Mesopontine rostromedial tegmental nucleus neurons projecting to the dorsal raphe and pedunculopontine tegmental nucleus: psychostimulant-elicited Fos expression and collateralization

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Abstract The mesopontine rostromedial tegmental nucleus (RMTg) is a GABAergic structure in the ventral midbrain and rostral pons that, when activated, inhibits dopaminergic neurons in the ventral tegmental area and substantia nigra compacta. Additional strong outputs from the RMTg to the pedunculopontine tegmental nucleus pars dissipata, dorsal raphe nucleus, and the pontomedullary gigantocellular reticular formation were identified by anterograde tracing. RMTg neurons projecting to the ventral tegmental area express the immediate early gene Fos upon psychostimulant administration. The present study was undertaken to determine if neurons in the RMTg that project to the additional structures listed above also express Fos upon psychostimulant administration and, if so, whether single neurons in the RMTg project to more than one of these structures. We found that about 50% of RMTg neurons exhibiting retrograde labeling after injections of retrograde tracer in the dorsal raphe or pars dissipata of the pedunculopontine tegmental nucleus express Fos after acute methamphetamine exposure. Also, we observed that a significant number of RMTg neurons project both to the ventral tegmental area and one of these structures. In contrast, methamphetamine-elicited Fos expression was not observed in RMTg neurons labeled with retrograde tracer following injections into the pontomedullary reticular formation. The findings suggest that the RMTg is an integrative modulator of multiple rostrally projecting structures.

Keywords RMTg · Psychostimulant · Fos · Addiction

Introduction

The ventral tegmental area (VTA) gives rise to the mesocorticolimbic dopaminergic system (Dahlstrom and Fuxe 1964) and is the substrate for the initiation of behavioral sensitization to psychostimulants (Kalivas and Webber 1988; Kalivas and Stewart 1991; Hooks et al. 1992; Perugini and Vezina 1994; Cador et al. 1995, 1999; Bijou et al. 1996; Vezina 1996; Vanderschuren and Kalivas 2000). Dopamine (DA) neurons in the VTA respond with increases or decreases in their firing rates to novelty, reward, aversive stimuli, cues predicting rewards or aversive stimuli, and omissions of expected rewards or aversive stimuli (Schultz 1998, 2007; Bromberg-Martin et al. 2010). Understanding of the mechanisms by which the activity of DA neurons is linked to circumstances, however, remains incomplete. The lateral habenula (LHb), which increases firing in response to aversive stimuli and reward omission (Christoph et al. 1986; Gao et al. 1996; Ji and Shepard 2007; Matsumoto and Hikosaka 2007; Shumake et al. 2010; Hong et al. 2011), could conceivably inhibit dopamine neurons in turn, due to its direct projections to the VTA (Herkenhan and Nauta 1979; Araki et al. 1988; Omelchenko et al. 2009). However, LHb projections to the ventral tegmental area are mainly glutamatergic (Geisler et al. 2007; Brischwitz et al. 2010), thus suggesting that LHb inhibition of DA neurons is mediated indirectly, possibly by a relay in another structure.

Evidence that such a mediator region exists was provided by Chou et al. (2004), who showed that a cluster of cells in the paramedian tegmentum behind the VTA projects to the VTA and influences fear-elicited behaviors.
Subsequently, Jhou and Gallagher (2007) (same person as Chou in Chou et al. 2004) showed that these neurons are activated by aversive stimuli. In definitive papers, Jhou and colleagues (Jhou et al. 2009a, b) showed that this structure is GABAergic, receives dense projections from the LHb, and projects strongly to the VTA and substantia nigra compacta (SNC). Earlier, Scammell et al. (2000) had identified in rats a cluster of neurons that express Fos after administration of modafinil, and designated it as the “retroVTA”. Later, Perrotti et al. (2005) showed a similar cluster that expressed deltaFosB, a long-lived splice variant of FosB, after the chronic administration of amphetamine and called it the “posterior tail of the VTA”. Geisler et al. (2008) demonstrated what appears to be the same cluster of

| Injection site | Retrogradely labeled neurons | Double-labeled neurons | Percent double-labeled neurons |
|----------------|-------------------------------|------------------------|-------------------------------|
|                | RMTg  | Outside | RMTg  | Outside | RMTg  | Outside |
| VTA            |       |         |       |         |       |         |
| 10192          | 152   | 118     | 74    | 17      | 48%   | 14%     |
| 11077          | 150   | 60      | 75    | 17      | 50%   | 28%     |
| 11080          | 59    | 18      | 32    | 1       | 54%   | 5%      |
| 11083          | 115   | 115     | 46    | 7       | 40%   | 6%      |
| 12051          | 176   | 131     | 198   | 28      | 47%   | 14%     |
| Mean ± SE      | 130 ± 35 | 88 ± 21 | 85 ± 29 | 14 ± 4 | 48 ± 2 | 13 ± 4 |
|                |       |         |       |         |       |         |
| PPTg           |       |         |       |         |       |         |
| 10193          | 84    | 101     | 33    | 10      | 40%   | 9%      |
| 10202          | 104   | 80      | 80    | 18      | 77%   | 30%     |
| 10203          | 84    | 20      | 35    | 6       | 42%   | 22%     |
| 11058          | 77    | 52      | 50    | 14      | 65%   | 26%     |
| 11059          | 72    | 72      | 46    | 18      | 64%   | 25%     |
| 12050          | 117   | 107     | 59    | 8       | 50%   | 7%      |
| Mean ± SE      | 89 ± 7 | 72 ± 13 | 50 ± 7 | 12 ± 2 | 54 ± 6 | 19 ± 4 |
|                |       |         |       |         |       |         |
| DR             |       |         |       |         |       |         |
| 10200          | 63    | 80      | 22    | 4       | 35%   | 5%      |
| 11060          | 91    | 140     | 44    | 32      | 48%   | 22%     |
| 11061          | 44    | 98      | 30    | 2       | 68%   | 2%      |
| 11119          | 66    | 92      | 34    | 3       | 51%   | 4%      |
| 12053          | 50    | 65      | 23    | 7       | 46%   | 11%     |
| Mean ± SE      | 71 ± 11 | 97 ± 10 | 35 ± 5 | 9 ± 4   | 47 ± 5 | 9 ± 4   |
|                |       |         |       |         |       |         |
| RtGi           |       |         |       |         |       |         |
| 11180          | 37    | 83      | 5     | 9       | 14%   | 11%     |
| 11184          | 23    | 14      | 2     | 2       | 8%    | 14%     |
| 11191          | 25    | 131     | 1     | 21      | 4%    | 16%     |
| 11192          | 38    | 129     | 8     | 27      | 21%   | 21%     |
| 11214          | 17    | 43      | 1     | 4       | 6%    | 9%      |
| 11215          | 32    | 72      | 2     | 6       | 6%    | 8%      |
| 11216          | 49    | 76      | 2     | 11      | 4%    | 14%     |
| 11217          | 27    | 73      | 2     | 15      | 7%    | 20%     |
| 11223          | 26    | 125     | 5     | 23      | 19%   | 18%     |
| 11224          | 40    | 99      | 3     | 15      | 7%    | 15%     |
| 11225          | 33    | 89      | 1     | 18      | 3%    | 20%     |
| Mean ± SE      | 31 ± 3 | 85 ± 10 | 3 ± 0.5 | 13 ± 2 | 8 ± 2 | 15 ± 1 |

All entries reflect data from the RMTg and region just surrounding it (see “Materials and methods”) from 3 sections/case

\( ^a \) Paired samples \( t \) test

Table 1 Numbers of retrogradely labeled and double-labeled neurons following brainstem tracer injections

Subsequently, Jhou and Gallagher (2007) (same person as Chou in Chou et al. 2004) showed that these neurons are activated by aversive stimuli. In definitive papers, Jhou and colleagues (Jhou et al. 2009a, b) showed that this structure is GABAergic, receives dense projections from the LHb, and projects strongly to the VTA and substantia nigra compacta (SNC). Earlier, Scammell et al. (2000) had identified in rats a cluster of neurons that express Fos after administration of modafinil, and designated it as the “retroVTA”. Later, Perrotti et al. (2005) showed a similar cluster that expressed deltaFosB, a long-lived splice variant of FosB, after the chronic administration of amphetamine and called it the “posterior tail of the VTA”. Geisler et al. (2008) demonstrated what appears to be the same cluster of
Table 2 Numbers of retrogradely labeled and double-labeled neurons for tracer (FG and CTb) injection combinations

| Injection combination | Retrogradely labeled neurons | Double-labeled neurons | Percent double-labeled neurons |
|-----------------------|-------------------------------|------------------------|-------------------------------|
|                       | RMTg Surround | RMTg Surround | RMTg Surround |
| PPTg–VTA              |                  |                        |                              |
| 11082                 | 230 (108, 122)a | 127 (64, 63) | 28 | 2 | 12% | 2% |
| 11083                 | 272 (129, 143) | 162 (67, 95) | 35 | 6 | 13% | 3% |
| 11090                 | 164 (71, 93)  | 108 (65, 43) | 8  | 0  | 5%  | 0% |
| 12051                 | 146 (57, 89)  | 112 (62, 50) | 9  | 1  | 6%  | 1% |
| Mean ± SE             | 203 ± 29      | 127 ± 12      | 20 ± 12 | 2 ± 1 | 9 ± 2 | 1 ± 0.5 |
| DR–VTA                |                  |                        |                              |
| 11118                 | 226 (115, 111) | 207 (92, 115) | 18 | 3 | 8%  | 1% |
| 11119                 | 188 (74, 114) | 145 (68, 77) | 20 | 6 | 10% | 4% |
| 11121                 | 191 (78, 113) | 147 (65, 82) | 23 | 4 | 12% | 3% |
| 12052                 | 203 (81, 122) | 123 (44, 79) | 18 | 4 | 9%  | 3% |
| Mean ± SE             | 202 ± 8       | 155 ± 18      | 20 ± 1 | 4 ± 0.5 | 10 ± 1 | 2 ± 0.5 |
| PPTg–DR               |                  |                        |                              |
| 11260                 | 98 (60, 38)   | 65 (33, 32)    | 6  | 0  | 6%  | 0% |
| 11261                 | 102 (42, 60)  | 36 (19, 17)    | 9  | 0  | 9%  | 0% |
| 11264                 | 124 (53, 71)  | 172 (74, 98)   | 4  | 3  | 3%  | 2% |
| 11265                 | 65 (49, 16)   | 46 (25, 21)    | 3  | 0  | 5%  | 0% |
| 12055                 | 70 (29, 41)   | 54 (20, 34)    | 3  | 1  | 4%  | 2% |
| Mean ± SE             | 91 ± 10       | 74 ± 24       | 5 ± 1 | 0.8 ± 0.5 | 5 ± 1 | 0.5 ± 0.5 |

All entries reflect data from the RMTg and region just surrounding it (see “Materials and methods”) from 3 sections/case

a Injection 1, injection 2

b Paired samples t test

Fos-expressing neurons after cocaine self-administration, and showed that the neurons comprising it project to the VTA. Upon further investigation, Jhou et al. (2009b) and Kaufling et al. (2009, 2010a) concluded that all these workers were studying the same structure, which Jhou et al. (2009b) named the mesopontine rostromedial tegmental nucleus (RMTg). This GABAergic midbrain structure has now been shown to mediate the LHb influence on the VTA (Hong et al. 2011): glutamatergic LHb neurons project to GABAergic neurons of the RMTg, which in turn exert an inhibitory influence on dopaminergic VTA/SNC neurons. Jhou et al. (2009b) utilized the anterogradely transported tracer, PHA-L, to characterize the efferent projections of the RMTg, and showed particularly robust projections to the pedunculopontine tegmental nucleus pars dissipata (PPTg), dorsal raphe nucleus (DR), and pontomedullary paramedian gigantocellular reticular formation (RtGi), indicating that important influences of the RMTg are not necessarily limited to the VTA.

At present, the criteria available to designate a neuron as belonging to the RMTg include input from the LHb, projection to the VTA/SNC, GABAergic phenotype, and the expression of Fos following some aversive stimuli (Jhou et al. 2009a), but invariably after administration of any psychostimulant drug (Kaufling et al. 2010b). The present study was undertaken to determine if neurons in the RMTg that project to the PPTg, DR, and RtGi also express Fos upon psychostimulant administration. At least for the DR and PPTg, this was found to be the case, which led us to examine if single neurons in the RMTg might project to more than one of these structures. We herein show that single neurons of the RMTg do indeed project to multiple targets, suggesting that RMTg neurons may be involved in modulating activity coordinately in several structures, including the PPTg, DR, and VTA.

Materials and methods

Male Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) weighing 225–250 g were used in accordance with the guidelines mandated in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The rats were housed on a 12-h light–dark cycle in groups of two to four until surgeries were performed, after which all were singly housed. Access to food and water was provided ad libitum. Unless otherwise stated, chemicals were purchased from Sigma (St. Louis, MO, USA).
Tracer injections

The rats were deeply anesthetized by intraperitoneal injections of a mixture of ketamine (72 mg/kg) and xylazine (11.2 mg/kg). The anesthetic was injected as a cocktail consisting of 45% ketamine (100 mg/ml), 35% xylazine (20 mg/ml), and 20% saline at a dose of 0.16 ml/100 g of body weight. The anesthetized rats were placed into a Kopf stereotaxic instrument and an incision was made to expose the skull, in which a small burr hole was made over the injection site. The retrograde tracers, cholera toxin β subunit (CTβ; List Biological Laboratories, Campbell, CA; 1% in ddH2O) or Fluoro-Gold (FG; Fluorochrome Inc., Englewood, CO; 1% in 0.1 M cacodylate buffer, pH 7.4) were injected into targeted brain structures (VTA, DR, PPTg or RtGi) using 1.0 mm filament-containing borosilicate glass pipettes pulled to tip diameters of 15–20 μm. The tracer solution was injected iontophoretically using positive current pulses (7 s on and 7 s off for 15 min) of 1 μA (for FG) and 3 μA (for CTβ). In order to generate more retrograde labeling in the vicinity of the RMTg, some injections of CTβ into RtGi were done with glass pipettes pulled to tip diameters of 30–40 μm from which the tracer was expelled by air pressure, resulting in an injected volume of approximately 0.1 μl. The incisions were closed with wound clips, and the rats were kept warm until they awakened.

Fixation of brains

Three days following tracer injections, the rats were given an intraperitoneal injection of methamphetamine (10 mg/kg). Two hours following methamphetamine injections, the rats were deeply anesthetized as described and perfused transaortically with 0.01 M Sorensen’s phosphate buffer (SPB; pH 7.4) containing 0.9% sodium chloride and 2.5% sucrose, followed by 0.1 M SPB (pH 7.4) containing 4% paraformaldehyde and 2.5% sucrose. The perfused brains were removed and placed in the same fixative for 4 h and then in a 30% sucrose solution overnight. Five adjacent series of 50 μm sections were collected, each representing an entire brain from the

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**Fig. 1** Fos-expressing retrogradely labeled neurons in the RMTg following injection of CTβ in the VTA. 

- **a** CTβ injection site in the VTA.
- **b** Immunoperoxidase detection of retrogradely labeled neurons (brown, yellow arrows) and Fos expression (black, white arrows) in the RMTg.
- **c–e** Immunofluorescence images of RMTg neurons showing Fos immunoreactivity (c, magenta), retrograde labeling (d, green), and a merged image (e). White arrows in c–e indicate double-labeled neurons. The round hole in the section at the left edge of the micrograph is a tissue punch marking the right hemisphere. IPN interpeduncular nucleus. Scale bars 400 μm in a; 100 μm in b and c.
frontal pole to the caudal medulla at sampled intervals of 250 μm. Each series of sections was stored in a separate glass vial at −20°C in cryoprotectant consisting of 0.01 M SPB containing 30% sucrose and 30% ethylene glycol.

Immunohistochemistry

Some series of sections from each case were processed for immunoperoxidase and others for immunofluorescence histochemistry. Sections processed for immunoperoxidase histochemistry, which produces permanent, non-fading preparations, were used for the evaluation of injection sites, efficacy of retrograde transport of the tracer and general distribution of retrograde labeling relative to the Fos-expressing RMTg. However, double-immunoperoxidase preparations show double labeling unreliably. Accordingly, additional sections processed for immunofluorescence histochemistry were used to reliably demonstrate and quantify double labeling.

Immunoperoxidase histochemistry

For immunoperoxidase processing, one series from each case was immersed in 0.1 M SPB containing 0.1% Triton X-100 (SPB-t) and anti-Fos antibody (Calbiochem, San Diego, CA, USA, catalog no. PC38) made in rabbit at a dilution of 1:3,000. The following day, the sections were rinsed in 0.1 M SPB-t and immersed for 1 h in 0.1 M SPB-t containing biotinylated antibody made in donkey against rabbit IgGs at a dilution of 1:200 (Jackson Immuno-Research Laboratories Inc., West Grove, PA, USA, catalog no. 711-065-152). Afterward, the sections were rinsed in 0.1 M SPB-t and then immersed for 1 h in 0.1 M SPB containing avidin–biotin-peroxidase complex (ABC) at a dilution of 1:200 (Vector Laboratories, Burlingame, CA, USA). After rinsing in 0.1 M SPB, the sections were reacted in 0.025 M Tris buffer (pH 8.0) containing 0.015% 3,3′-diaminobenzidine (DAB), 0.4% nickel ammonium sulfate, and 0.003% hydrogen peroxide (NiDAB), which generates an insoluble black reaction product. The sections

**Fig. 2** Fos-expressing retrogradely labeled neurons in the RMTg following injection of CTβ in the PPTg. **a** CTβ injection site in the PPTg. **b** Adjacent section processed for Nos immunoreactivity (black) verifies that the injection is in the PPTg, pars dissipata. **c** Retrogradely labeled neurons (yellow arrows) and Fos expression (white arrows) in the RMTg. **d–f** Immunofluorescence images of RMTg neurons showing Fos immunoreactivity (d, magenta), retrogradely labeled neurons (e, green), and a merged image (f). White arrows indicate double labeling (d–f). **PPTg c** pedunculopontine tegmental nucleus, pars compacta; **PPTg d** pedunculopontine tegmental nucleus, pars dissipata; **IPN** interpeduncular nucleus. Scale bars 400 μm in a and b; 100 μm in c and d.
were then rinsed in 0.1 M SPB and immersed overnight in 0.1 M SPB containing polyclonal antibodies against CTβ (anti-CTβ, List Biological Laboratories, Cambell, CA, USA, catalog no. 7032A5) made in goat and used at a dilution of 1:4,000. The following day, after rinsing in 0.1 M SPB, the sections were immersed for 1 h in 0.1 M SPB containing biotinylated antibody made in donkey against goat IgGs at a dilution of 1:200 (Jackson). Afterward, the sections were rinsed in 0.1 M SPB-t and then immersed for 1 h in 0.1 M SPB containing ABC at a dilution of 1:200 (Vector). After rinsing in 0.1 M SPB, the sections were reacted in NiDAB. The sections were then mounted onto gelatin-coated slides and coverslipped with Permount (Fisher, Pittsburgh, PA, USA).

For immunoperoxidase in cases involving injection of FG into one brain area and CTβ into a different site, one series from each case was immersed overnight in 0.1 M SPB-t containing anti-FG (Chemicon International, Temecula, CA, USA, catalog no. AB153), made in rabbit at a dilution of 1:3,000. The following day, the sections were rinsed in 0.1 M SPB-t and immersed for 1 h in 0.1 M SPB containing biotinylated antibody made in donkey against rabbit IgGs at a dilution of 1:200 (Jackson). Afterward, the sections were rinsed in 0.1 M SPB-t and then immersed for 1 h in 0.1 M SPB containing ABC at a dilution of 1:200 (Vector). After rinsing in 0.1 M SPB, the sections were reacted in NiDAB. The sections were then mounted in SPB and immersed overnight in SPB containing polyclonal antibodies against CTβ.
anti-CTβ used at a dilution of 1:4,000. The following day, after rinsing in 0.1 M SPB, the sections were immersed for 1 h in 0.1 M SPB containing biotinylated antibody made in donkey against goat IgGs at a dilution of 1:200 (Jackson). Afterward, the sections were rinsed in 0.1 M SPB and then immersed for 1 h in 0.1 M SPB containing ABC at a dilution of 1:200 (Vector). After more rinsing in 0.1 M SPB, the sections were reacted in NiDAB.

For injections into the DR, one series of each case was immersed overnight in 0.1 M SPB-t containing anti-5-hydroxytryptophan (5-HT, Immunostar, Hudson, WI, USA, catalog no. 2446), made in rabbit at a dilution of 1:1,000. For injections into the PPTg, one series from each case was immersed overnight in 0.1 M SPB-t containing anti-nitric oxide synthase (Nos, Sigma, catalog no. 6715) made in rabbit at a dilution of 1:3,000. The following day, the sections that had been immersed in either anti-5-HT or anti-Nos were further processed similarly. They were rinsed in 0.1 M SPB-t and immersed for 1 h in 0.1 M SPB containing ABC at a dilution of 1:200 (Vector). After more rinsing in 0.1 M SPB, the sections were reacted in NiDAB, as described above.

**Fluorescence immunohistochemistry**

For immunofluorescence processing, one series from each case was immersed overnight in 0.1 M SPB-t containing anti-CTβ used at a dilution of 1:4,000. The following day, the sections were rinsed in 0.1 M SPB containing biotinylated antibody made in donkey against goat IgGs at a dilution of 1:200 (Jackson). Afterward, the sections were immersed for 1 h in 0.1 M SPB containing ABC at a dilution of 1:200 (Vector). After more rinsing in 0.1 M SPB, the sections were reacted in NiDAB.

**Fig. 4** Fos-expressing retrogradely labeled neurons in the RMTg following injection of CTβ in the DR. a CTβ injection site in the DR. b Adjacent section processed for 5-HT immunoreactivity (black) verifies that the injection is in the DR. c Retrogradely labeled neurons (yellow arrows) and Fos expression (white arrows) in the RMTg.

d–f Immunofluorescence images of RMTg neurons showing Fos immunoreactivity (d, magenta), retrogradely labeled neurons (e, green), and a merged image (f). White arrows indicate double labeling (d–f). IPN interpeduncular nucleus. Scale bars: 400 μm in a and b; 100 μm in c and d.

For immunofluorescence processing, one series from each case was immersed overnight in 0.1 M SPB-t and polyclonal anti-CTβ used at a dilution of 1:4,000. The following day, the sections were rinsed in 0.1 M SPB-t and immersed for 1 h in 0.1 M SPB-t containing DyLight 488 conjugated to anti-sheep IgG made in donkey (Jackson, catalog no. 713-485-14), which recognizes the anti-CTβ made in goat, used at a concentration of 1:200. The sections then were rinsed in 0.1 M SPB and immersed overnight in 0.1 M SPB containing anti-Fos at a concentration of 1:3,000. The following day, the sections were rinsed in 0.1 M SPB and immersed for 1 h in 0.1 M SPB containing DyLight 594 conjugated to anti-rabbit IgG made in donkey (Jackson, catalog no. 711-515-152) used at 1:200. The sections were rinsed in 0.1 M SPB and mounted and coverslipped with ProLong Gold antifade reagent (Invitrogen).
For immunofluorescence in cases involving injection of FG into one brain site and CTβ into another, one series from each case was first rinsed in 0.1 M SPB-t and immersed overnight in 0.1 M SPB-t containing anti-CTβ at a concentration of 1:4,000. The following day, the sections were rinsed in 0.1 M SPB-t and immersed for 1 h in 0.1 M SPB-t containing DyLight 488-anti-sheep IgG conjugate (Jackson) at a concentration of 1:200. The sections were rinsed in 0.1 M SPB and immersed overnight in 0.1 M SPB containing anti-FG at a concentration of 1:5,000. The following day, the sections were rinsed in 0.1 M SPB and immersed for 1 h in 0.1 M SPB containing DyLight 594-anti-rabbit conjugate (Jackson) at a concentration of 1:200. The sections were rinsed in 0.1 M SPB and mounted onto gelatin-coated slides and coverslipped with ProLong Gold.

Analysis of labeling

Sections were viewed with an Olympus BX51 microscope in the brightfield or immunofluorescence modes, and digital micrographs were generated using a DVC 2000C-00-GE-MBF digital camera. Neurons were plotted with the aid of the Neurolucida hardware–software platform (MBF Bioscience, Williston, VT, USA, version 5.65) such that retrogradely labeled neurons, Fos-containing neurons and double-labeled neurons were designated with distinct markers. These plots were exported into Adobe Illustrator CS2 to generate maps showing three sections of the RMTg spaced at 250 μm for each case. Quantification of the markers for Fos, retrograde labeling and double labeling was performed with the aid of the Neurolucida NeuroExplorer program (MBF Bioscience, version 4.70.3). Counts were generated uniformly across all cases by delineating the RMTg with a circle of arbitrarily designated area (0.32 mm²) surrounding the spot of densest Fos labeling. The region just surrounding the RMTg, was designated by a larger (1.15 mm²) circle from which the area of the smaller circle was subtracted, leaving 0.83 mm². The numbers of double-labeled neurons, i.e. Fos-expressing retrogradely labeled neurons within these two arbitrarily designated regions were expressed as percentages of total retrogradely labeled neurons therein. In order to determine if enrichments of double labeling were present in the RMTg, the mean percentages of double-labeled neurons within the RMTg circle were compared with those in the arbitrarily

![Fig. 5](image-url)
defined area just outside of the RMTg using a paired t test with a 95% confidence interval. For each injection site or combination of injection sites, 4–11 cases were used (Tables 1, 2). Statistics were done with the aid of SPSS software.

**Results**

**Single CTβ injections**

**VTA**

Consistent with a published report in which the retrograde tracer Fluoro-Gold was used (Jhou et al. 2009b), injections of CTβ into the VTA exhibited double labeling with Fos immunoreactivity in 48 ± 2% of retrogradely labeled neurons (n = 5) within the cluster of Fos-immunoreactive neurons designated as the RMTg, as compared to 13 ± 4% double labeling of retrogradely labeled neurons in the area immediately surrounding the RMTg (Figs. 1, 3a–c, 7). A representative plot of the results for one VTA case is shown in Fig. 3, which illustrates rostral (Fig. 3a) and more caudal (Fig. 3b) levels of the RMTg. The maps in Fig. 3 and similar maps to follow (i.e., Figs. 6, 11) show localization of the retrograde tracer, Fos expression, and, if present, double-labeled neurons. In contrast, in cases in which no methamphetamine was administered, there were few Fos-immunoreactive neurons (data not shown), and in cases in which the injection sites were misplaced, there was no
Fig. 7 Graph showing the percentages of double-labeled neurons in the RMTg compared to the region surrounding it for four different injection sites: PPTg (n = 6), DR (n = 5), VTA (n = 5), and RtGi (n = 11). *p < 0.01 (paired t test).

Fig. 8 Retrogradely labeled neurons in the RMTg following injections of CTβ in the PPTg and FG in the VTA. a CTβ injection site in the PPTg. b Adjacent section processed for Nos immunoreactivity (black) verifies that the injection shown in ‘a’ is in the PPTg, pars dissipata. c FG injection site in the VTA. d CTβ-labeled neurons (yellow arrows) and FG-labeled neurons (white arrows) in the RMTg. e–g Immunofluorescence images of RMTg neurons showing FG-labeled RMTg neurons (e, magenta), CTβ-labeled RMTg neurons (f, green), and a merged image (g). White arrows indicate double labeling (e–g). PPTg c pedunculopontine tegmental nucleus, pars compacta; PPTg d pedunculopontine tegmental nucleus, pars dissipata; IPN interpeduncular nucleus. Scale bars 400 μm in a–c; 100 μm in d and e.
enrichment of retrogradely labeled or double-labeled neurons in the RMTg as compared to the surrounding region.

**PPTg**

Following injections of CTβ into the pars dissipata of the PPTg, 56 ± 6% of the retrogradely labeled neurons (n = 6) within the RMTg exhibited Fos-immunoreactivity compared to 19 ± 4% in the area immediately surrounding the RMTg (Figs. 2, 3d–f, 7). The locations of injection sites (Fig. 2a) within the PPTg dissipata were verified by demonstrating Nos expression (Fig. 2b), which co-localizes in cholinergic neurons that characterize the PPTg region (Armstrong et al. 1983). A representative plot of the results for one PPTg case is shown in Fig. 3, which illustrates rostral (Fig. 3d) and caudal (Fig. 3f) levels of the RMTg.

**DR**

47 ± 5% of retrogradely labeled neurons (n = 5) in the RMTg were double labeled following injections of CTβ in the DR compared to 9 ± 4% in the area immediately surrounding the RMTg (Figs. 4, 6a–c, 7). DR injection sites (Fig. 4a) were verified by demonstrating 5-HT immunoreactivity in adjacent sections (Fig. 4b), which identified serotonergic neurons that characterize that structure (Aghajanian et al. 1967).

**RtGi**

In contrast, injections of CTβ into the RtGi region (Fig. 5) resulted in few retrogradely labeled neurons in the RMTg (n = 11), of which 8 ± 2% were double labeled. Greater numbers of retrogradely labeled neurons were present in the area surrounding the RMTg following injections in the RtGi and the percentage of these that were double labeled (15 ± 1%) was comparable to that observed within the RMTg (Figs. 5, 6d–f, 7). A representative plot of the results from one RtGi injection is shown in Fig. 6, which illustrates rostral (Fig. 6d) and caudal (Fig. 6e) RMTg levels.
Double-injection tracer studies

In a series of cases in which FG was injected into the VTA and CTβ into the PPTg, 9 ± 2% of retrogradely labeled neurons within the RMTg (n = 4) exhibited both tracers, indicating RMTg neurons that project to both the VTA and PPTg, compared to 1 ± 0.5% in the area immediately surrounding the RMTg (Fig. 8). Similarly, injections of FG into the VTA and CTβ into the DR produced 10 ± 1% of retrogradely labeled neurons (n = 4) within the RMTg exhibiting both markers compared to 2 ± 0.5% in the area immediately surrounding the RMTg (Fig. 9). Injections of FG into PPTg and CTβ into DR produced 5 ± 1% of retrogradely labeled neurons (n = 5) within the RMTg exhibiting both markers compared to 0.5 ± 0.5% in the area immediately surrounding the RMTg (Fig. 10). Representative plots of these results for the PPTg/VTA, DR/VTA, and DR/PPTg injection combinations showing rostral (Fig. 11a, d, g) and more caudal (Fig. 11b, e, h) RMTg levels are shown in Fig. 11a–i

Discussion

Neuroanatomical characterization of the RMTg has revealed that it is a GABAergic structure with dense afferent inputs from the LHb and SN and outputs to the VTA/SNC and other brainstem structures, including the PPTg, DR, and RtGi (Jhou et al. 2009b). The present study was undertaken to identify if RMTg neurons that project to the PPTg and DR also express Fos upon acute psychostimulant drug administration, as do RMTg neurons that project to the VTA/SNC (Geisler et al. 2008; Jhou et al. 2009b). VTA-projecting RMTg neurons that express Fos in response to psychostimulant administration are concentrated within the dense focus of Fos-expressing neurons...
that has come to be recognized as the RMTg per se, but such neurons were also present in the region immediately surrounding the RMTg, diminishing in number with increasing distance from the RMTg (Geisler et al. 2008). Presumably, RMTg neurons projecting to the PPTg and DR and expressing Fos after psychostimulants could be similarly distributed. Accordingly, double labeling in the RMTg was contrasted in the present study with that in the immediately surrounding region in order to emphasize the distinct character of the RMTg (Fig. 7).

Our current results for the VTA projections replicate those of our earlier study (Geisler et al. 2008) indicating that 48% of RMTg neurons that project to the VTA, as shown by retrograde labeling, express Fos following administration of methamphetamine (10 mg/kg), whereas only 13% of VTA-projecting neurons expressed Fos in the region just outside the RMTg. Also consistent with the previous study, the abundance of retrogradely labeled, Fos-expressing neurons in the rest of brainstem was negligible by comparison, although this was not objectively quantitated as was done in the previous study (Geisler et al. 2008). In turn, we found that 56% of RMTg neurons that project to the PPTg pars dissipata expressed Fos after methamphetamine, compared with 19% of PPTg-projecting neurons just outside the RMTg. Similarly, 47% of RMTg neurons that project to the DR expressed Fos, compared with 9% of DR-projecting neurons just outside the RMTg. The results for injections into the RtGi were surprising, however, with only 8% of retrogradely labeled RMTg neurons expressing Fos, compared with 15% of RtGi-projecting neurons just outside the RMTg. Overall, these results indicate that neurons in the RMTg that express Fos following methamphetamine administration project to the VTA, PPTg pars dissipata, and DR, but not

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**Fig. 11** Representative plots at rostral (a, d, g) and caudal (b, e, h) levels of the RMTg (small black circles) from cases that received injections of CTβ tracer in the PPTg and FG in the VTA (a–e), cases that received injections of CTβ tracer in the DR and FG in the VTA (d–f), and cases that received injections of CTβ tracer in the DR and FG in the PPTg (g–i) and were processed to exhibit retrogradely labeled neurons. Brains were injected on the sides indicated by the asterisks. Large blue circles indicate the regions bordering the RMTg. c, f, and i Show enlargements of the caudal levels. Black triangles FG-containing neurons, blue dots CTβ-containing neurons; red circles double-labeled neurons.
substantially to the RtGi. In view of these results, we speculated that single neurons in the RMTg may project to more than one of these structures. Indeed, when the VTA and PPTg were injected in the present study with different retrograde tracers, 9% of retrogradely labeled neurons in the RMTg carried both tracers. When the VTA and DR were injected with different retrograde tracers, 10% of the retrogradely labeled neurons in the RMTg were double labeled. When the PPTg and DR were injected with different retrograde tracers, 5% of retrogradely labeled neurons in the RMTg carried both tracers. These results indicate that a significant number of RMTg neurons project to multiple targets.

RMTg projections to the PPTg and DR have received little consideration by comparison with those to the VTA/SNC. The PPTg has cholinergic (Oakman et al. 1995; Omelchenko and Sesack 2006), glutamatergic (Charara et al. 1996; Clements and Grant 1990; Geisler et al. 2007), and GABAergic (Charara et al. 1996; Jia et al. 2003; Hui-Ling and Morales 2009) projections to the VTA, and the data reported herein indicate that some or all of these are likely subject to RMTg modulation. It is well established that the VTA plays a prominent role in the initiation of behavioral sensitization (Vanderschuren and Kalivas 2000; Colussi-Mas et al. 2007) and increasing evidence implicates PPTg involvement as well (Alderson et al. 2003; Nelson et al. 2007). The present data indicate that the RMTg is also likely to influence the initiation of sensitization insofar as it appears anatomically positioned to coordinate activity in these structures. However, an alternative possibility exists that RMTg projections to the PPTg may exert less influence on VTA-projecting PPTg neurons than on PPTg neurons with other functional consequences, such as, e.g., modulation of spinal motor neuron excitability (Scarnati et al. 2011).

Neurons of the DR are primarily serotonergic (Aghajanian et al. 1967; Baker et al. 1991; Molliver 1987), and, like the VTA/SNC (Christoph et al. 1986; Jhou et al. 2009a; Matsumoto and Hikosaka 2007) are inhibited by stimulation of LHB (Stern et al. 1979), as is release of serotonin by ascending projections from the raphe (Nishikawa and Scatton 1985). Due to the strong neuronal projections from the RMTg to the DR, it seems likely that the RMTg acts to modulate the signal from the LHB to the DR (Herkenham and Nauta 1979). Hikosaka (2010) has shown the inhibition of RMTg neurons in response to psychostimulant administration in which case the Fos expression can be better explained as accompanying transcriptional regulation associated with neuronal inhibition. Alternatively, systemically administered psychostimulants may produce an initial depression of RMTg neuronal activity (associated with disinhibition of the DA neurons, DA release in the Acb, and euphoria) followed by a rebound excitation, during which Fos is expressed, that would depress DA release and be aversive (TC Jhou, personal communication). This idea is consistent with the observation that both appetitive and aversive sensations commonly accompany administration of psychostimulant drugs of abuse (Hutchison and Riley 2008; Lecca et al. 2011; Simpson and Riley 2005; Wise et al. 1976). Our evidence for single RMTg neurons projecting to multiple
structures involved in the regulation of VTA/SNC neuronal activity and thus DA release supports this idea. This system of brainstem connections warrants further consideration when one thinks about how appetitively and aversively affective stimuli enlist opponent processes that underlie adaptive responses to environmental stimuli and maladaptive ones following repeated exposure to drugs of abuse (Daw et al. 2002; Fields 2007; Koob and Le Moal 2008; Hikosaka 2010; Jhou et al. 2009a, b; Matsumoto and Hikosaka 2007).

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