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ORIGINAL ARTICLE

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.

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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; Bjijou 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; Brinschwitz 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.

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

Injection site	Retrogradely labeled neurons		Double-labeled neurons		Percent double-labeled neurons	
Case	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
					$p = 0.001$[a]	
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
					$p = 0.001$	
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
					$p = 0.005$	
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
					$p = 0.008$	

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

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 CTβ) injection combinations

Injection combination	Retrogradely labeled neurons		Double-labeled neurons		Percent double-labeled neurons	
Case	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
					$p = 0.01$[b]	
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
					$p = 0.02$	
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
					$p = 0.02$	

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).

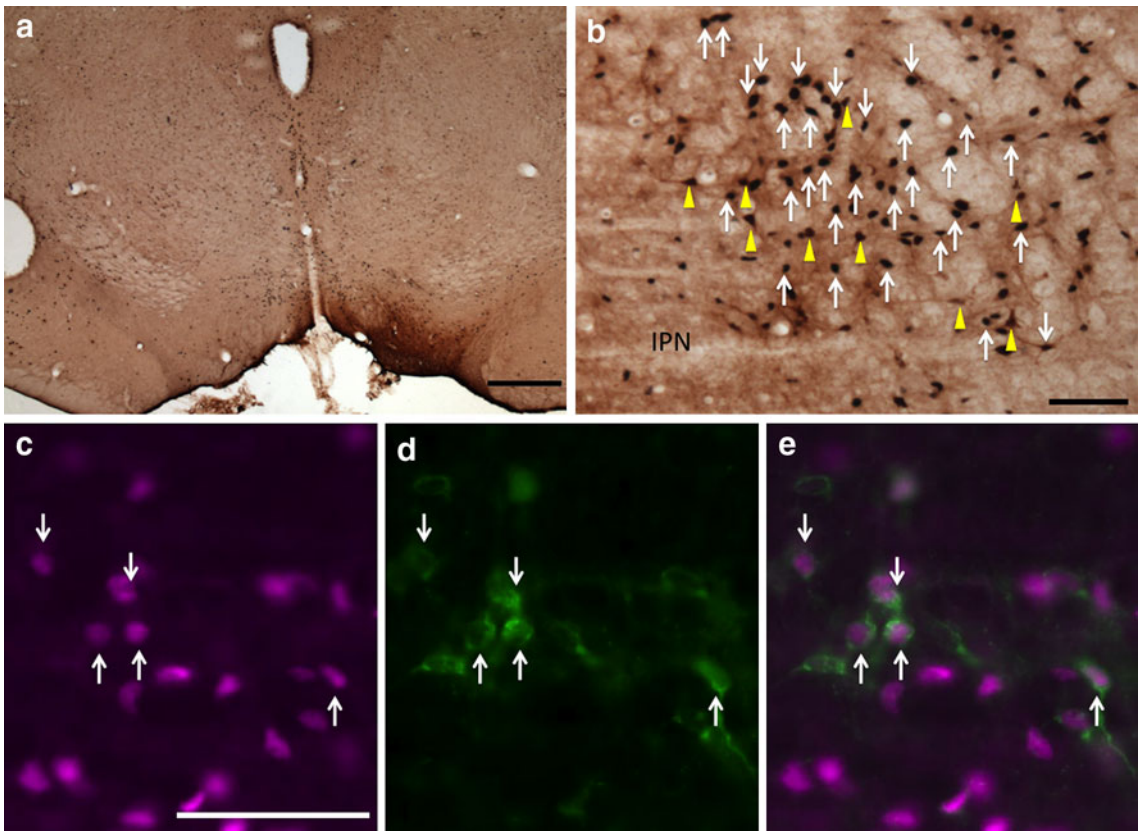

Fig. 1 Fos-expressing retrogradely labeled neurons in the RMTg following injection of CTβ in the VTA. **a** CTβ injection site in the VTA. **b** Immunoperoxidase detection of retrogradely labeled neurons (*brown, yellow arrows*) and Fos expression (*black, white arrows*) in the RMTg. **c**–**e** Immunofluorescence 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**

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 scull, 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 ddH_2O) 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 ionotophoretically 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

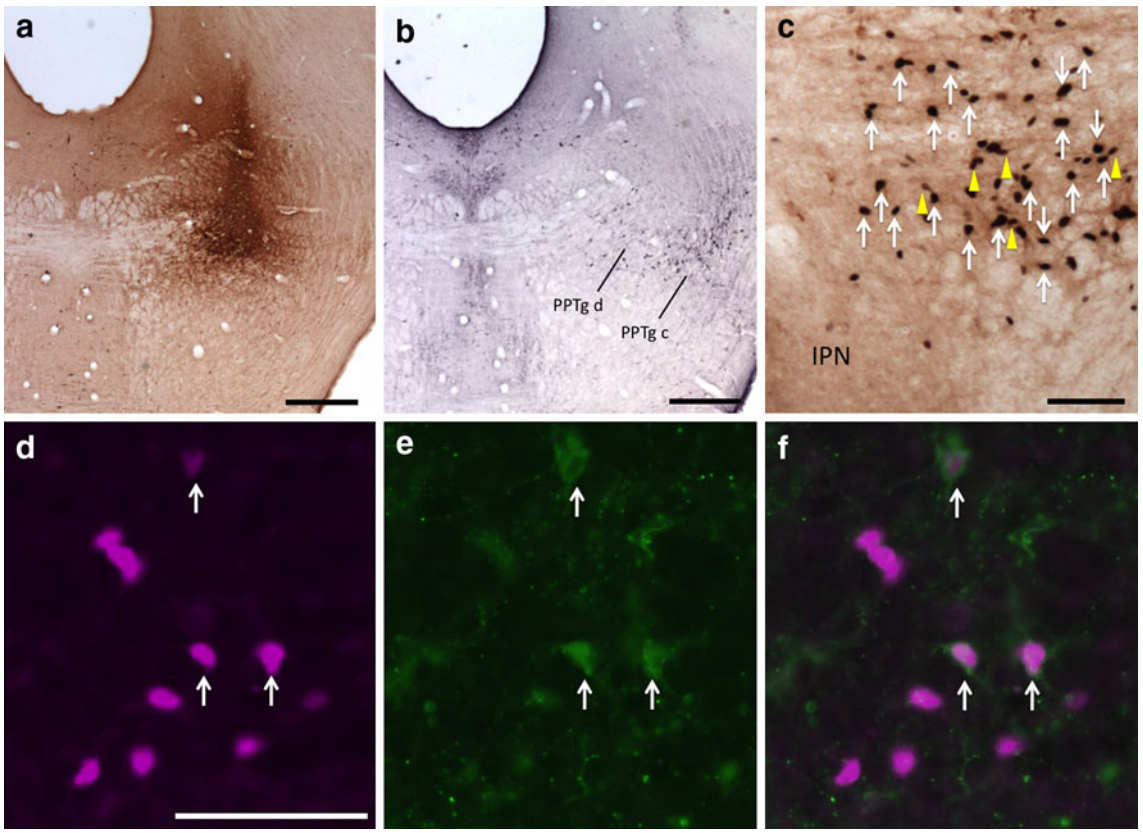

Fig. 2 Fos-expressing retrogradely labeled neurons in the RMTg following injection of CTβ in the PPTg. **a** CTβ injection site in the PPTg. **b** Adjacent section processed for Nos immunoreactivity (*black*) verifies that the injection is in the PPTg, pars dissipata. **c** Retrogradely labeled neurons (*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**

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

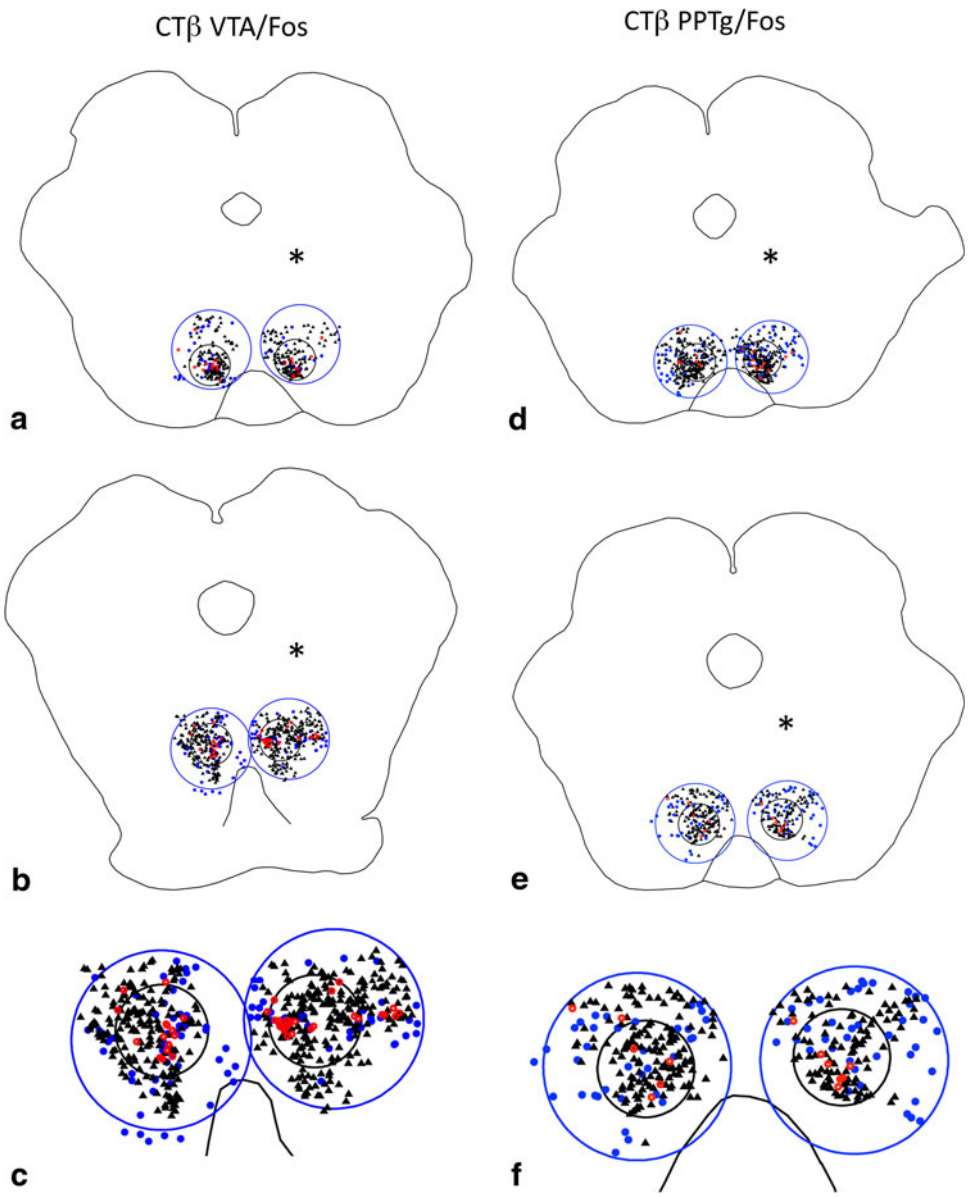

Fig. 3 Representative plots at rostral (**a**, **d**) and caudal (**b**, **e**) levels of the RMTg (*small black circles*) from cases that received injections of tracer in the VTA (**a**–**c**) and PPTg (**d**–**f**) and were processed to exhibit retrogradely labeled neurons and Fos expression. Brains were injected on the sides indicated by the *asterisks*. *Large blue circles* indicate the regions bordering the RMTg. **c** and **f** Show enlargements of the caudal levels. *Black triangles* Fos expressing neurons, *blue dots* retrogradely labeled neurons, *red circles* double-labeled neurons

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, catalog no. 705-065-003). 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 rinsing in 0.1 M SPB, the sections were reacted in 0.1 M SPB containing 0.05% 3,3′-diaminobenzidine and 0.003% hydrogen peroxide (DAB), which generates an insoluble brown reaction product. In this variation of the immunoperoxidase protocol and those to follow, 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 rinsed in SPB and immersed overnight in SPB containing polyclonal

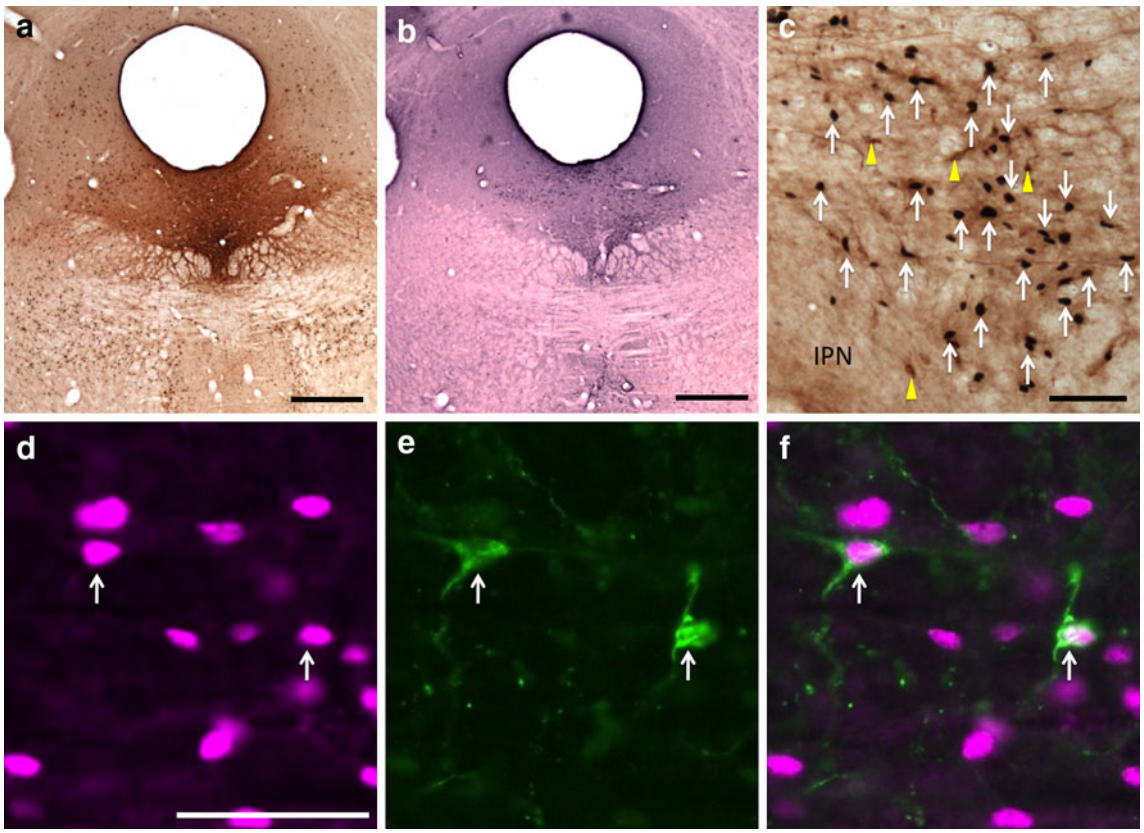

Fig. 4 Fos-expressing retrogradely labeled neurons in the RMTg following injection of CTβ in the DR. **a** CTβ injection site in the DR. **b** Adjacent section processed for 5-HT immunoreactivity (*black*) verifies that the injection is in the DR. **c** Retrogradely labeled neurons (*yellow arrows*)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

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, as described above.

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 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 more rinsing in 0.1 M SPB, the sections were reacted in NiDAB.

Fluorescence immunohistochemistry

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).

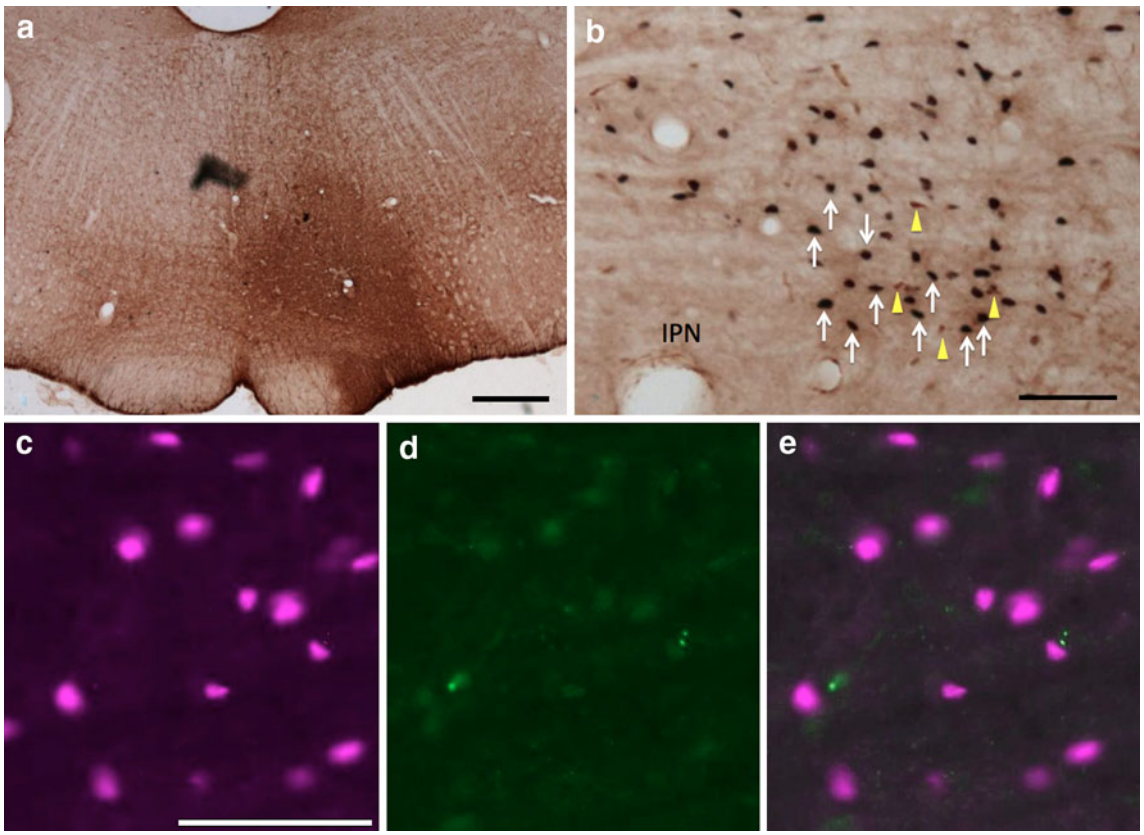

Fig. 5 Fos-expressing retrogradely labeled neurons in the RMTg following injection of CTβ in the RtGi. **a** CTβ pressure injection site (see "Materials and methods" section) in the RtGi. **b** Immunoperoxidase detection of uncharacteristically numerous retrogradely labeled neurons (*yellow arrows*) and Fos expression (*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**), confirming the absence of double labeling. *IPN* interpeduncular nucleus. *Scale bars* 400 μm in **a**; 100 μm in **b** and **c**

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 mm^2) surrounding the spot of densest Fos labeling. The region just surrounding the RMTg, was designated by a larger (1.15 mm^2) circle from which the area of the smaller circle was subtracted, leaving 0.83 mm^2. 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

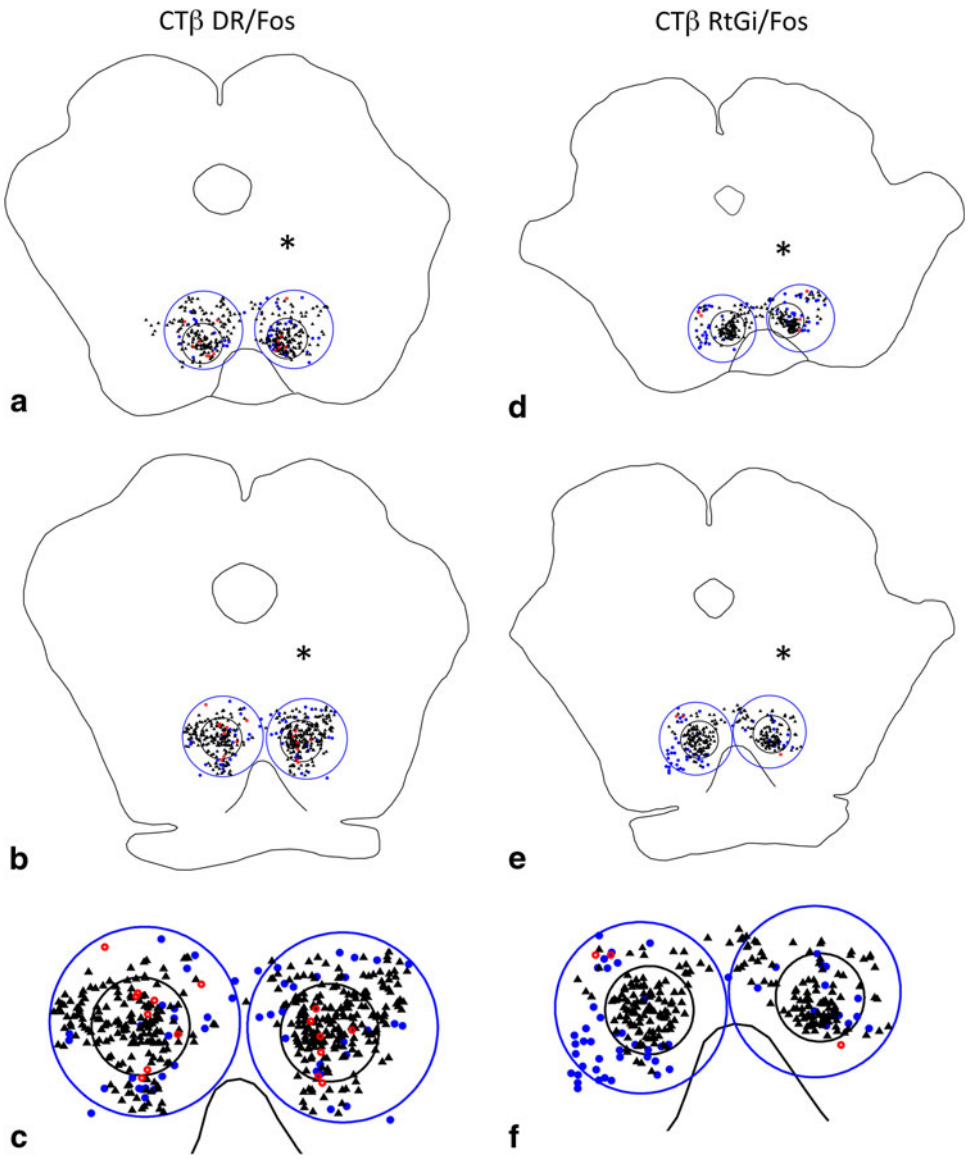

Fig. 6 Representative plots at rostral (**a**, **d**) and caudal (**b**, **e**) levels of the RMTg (*small black circles*) from cases that received injections of tracer in the DR (**a**–**c**) and RtGi (**d**–**f**) and were processed to exhibit retrogradely labeled neurons and Fos expression. Brains were injected on the sides indicated by the *asterisks*. *Large blue circles* indicate the regions bordering the RMTg. **c** and **f** Show enlargements of the caudal levels. *Black triangles* Fos expressing neurons, *blue dots* retrogradely labeled neurons, *red circles* double-labeled neurons

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 $48 \pm 2\%$ of retrogradely labeled neurons ($n = 5$) within the cluster of Fos-immunoreactive neurons designated as the RMTg, as compared to $13 \pm 4\%$ 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

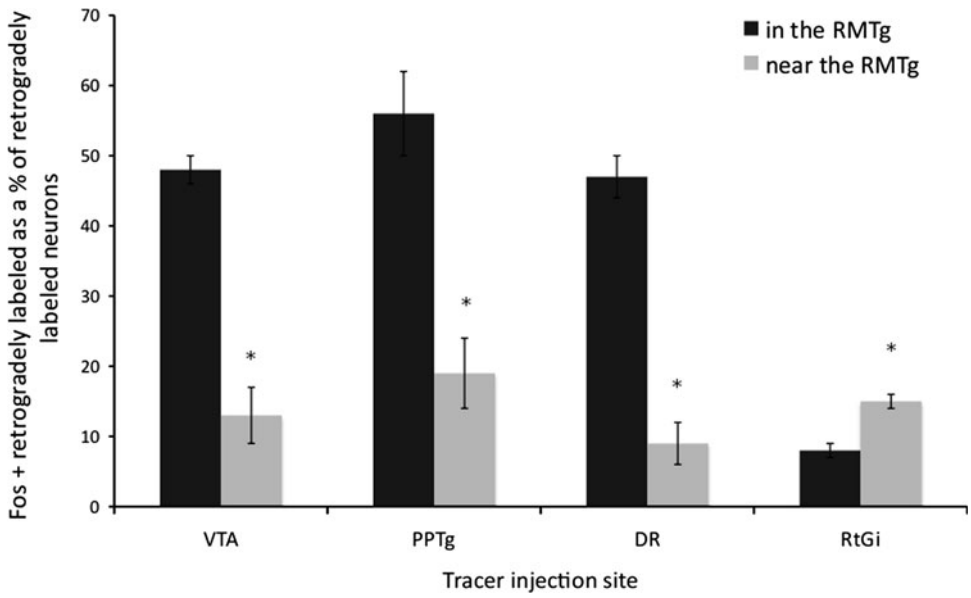

Fig. 7 Graph showing the percentages of double-labeled neurons in the RMTg compared to the region surrounding it for four different injection sites: PPTg ($n = 6$), DR ($n = 5$), VTA ($n = 5$), and RtGi ($n = 11$). *$p < 0.01$ (paired t test)

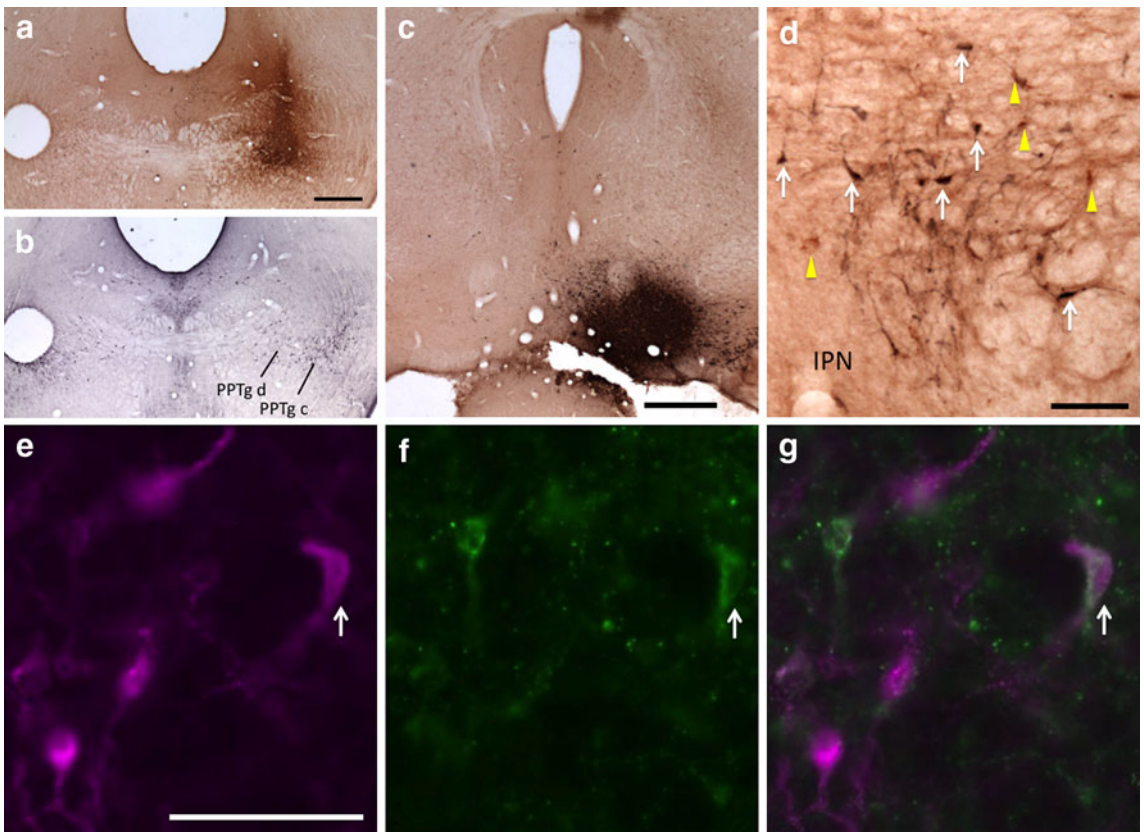

Fig. 8 Retrogradely labeled neurons in the RMTg following injections of CTβ in the PPTg and FG in the VTA. **a** CTβ injection site in the PPTg. **b** Adjacent section processed for Nos immunoreactivity (*black*) verifies that the injection shown in '**a**' is in the PPTg, pars dissipata. **c** FG injection 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**

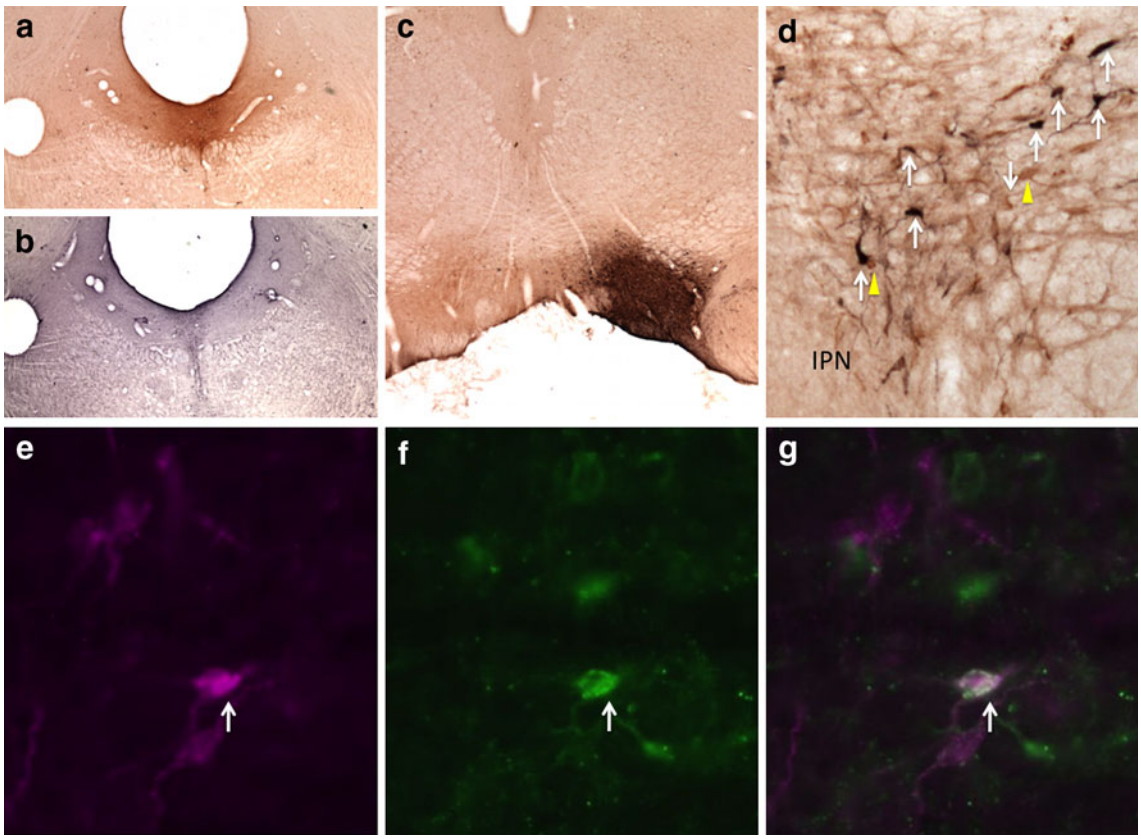

Fig. 9 Retrogradely labeled neurons in the RMTg following injections of CTβ in the DR and FG in the VTA. **a** CTβ injection site in the DR. **b** Adjacent section processed for 5-HT immunoreactivity (*black*) verifies that the injection shown in **a** is in the DR. **c** FG injection site in the VTA. **d** CTβ-labeled neurons (*yellow arrows*), 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**). *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 serotoninergic 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.

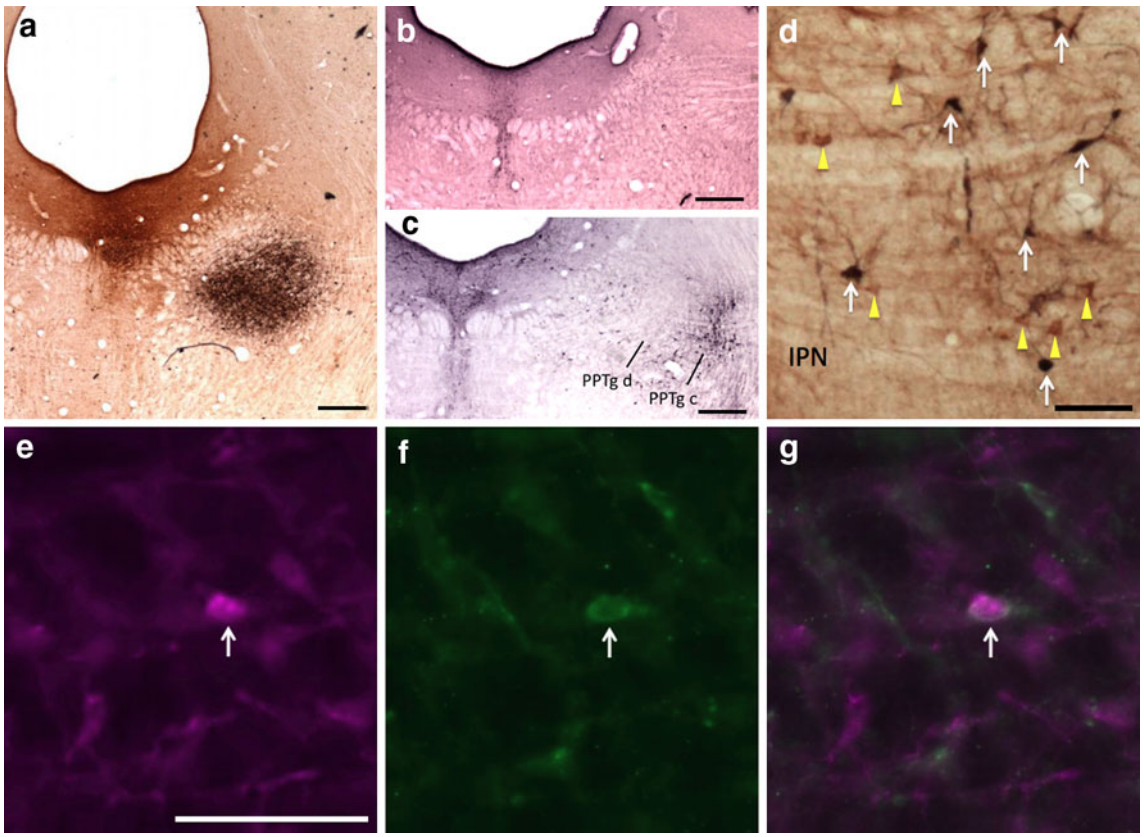

Fig. 10 Retrogradely labeled neurons in the RMTg following injections of CTβ in the DR and FG in the PPTg. **a** FG injection site in the PPTg (*black*); CTβ injection site in the DR (*brown*). **b** Adjacent section processed for 5-HT immunoreactivity (*black*) verifies that the *brown* injection shown in **a** is in the DR. **c** Adjacent section processed for Nos immunoreactivity (*black*) verifies that the *black* injection shown in **a** is in the PPTg, pars dissipata. **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**

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

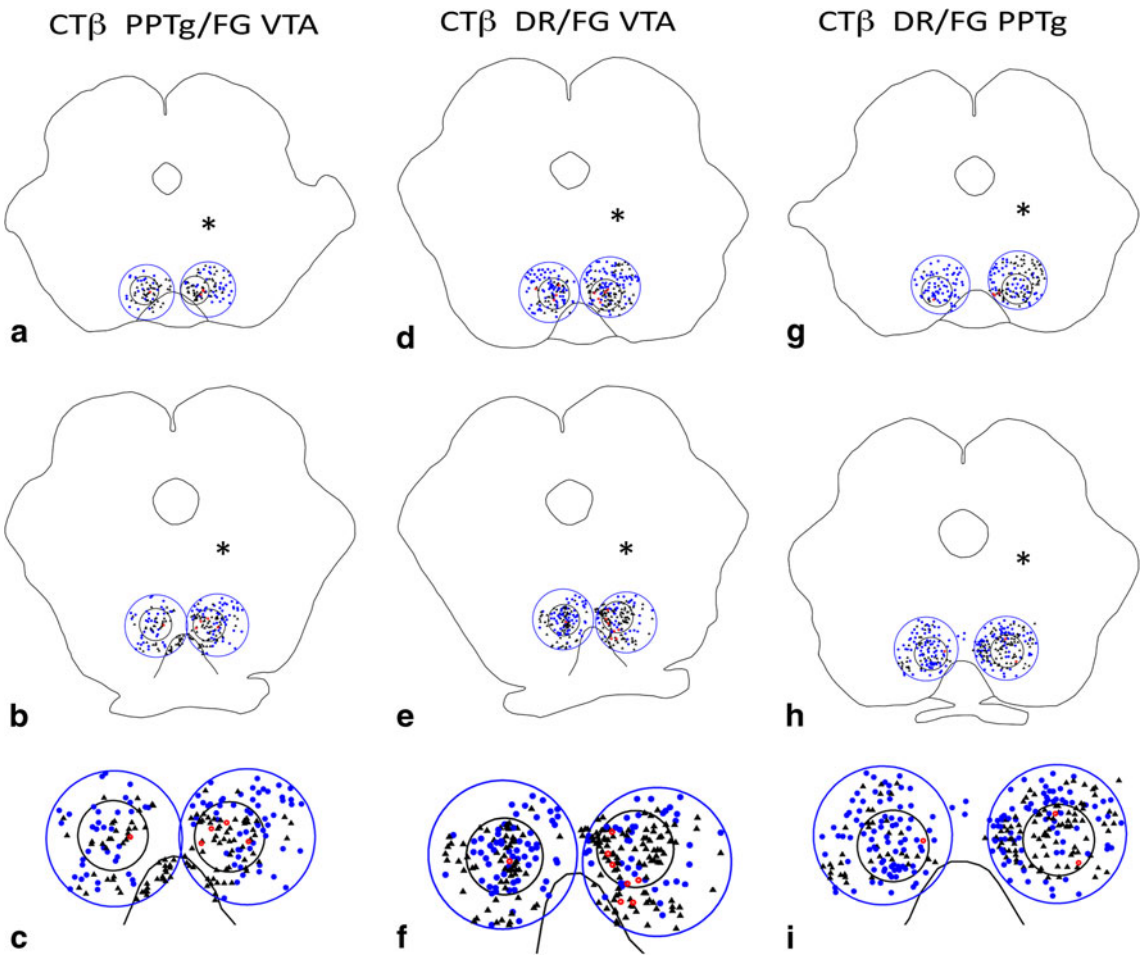

Fig. 11 Representative plots at rostral (**a**, **d**, **g**) and caudal (**b**, **e**, **h**) levels of the RMTg (*small black circles*) from cases that received injections of CTβ tracer in the PPTg and FG in the VTA (**a–c**), cases that received injections of CTβ tracer in the DR and FG in the VTA (**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

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

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 coordinately regulate both the VTA/SNC and PPTg. As single neurons in the RMTg project to both the VTA and the PPTg, it seems reasonable to speculate that the RMTg may serve 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 serotoninergic (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 suggested that DR neurons may monitor the stressfulness of situations in the service of developing appropriate behavioral responses. The RMTg may convey information integrated from a variety of different descending pathways (Jhou et al. 2009b; Kaufling et al. 2009), and conduct it to the DR in order to facilitate the synthesis of appropriate responses by that structure. As was observed for RMTg projections to the PPTg, single RMTg neurons project both to the DR and VTA, suggesting again that single RMTg neurons may coordinate utilization of multiple neurotransmitters in several structures.

Despite convincing evidence from PHA-L injections that the RtGi is a target of strong RMTg projection (Jhou et al. 2009b), the present results shed doubt on this conclusion. The percentage of retrogradely labeled neurons that expressed Fos in the RMTg following CTβ injections into the RtGi was no different than in areas surrounding the RMTg and no focal accumulation of retrogradely labeled neurons in the RMTg was observed. To the contrary, retrogradely labeled neurons were relatively dense following these injections in much of the midbrain tegmentum including the retrorubral field, which is known to give rise to a robust descending projection to the vicinity of the RtGi (von Krosigk and Smith 1991; von Krosigk et al. 1992). Presumably in the earlier study (Jhou et al. 2009b), PHA-L labeled these descending projections or ones akin to them. The present result leads to the idea that the RMTg may be more involved in the modulation of structures that give rise to strong ascending projections, such as the VTA/SNC, PPTg, and DR, than in structures that have mainly descending outputs, such as the RtGi.

Recent studies of the RMTg demonstrate that it is activated and exhibits Fos expression in response to aversive stimuli or reward omission and inhibited by rewards and reward-predictive stimuli (Jhou et al. 2009a; Hong et al. 2011). RMTg neurons also express Fos in response to acute and repeated exposure to psychostimulants (Colussi-Mas et al. 2007; Geisler et al. 2008; Jhou et al. 2009a, b; Kaufling et al. 2010a, b; Perrotti et al. 2005; Scammell et al. 2000), but this does not necessarily indicate that psychostimulants activate RMTg neurons (Zahm et al. 2010). To the contrary, Lecca et al. (2011) have 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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