurons through a collateral that terminates in DR and NRM.

Cholinergic modulation of 5HT systems

Brainstem structures implicated in the control of wakefulness and sleep (including paradoxical sleep; Jones, 1991) contained large numbers of cells labeled with GFP. These regions, which include the LDTg and PPTg nuclei, project to the LC, NRM, DR, and medullary reticular nuclei (Jackson and Crossman, 1983; Rye et al., 1988; Woolf and Butcher, 1989; Jones, 1990). Here we found that neurons in both the cholinergic PPTg and LDTg cell groups contained GFP following injections of the BA2001 in the DR. We conclude that 5HT neurons of the DR receive inputs from both cholinergic loci. In contrast, injections in RVM resulted in GFP labeling in the PPTg only, indicating that 5HT neurons of the NRM receive selective cholinergic inputs from PPTg only. Because the LDTg also projects to the NRM, we conclude that cholinergic neurons of the LDTg likely target the non-5HT population of neurons of the RVM. This latter observation is in agreement with the study of Brodie and Proudfit (1986), which showed that 5HT antagonists have no effect on the analgesia induced by an injection of cholinergic agonists (carbachol) in the rat NRM. Furthermore, and because we never found spinal cord neurons retrogradely labeled after injection of BA2001 in the DR, our results suggest that the cholinergic neurons of the LDTg and PPTg that respond to noxious stimuli (Carlson et al., 2004, 2005) do not contact 5HT neurons of either the DR or the NRM.

In conclusion, this conditional viral retrograde tracing study indicates that a diverse array of neuronal networks in the brainstem influences serotonergic neurons of both the DR and the NRM. These networks include catecholaminergic and cholinergic cell groups. This pattern of connectivity underlies the extensive 5HT contribution to the modulation of a wide spectrum of behavioral and physiological processes, from cognitive and neuroendocrine functions to sleep-wakefulness states and pain. We have provided evidence for the existence of a spinoreticular ascending pathway through which 5HT neurons of the NRM, but not of the DR, can be activated by noxious stimuli. This in turn could trigger serotonergic descending (inhibitory or facilitatory) controls of the transmission of nociceptive messages.

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