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ORIGINAL ARTICLE

Dopamine receptors in the rat entopeduncular nucleus

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Abstract
Dopamine is critical for the normal functioning of the basal ganglia, modulating both input and output nuclei of this system. The distribution and function of each of the five dopamine receptor subtypes have been studied extensively in the striatum. However, the role of extrastriatal dopamine receptors in basal ganglia information processing is less clear. Here, we studied the anatomical distribution of dopamine receptors in one of the output nuclei of the rodent basal ganglia, the entopeduncular nucleus (EP). The presence of all dopamine receptor subtypes was verified in the EP using immunostaining. We detected co-localization of dopamine receptors with VGAT, which suggests presynaptic expression on GABAergic terminals. D1R and D2R were strongly colocalized with VGAT, whereas DR3-5 showed only sparse co-localization. We further labeled striatal or pallidal neurons with GFP and showed that only D1 receptors were co-localized with striatal terminals, while only D2R and D3R were co-localized with pallidal terminals. Dopamine receptors were also strongly co-localized with MAP2, indicating postsynaptic expression. Overall, these findings suggest that the dopaminergic system modulates activity in the EP both directly via postsynaptic receptors, and indirectly via GABAergic synapses stemming from the direct and indirect pathways.

Keywords Entopeduncular nucleus · Globus pallidus · Striatum · Basal ganglia · Dopamine receptors

Introduction

The basal ganglia are a group of subcortical nuclei implicated in motor and cognitive functions (Alexander 1986; Mink 1996). Dopaminergic modulation of the input to these nuclei is responsible for balancing the information flow between the direct and indirect pathways from the striatum to the output nuclei (Gerfen and Surmeier 2011) leading to modulation of learning, reinforcement signaling and the execution of motor actions (Beninger 1983; Schultz et al. 1992; Nakahara et al. 2004; Wickens et al. 2007; Maia and Frank 2011). The importance of dopamine has led to extensive investigation of dopaminergic modulation of striatal activity. However, less attention has been directed towards dopaminergic modulation outside the striatum, despite the fact that dopaminergic innervation of other basal ganglia structures is well established (Smith et al. 1989; Caille et al. 1996; Gauthier et al. 1999; Bolam et al. 2000; Watabe-Uchida et al. 2012; Lavian et al. 2017). Furthermore, dopamine receptors have been detected in many other structures of the basal ganglia, such as the globus pallidus (GP), the substantia nigra pars reticulata (SNr) and the entopeduncular nucleus (EP) (Lindvall et al. 1974; Beckstead et al. 1979; Yung et al. 1995; Rivera et al. 2003; Fuchs and Hauber 2004; Kliem et al. 2010; Mitkovski et al. 2012).

The EP, a major output nucleus of the rodent basal ganglia, and the homologue of the human globus pallidus internus (GPi), is a key structure in basal ganglia information processing. The importance of the EP comes to the fore in clinical cases, where it has been shown that high frequency stimulation of the GPi can alleviate symptoms of patients with Parkinson's disease (Siegfried and Lippitz 1994; Kumar et al. 1998) or Tourette syndrome (Schrock et al. 2014). EP neurons integrate information stemming from all three basal ganglia pathways: individual neurons receive input from GABAergic striatal spiny projection

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neurons (SPNs) via the direct pathway, from GABAergic neurons of the GP via the indirect pathway, and from glutamatergic neurons of the subthalamic nucleus (STN) via the hyperdirect pathway (Deniau et al. 1978; Nagy et al. 1978; Bolam and Smith 1992). Recently, we showed that in the EP, dopamine differentially modulates transmission of GABAergic synapses from the striatum and GP (Lavian et al. 2017). We found that striatal inputs are potentiated by dopamine via D1-like dopamine receptors (D1LR) and that pallidal inputs are depressed by dopamine via D2LR. Others have shown that dopamine and dopamine antagonists induce changes in GABA concentration in the EP and in the firing rate of EP neurons (Floran et al. 1990; Aceves et al. 1995; Ruskin et al. 2002; Kliem et al. 2007). Together, these findings suggest that dopamine acts indirectly in the EP, by modulating activity of EP neurons via GABAergic synapses.

Previous anatomical studies have detected D1R, D4R and D5R in the EP and GPi (Yung et al. 1995; Ciliax et al. 2000; Rivera et al. 2003; Kliem et al. 2010). These studies used immunohistochemistry and electron microscopy to show that these receptors are co-localized with axons and axon terminals, and suggested that these axons are GABAergic and arise from the striatum. However, D4R and D5R have also been detected postsynaptically in the EP (Ciliax et al. 2000; Rivera et al. 2003). Furthermore, RNA for all five dopamine receptors was found to be expressed by EP neurons (Lavian et al. 2017). These results suggest that the effects of dopamine on EP activity may not be completely mediated by the striatum, and that dopamine could have a potent effect in directly modulating neuronal activity in the EP. Here, we tested this hypothesis and investigated the distribution of the five dopamine receptors in the EP and their expression on different neuronal elements. We used viral labeling and immunohistochemistry to investigate the expression of each of the dopamine receptors on striatal and pallidal axons in the EP, as well as their expression on the somata and dendrites of EP neurons.

Materials and methods

Animals

All procedures were approved and supervised by the Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Bar-Ilan University Guidelines for the Use and Care of Laboratory Animals in Research. This study was approved by the Israel National Committee for Experiments in Laboratory Animals at the Ministry of Health.

Viral injections

Eight adult female rats (four Long-Evans, four Wistar), weighing 247 ± 18 g (mean ± SD) were administered viral injections into the dorsolateral striatum and GP. The rats were anaesthetized with isoflurane, followed by an intramuscular injection of ketamine HCl (100 mg/kg) and xylazine HCl (10 mg/kg). The rat's head was fixed in a stereotaxic frame and the AAV9-CAG-GFP virus (1–2 µl; titer: 3.9×10^{12} vg/ml, University of North Carolina Gene Therapy Center) was injected bilaterally either into the dorsolateral striatum ($n = 4$) (AP, 1 mm; ML, ± 3 mm; DV, 4 mm and AP, 1 mm; ML, ± 3 mm; DV, 4.5 mm) or into the GP ($n = 4$) (AP, − 0.95 mm; ML, ± 3 mm; DV, 6 mm) (Paxinos and Watson 2007). The virus was injected using a syringe pump (World Precision Instruments, USA) at a rate of 0.1 µl/min, and left in place for 10 min to allow viral particle diffusion before needle removal. Experiments were performed 4 weeks after viral injection.

Immunohistochemistry

The rats were deeply anesthetized using ketamine/xylazine (100 and 10 mg/kg, respectively) and perfused transcardially, with cold 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. The brains were quickly removed, placed in 4% PFA overnight and then sequentially cryoprotected in 20 and 30% sucrose in 0.1 M PBS until the brains sank in the solution. The brains were then sectioned into 40-µm-thick sagittal slices on a freezing microtome. The slices were kept in a tissue-collecting solution (TCS; 25% glycerol, 30% ethylene glycol and Na_2PO_4 0.1M, pH 7.2) at − 20 °C. All immunohistochemistry was completed on free-floating sections and mounted on slides for analysis. The slices were washed five times in 0.1% Triton X-100 in PBS for a total of 25 min. Nonspecific binding was blocked with 20% normal goat serum and 0.1% Triton X-100 in PBS for 60 min. Slices were then incubated for 24–72 h at 4 °C with primary antibody diluted in 2% normal goat serum and 0.1% Triton X-100 in PBS. The primary antibodies were: rat anti-D1R (1:200, Sigma-Aldrich, Cat# D2944, RRID: AB_1840787), rabbit anti-D2R (1:400, Millipore, Cat# AB5084P, RRID: AB_2094980), rabbit anti-D3R (1:100, Abcam, Cat# ab42114, RRID: AB_732004), rabbit anti-D4R (1:200, Abcam, Cat# ab20424, RRID: AB_445574) and rabbit anti-D5R (1:800, Abcam, Cat# ab40656, RRID: AB_732008). We used chicken anti-MAP2 for neuronal labeling (1:1000, Abcam, Cat# ab5392, RRID: AB_2138153) and mouse anti-VGAT for labeling of GABAergic presynaptic terminals (1:200,

Synaptic Systems, Cat# 131 011, RRID: AB_887872). In each immunostaining experiment we omitted the primary antibody in one slice as a control. Following incubation with the primary antibody, slices were washed five times in 0.1% Triton X-100 in PBS for a total of 25 min. For secondary antibody staining, slices were incubated for 1 h at room temperature with secondary antibodies. The secondary antibodies were: goat anti-rat igG conjugated to Alexa 568 for D1R staining (1:1000, Thermo Fisher Scientific, Cat# A-11077, RRID:AB_2534121), goat anti-rabbit igG conjugated to Alexa 568 for DR2-5 staining (1:1000, Thermo Fisher Scientific, Cat# A-11011, RRID: AB_143157), goat anti-chicken igG conjugated to Alexa 647 for MAP2 staining (1:1000, Abcam, Cat# ab150175, RRID: N/A) and goat anti-mouse conjugated to Alexa 488 for VGAT staining (1:1000, Thermo Fisher Scientific, Cat# A-11001, RRID: AB_2534069). To label nuclei, slices were incubated with Hoechst 33342 (1:1000, Invitrogen) for 10 min and then washed three times in 0.1% Triton X-100 in PBS for a total of 15 min. Slices

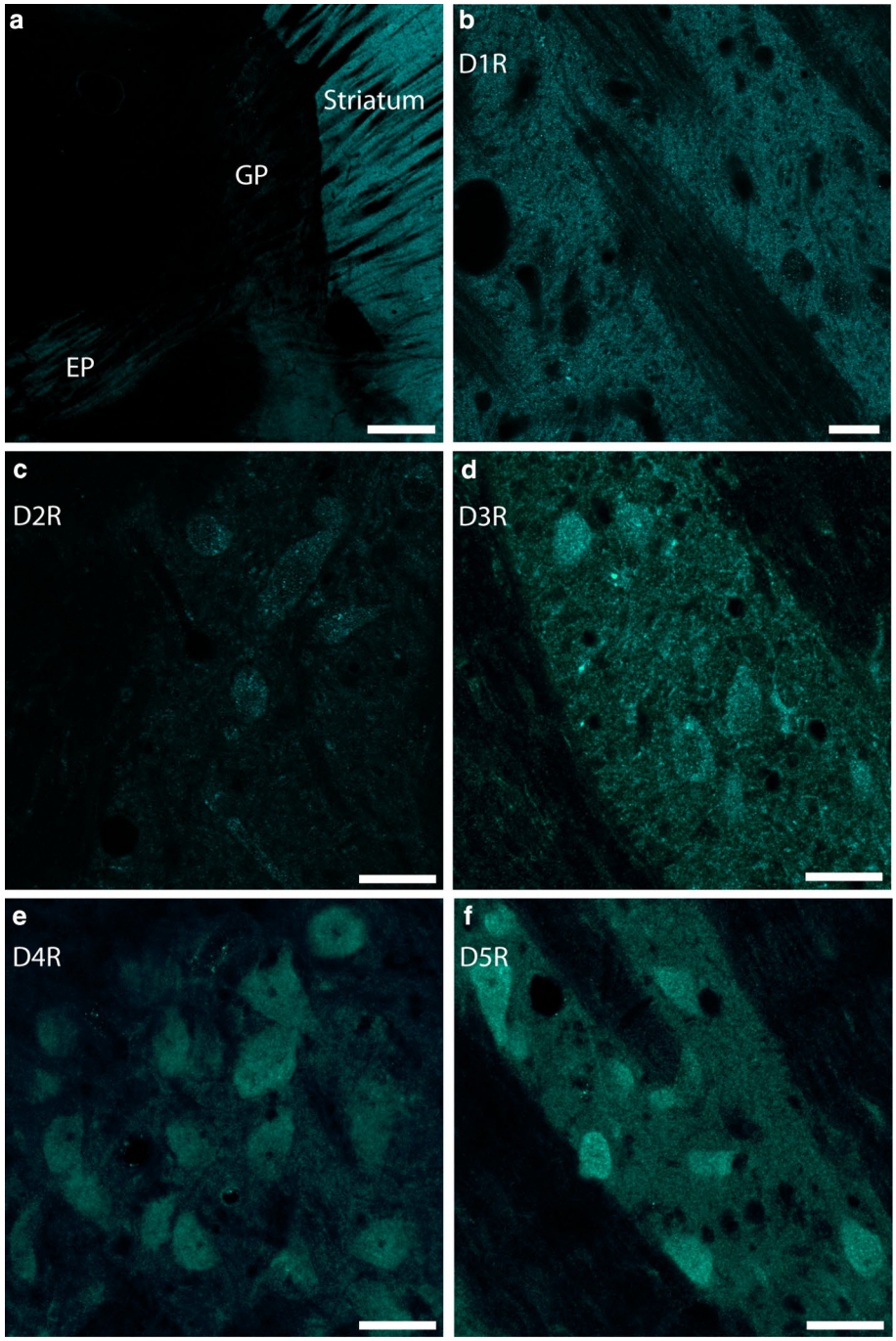

Fig. 1 All dopamine receptors are expressed in the EP. **a** A sagittal slice from a rat brain containing the striatum, GP and EP, stained for D1R. ×4 magnification, scale bar = 500 µm. **b**–**f** Confocal images of EP sections immune stained for D1R (**b**), D2R (**c**), D3R (**d**), D4R (**e**) and D5R (**f**) (blue). ×63 magnification, scale bar = 20 µm

were then placed on glass slides and dried for 15 min before being immersed in a mounting solution (Aqua Poly/Mount, Polyscience Inc., Pennsylvania, USA) and covered with a cover slip. Whole slice images were obtained with the neuro-explorer system (MicroBrightField). Confocal images were obtained with a Leica SP8 STED microscope (100×/1.4 N.A. oil objective) or with a Leica SP8 confocal microscope (63×/1.4 N.A. oil objective).

Primary antibodies against D1R and D2R were tested previously on knockout mice and were found to be highly specific for immunohistochemistry (Stojanovic et al. 2017). The specificity of primary antibody against D4R was tested previously using a preabsorption protocol with an immunogen peptide (Wang et al. 2012). To ensure specificity of primary antibodies against D3R, D4R and D5R, we performed control experiments in which each antibody was preabsorbed with the corresponding immunogen peptide. The immunogen peptide sequences of antibodies against D3R, D4R and D5R were, respectively: CQACHVSPELYRATTWGY, DVRGRDPAVC and GNAVGGSAGAPP (Shanghai Hanhong Scientific). Each primary antibody was incubated with the immunogen peptide for 24 h at 4 °C. These experiments showed that preabsorption with a control peptide abolished the immunostaining, thus validating the specificity of these antibodies (data not shown).

Data analysis

All offline analyses were carried out using MATLAB R2013a (Mathworks, RRID: SCR_001622) and IgorPro 6.0 (WaveMetrics, RRID: SCR_000325). Data for each experiment were obtained from at least three rats. All the results for each experiment were pooled and are displayed as the mean ± SEM, unless mentioned otherwise.

2D correlation maps were constructed for verification of co-localization sites. For each image, the 2D correlation was calculated between the stained dopamine receptor and MAP2, and between the stained dopamine receptor and the VGAT/GFP-labeled axons. Each image was divided into bins, the 2D correlation was calculated for each bin, followed by linear interpolation. The Pearson's correlation coefficient r between channels X and Y was calculated as follows:

$$r = \frac{\sum_m \sum_n (X_{mn} - \bar{X})(Y_{mn} - \bar{Y})}{\sqrt{(\sum_m \sum_n (X_{mn} - \bar{X})^2)(\sum_m \sum_n (Y_{mn} - \bar{Y})^2)}}.$$

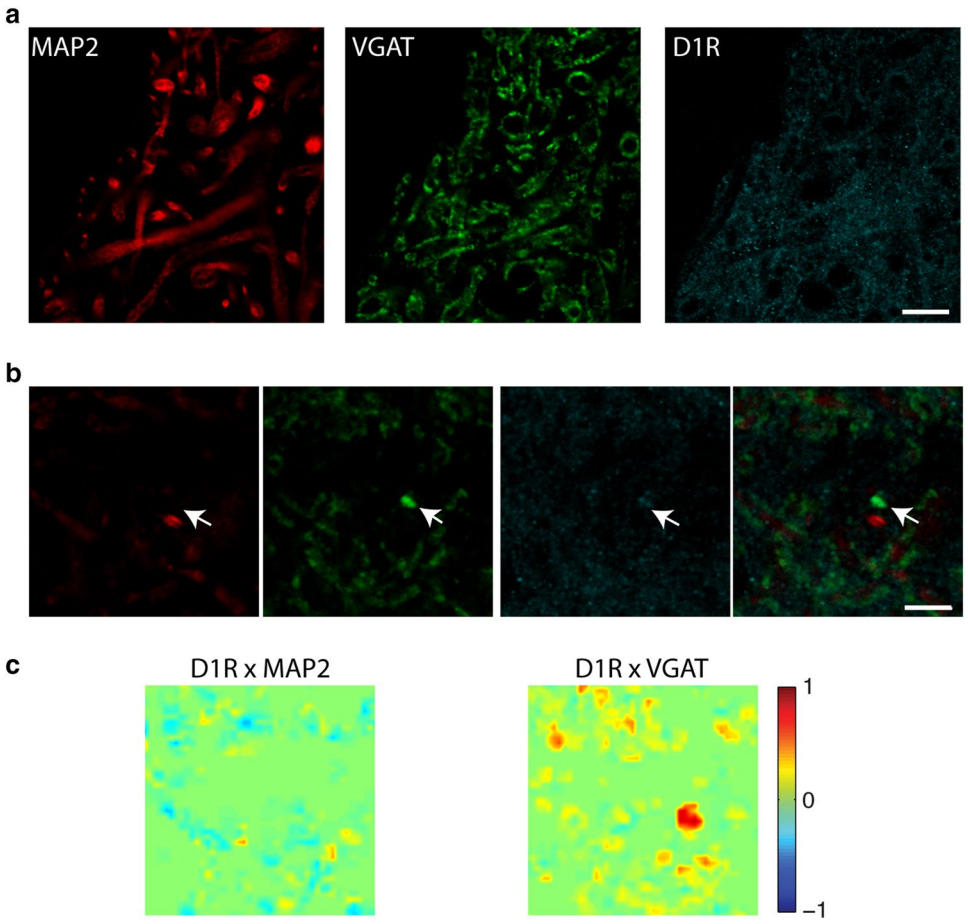

Fig. 2 D1 receptors co-localize with presynaptic terminals of GABAergic synapses in the EP. **a** Confocal images of EP sections immune-stained for D1R (blue), MAP2 (red) and VGAT (green). ×63 magnification, scale bar = 10 µm. **b** Higher magnification of confocal images of EP sections immunostained for D1R (blue), MAP2 (red) and VGAT (green). Scale bar = 5 µm. White arrows indicate VGAT puncta co-localized with the D1R. **c** 2D correlation map constructed for the image presented in **b**. 2D correlation between MAP2 and D1R indicates no co-localization of D1R with dendrites of EP neurons. 2D correlation map between VGAT and D1R indicates sites of co-localization of D1R with GABAergic presynaptic terminals

Results

Dopamine receptors 1–5 are expressed in the EP

To investigate the distribution of dopamine receptors in the EP, we immuno-stained EP sections for each of the dopamine receptors (Fig. 1). Figure 1a shows a sagittal section of a brain slice stained for D1R. As expected, and serving as an internal control, D1R was strongly expressed in the striatum and EP, but not in the GP (Boyson et al. 1986; Dawson et al. 1986; Yung et al. 1995). Confocal images suggested that D1 receptors were mainly detected on cellular processes in the EP (Fig. 1b). In addition to strong expression of D1R, we also detected the expression of all other dopamine receptor subtypes in the EP, in line with our previous findings showing the mRNA of all dopamine receptors in the EP (Lavian et al. 2017). Confocal images revealed that unlike D1R, DR2-5 were expressed primarily on somata of EP neurons, with weaker staining of cellular processes (Fig. 1c–f). Thus, all five dopamine receptor subtypes were expressed in the EP, but on different neuronal elements.

Dopamine receptors are expressed on GABAergic terminals in the EP

Previous studies have shown that D1R and D4R are sparsely expressed on presynaptic terminals, with a larger expression on axons in the EP (Yung et al. 1995; Rivera et al. 2003). Based on earlier observations, it has been suggested that these are GABAergic terminals and axons originating in the striatum. However, this suggestion has not been verified, leaving open the possibility that the stained terminals were not GABAergic. To illuminate the functional identity of the stained terminals in the EP we co-stained slices for each of the dopamine receptors, for VGAT to label GABAergic presynaptic terminals, and for MAP2 to label neuronal somata and dendrites (Figs. 2, 3, 4). To detect GABAergic terminals labeled with dopamine receptors, we conducted confocal imaging of stained EP sections and searched for sites with high co-localization of dopamine receptors with VGAT and no co-localization with MAP2. We found that D1 receptors were strongly co-localized with VGAT, supporting their expression on GABAergic terminals (Yung et al. 1995) (Fig. 2). Figure 2a shows a confocal image of a stained EP section, in which the expression pattern of D1R is similar

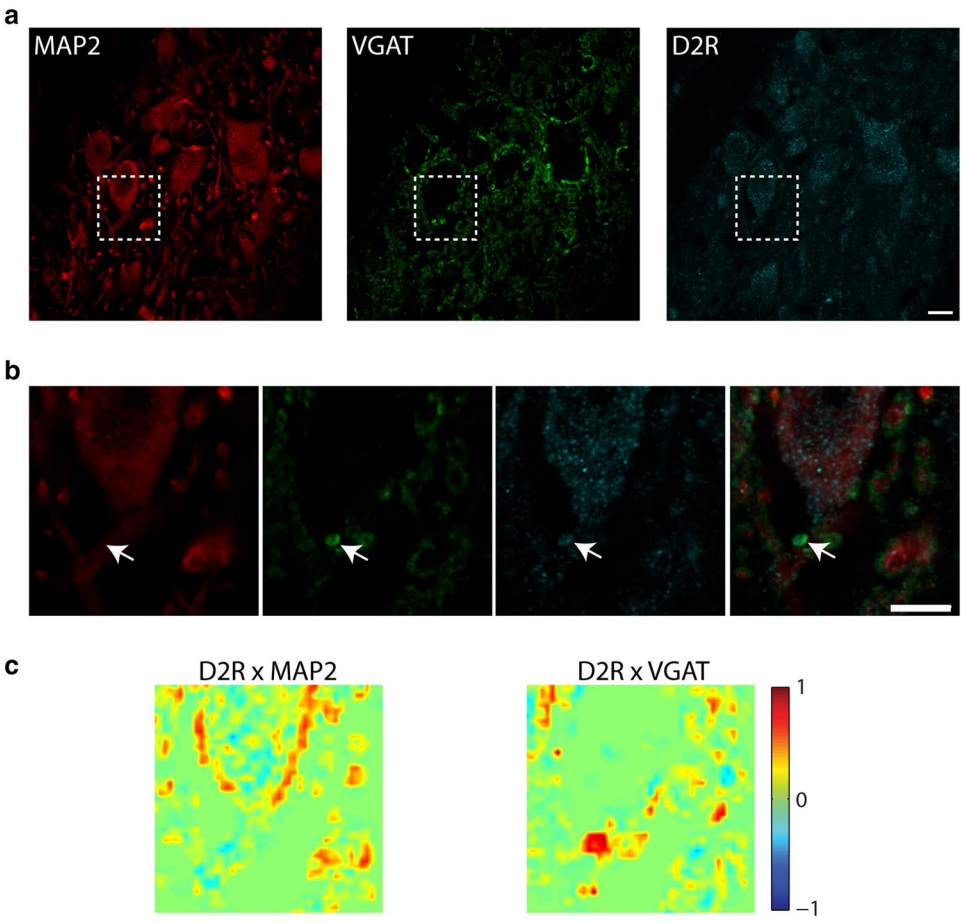

Fig. 3 D2R are co-localized with presynaptic terminals of GABAergic synapses in the EP. **a** Confocal images of EP sections immuno-stained for D2R (blue), MAP2 (red) and VGAT (green). ×63 magnification, scale bar = 25 µm. **b** Higher magnification of confocal images of EP sections immuno-stained for D2R (blue), MAP2 (red) and VGAT (green). Scale bar= 5 µm. White arrows indicate VGAT puncta co-localized with the D2R. **c** 2D correlation map constructed for the image presented in **b**. 2D correlation maps indicate co-localization of D2R with MAP2 as well as with VGAT

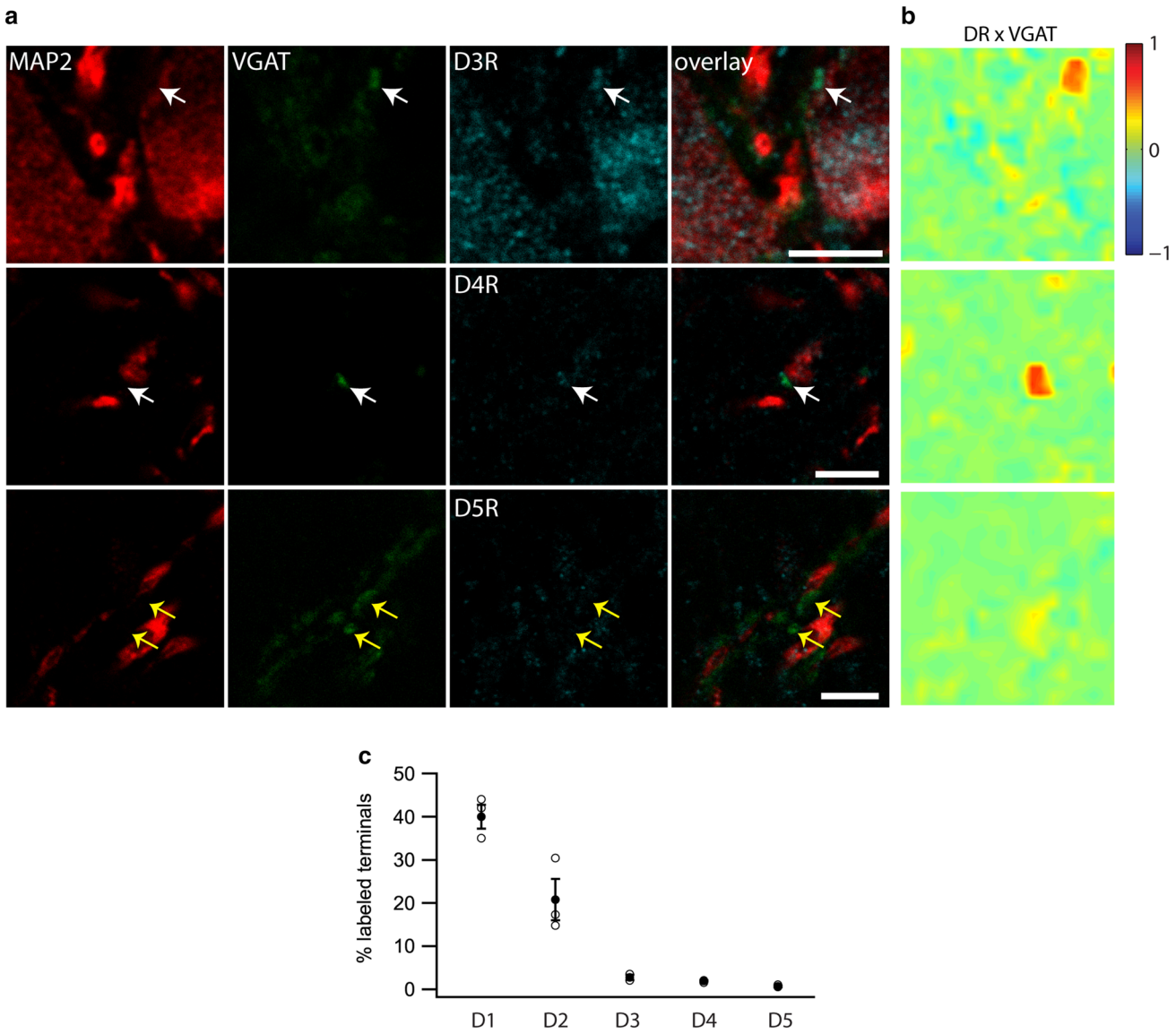

Fig. 4 Sparse co-localization of GABAergic terminals with DR3-5 in the EP. **a** Confocal images of EP sections triple immuno-stained for MAP2 (red), VGAT (green) and DR3-5 (blue). White arrows indicate VGAT puncta co-localized with the stained dopamine receptor (DR). Yellow arrows indicate VGAT puncta not co-localized with the stained dopamine receptor. Scale bar = 5 µm. **b** 2D correlation between VGAT and DR3-5 was calculated for each image. **c** percentage of VGAT puncta co-localized with each of the dopamine receptors in the EP (means ± SEM, $n_{(animals)} = 3$, $n_{(VGAT\ puncta)} = 1112 \pm 221$)

to the expression of VGAT, but not MAP2. Figure 2b shows a higher magnification of a different EP section, in which a GABAergic terminal co-localized with D1R can clearly be seen. This co-localization between VGAT and D1R also emerged in the 2d correlation map of the images as displayed in Fig. 2b, c.

D2 receptors were also strongly co-localized with VGAT (Fig. 3). Figure 3b shows an EP neuron and a GABAergic terminal, both of which are co-localized with D2R. Unlike D1R and D2R, DR3-5 showed only sparse co-localization with VGAT (Fig. 4). To quantify our results, we counted all VGAT puncta, and calculated the percentage of VGAT puncta co-localized with each of the dopamine receptors. We found that the number of VGAT puncta co-localized with D1R was higher than that of all other dopamine receptors ($n = 3$, one way ANOVA, $F_{(4,9)} = 39$, $p < 0.001$; Tukey–Kramer post hoc test, $p < 0.01$), and that the number of VGAT puncta co-localized with D2R was higher than that of DR3-5 (Fig. 4c, Tukey–Kramer post hoc test: $p < 0.05$). These findings support the current model, which

suggests that the effects of dopamine on activity in the EP are not direct, but mediated by the modulation of presynaptic GABAergic terminals.

D1 receptors are expressed on striatal axons

Our results so far have indicated that within the EP, dopamine receptors are co-localized with GABAergic presynaptic terminals. However, immunohistochemistry is not sufficient for the identification of the origin of these presynaptic terminals. Thus, we next used AAV-induced GFP expression to pinpoint the origin of the GABAergic terminals co-localized with dopamine receptors in the EP. There are two major known sources for GABAergic input to the EP: the striatum and GP (Alexander and Crutcher 1990). Previous studies have suggested that in the EP, dopamine receptors are expressed on striatal axons and axon terminals (Yung et al. 1995; Rivera et al. 2003). To test this hypothesis, we labeled striatal axons terminating in the EP by virally transfecting striatal neurons using AAV-induced GFP expression (Fig. 5a). We stained these slices for MAP2 and each of the dopamine receptors, and obtained confocal images of striatal axons and axon terminals in the EP (Fig. 5b). Confocal images of these sections revealed synaptic terminals adjacent to dendrites of EP neurons, a characteristic of striatal synapses in the EP (Bolam and Smith 1992). In line with previous suggestions, we detected sparse co-localization between D1R and striatal axon terminals (Fig. 5b, c). However, in contrast to reports in the literature, we found that D5 receptors were completely absent from striatal axons and axon terminals, and detected only negligible co-localization between DR2-4 and striatal axons (Fig. 5d). Figure 5b shows a confocal image of an EP section, in which striatal axons and axon terminals are not co-localized with D4R. Thus, in the EP only D1 receptors are expressed by striatal terminals.

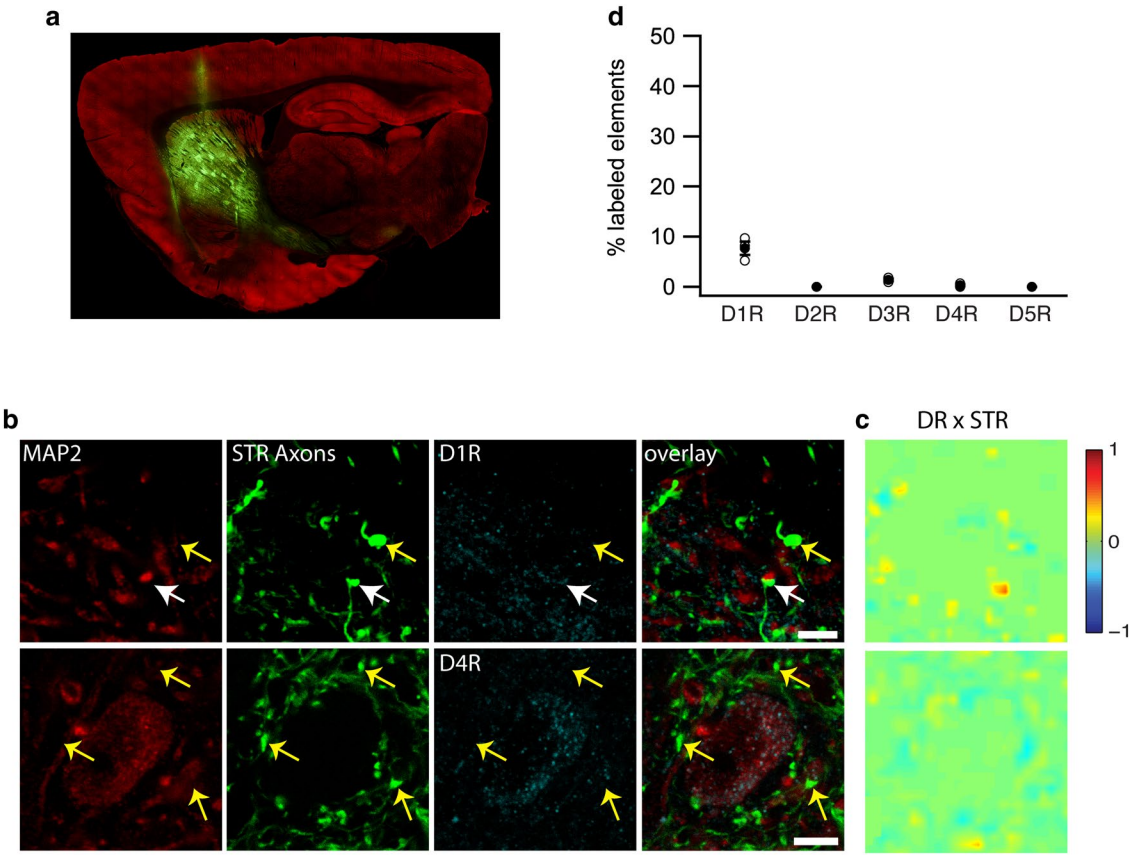

Fig. 5 Dopamine receptor expression on striatal GABAergic terminals. **a** A sagittal slice from a rat brain with AAV9-CAG-GFP injected to the striatum. The slices were stained for MAP2 (seen in red in the images) to stain somata and dendrites to enable visualization of the ultrastructure of the slice. **b** Confocal images of EP sections double immuno-stained for MAP2 (red) and D1R/D4R (blue). Striatal axons virally labeled with GFP are shown in green. White arrows indicate striatal terminals co-localized with the stained dopamine receptor (DR). Yellow arrows indicate striatal terminals not co-localized with the stained dopamine receptor. Scale bar = 5 µm. **c** 2D correlation maps show striatal axon terminals co-localized with D1R, but not with D4R. **d** Percentage of striatal elements co-localized with DR1-5 ($n_{(\text{animals})} = 3–5$, $n_{(\text{STR axons})} = 2279 \pm 422$)

D2R and D3R are expressed on GP axons and axon terminals

The GP is the second major source of GABAergic input to the EP, accounting for 15% of the total input to the EP (Bolam and Smith 1992; Nambu 2007). Thus, we labeled axons reaching the EP from the GP by virally transfecting GP neurons using AAV-induced GFP expression (Fig. 6a). EP sections were stained for each of the dopamine receptors and MAP2 and visualized with a confocal microscope. Confocal images of these sections revealed synaptic terminals adjacent to EP soma, a characteristic of GP synapses in the EP (Bolam and Smith 1992) (Fig. 6b). Similar to the results from the striatum, confocal images did not indicate significant co-localization between most dopamine receptors and GP axons in the EP (Fig. 6d). D1R, D4R and D5R showed no co-localization with GP axons. However, D2R and D3R were co-localized with GP axons and axon terminals. Figure 6b shows several GP terminals co-localized with either D2R or D3R. This co-localization also emerged in the 2D correlation map of the images as displayed in Fig. 6c. Thus, in the EP, only D2R and D3R are expressed by GP terminals. These results are in line with our previous findings and suggest that D2LR modulate transmission in GP-EP synapses via a presynaptic mechanism (Lavian et al. 2017).

Dopamine receptors 1–5 are expressed by EP neurons

Thus far, our findings have indicated that dopamine receptors are expressed presynaptically in the EP, suggesting that dopaminergic modulation of EP activity is mediated by presynaptic dopamine receptors expressed on GABAergic terminals. However, previous studies have detected D4R and D5R on postsynaptic structures in the EP (Ciliax et al. 2000; Rivera et al. 2003), as did our initial observations (Fig. 1). Furthermore, we recently used qRT-PCR to show that the mRNA of all five dopamine receptors is expressed by EP neurons (Lavian et al. 2017). These findings suggest that dopamine receptors are also expressed postsynaptically in the EP. To test this hypothesis, we

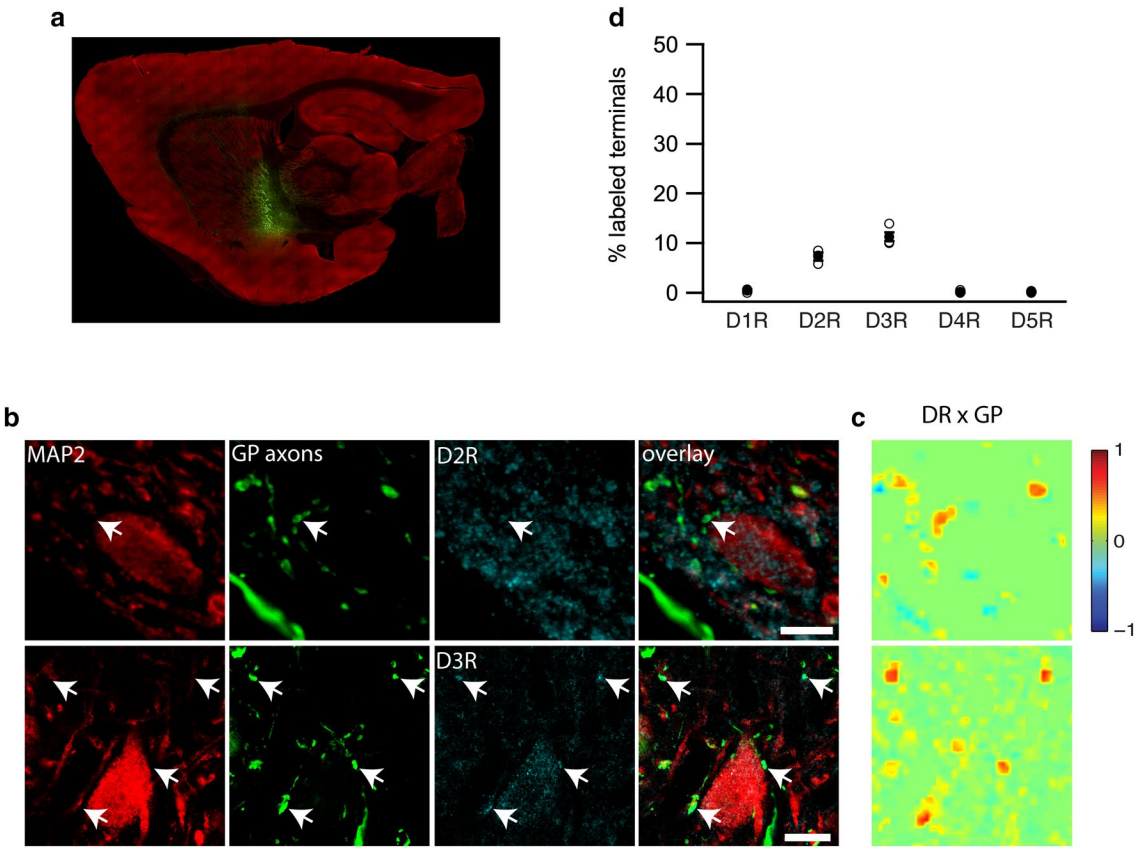

Fig. 6 Dopamine receptors expression on pallidal GABAergic terminals. **a** A sagittal slice from a rat brain with AAV9-CAG-GFP injected into the GP. **b** Confocal images of an EP section double immuno-stained for MAP2 and D2R/D3R. White arrows indicate GP terminals co-localized with the stained dopamine receptor (DR). Scale bar = 5 µm. **c** 2D correlation maps show co-localization of GP terminals with D2R or D3R. **d** percentage of GP elements co-localized with DR1-5 ($n_{(animals)} = 3$, $n_{(GP\ axons)} = 720 \pm 121$)

further immunostained EP sections for each of the dopamine receptors and for MAP2 (Fig. 7). As indicated by qRT-PCR, all five dopamine receptors were co-localized with MAP2, indicating their expression by EP neurons. The staining for MAP2 allowed us to count the total number of neurons and to calculate the percentage of neurons co-localized with each of the dopamine receptors. Whereas D1 receptors were detected in 41% of the neurons, DR2-5 were all expressed by the majority of neurons (Fig. 7), suggesting a potent effect of dopamine on EP activity. These findings show that in the EP, DR2-5 are primarily postsynaptic. Taken together, our findings indicate that in the EP dopamine receptor subtypes are expressed both on presynaptic sites and by EP neurons, hence supporting the working hypothesis that dopamine plays a role in pre- and post-synaptic information processing in the EP (Fig. 8).

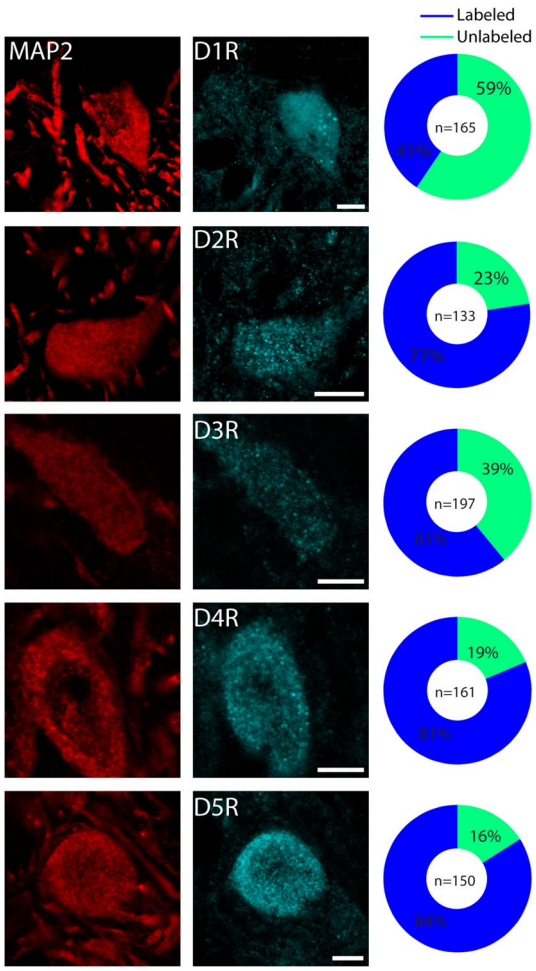

Fig. 7 Postsynaptic expression of dopamine receptors in the EP. Confocal images of EP sections immuno-stained for MAP2 (red) and DR1-5 (blue). Scale bar = 5 µm. Pie charts show the proportion of neurons expressing each one of the dopamine receptors out of the total number of neurons stained with MAP2 ($n_{(animals)} = 4$)

Discussion

In the present study, we used immunostaining and viral labeling to investigate the distribution of the different types of dopamine receptors in the EP. We show that all dopamine receptor subtypes are expressed in the EP. Furthermore, we show that D1R and D2R are strongly co-localized with GABAergic presynaptic sites, whereas all other dopamine receptors are sparsely co-localized. Viral labeling experiments indicated that only D1 receptors are expressed on striatal axon terminals, and only D2R and D3R are expressed on GP axons and axon terminals. Finally, we found that all dopamine receptor subtypes are expressed by EP neurons. These results are summarized in the scheme illustrated in Fig. 8. Overall, these findings suggest that the activity of EP neurons is modulated directly by dopamine via postsynaptic receptors, as well as indirectly via modulation of GABAergic synaptic transmission.

In previous studies, D1 receptors have been observed on axons within the EP, which prompted the suggestion that these axons originated in the striatum (Mansour et al. 1992; Yung et al. 1995). Our results here show strong co-localization of D1R with GABAergic terminals in the EP (Figs. 2, 4). However, whereas 40% of the VGAT puncta were co-localized with D1R, only 8% of the striatal terminals labeled with GFP were co-localized with D1R (Fig. 5). Since the striatum accounts for ~ 75% of the input to the EP, these findings seem contradictory. Furthermore, none of the GP terminals showed co-localization with D1R (Fig. 6). It should be noted that our analysis did not allow us to completely separate GFP-labeled axons from GFP-labeled axon terminals. Thus, the fraction of striatal terminals co-localized with D1R was probably larger. Another possible explanation would be inhomogeneous expression of D1R on SPN axon terminals. In our experiments, the GFP labeling of striatal axons involved injections into the dorsolateral striatum. Thus, the expression of D1R on SPN axon terminals may not have been homogenous, and potentially higher in the ventral striatum. This explanation should be further explored. D1R and D5R were also co-localized with MAP2, possibly indicating postsynaptic expression (Fig. 7). Thus, while D1R are expressed in a small fraction of striatal terminals, D1LR are primarily expressed postsynaptically, suggesting that the main site of dopaminergic modulation of striatal input to the EP is postsynaptic. This suggestion is supported by our previous electrophysiological results, which showed that application of SCH23390, a D1LR blocker, has no effect on the paired pulse ratio (PPR) of striatal-evoked IPSCs (Lavian et al. 2017). Changes in the PPR are an indicator for changes in transmitter release, and hence serve as evidence for presynaptic modulation. The lack of such changes indicates, in

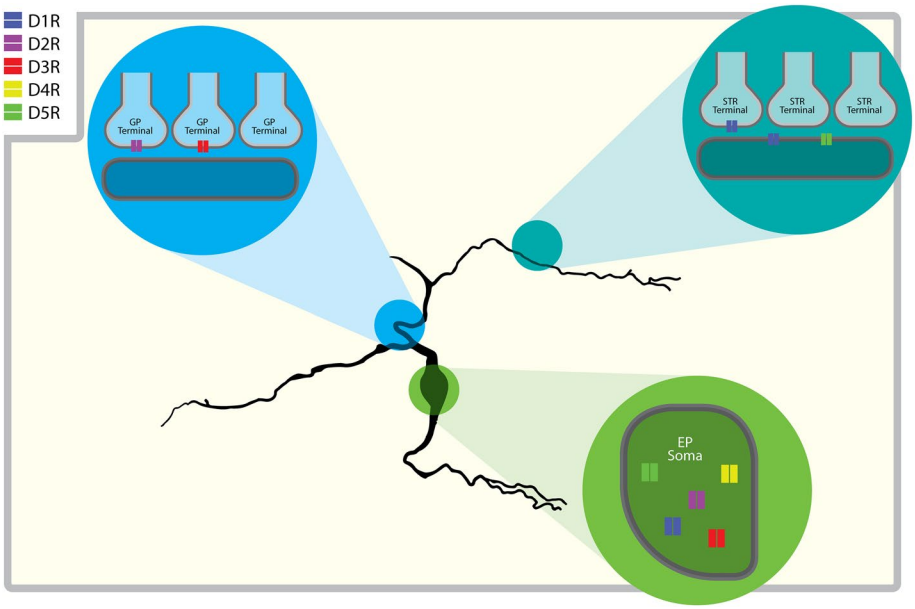

Fig. 8 Schematic illustrating the expression of all dopamine receptors in the EP. D1 receptors are sparsely co-localized with GABAergic presynaptic terminals originating in the striatum. Previous electrophysiological findings indicate that D1R and D5R modulate striatal synapses postsynaptically. D2R and D3R are sparsely co-localized with GABAergic presynaptic terminals originating in the GP. All dopamine receptors are co-localized with the somata of EP neurons. Our results cannot determine whether dopamine receptors are only produced by EP neurons, or are further expressed postsynaptically in the EP

line with our current findings, that dopaminergic modulation of striatal-EP transmission is primarily postsynaptic.

We also showed that D2 receptors are strongly co-localized with GABAergic terminals, and that D3 receptors are sparsely expressed (Figs. 3, 4). Furthermore, in brain slices expressing GFP in the GP only D2R and D3R were observed to be co-localized with presynaptic elements (Fig. 6). These results are consistent with our previous observation that the PPR of GP evoked IPSCs decreased following the application of sulpiride, a D2LR blocker, hence indicating a presynaptic mechanism (Lavian et al. 2017). Since both D2R and D3R were stained presynaptically, changes to the PPR may have been mediated by either one or both of these receptors.

D4R and D5R have been found to be expressed postsynaptically by EP neurons, as well as on axon terminals (Ciliax et al. 2000; Rivera et al. 2003; Kliem et al. 2010). In line with these results, we found that these dopamine receptors were sparsely expressed on GABAergic axon terminals in the EP. However, D4R and D5R showed no co-localization with striatal or GP axons or axon terminals (Figs. 5, 6). These findings do not preclude the possibility that in the EP, dopamine receptors are expressed on axons with another origin. EP afferents originate not only from the striatum and GP; the EP receives glutamatergic input from the STN (Deniau et al. 1978) and the rostromedial tegmental nucleus (Clarke et al. 1996), and dopaminergic input from the SNc (Beckstead et al. 1979).

In addition to presynaptic expression, we found that all dopamine receptors displayed postsynaptic staining in the EP (Fig. 7). These results support the notion that dopamine receptors are expressed by EP neurons, as was found previously by qRT-PCR (Lavian et al. 2017). It should be noted that our results indicate that EP neurons produce all dopamine-receptor subtypes, but not necessarily that these receptors are postsynaptically expressed by these neurons. It is possible that EP neurons express dopamine receptors, which are further transported to EP axon terminals. This is merely a speculation and should be further investigated.

The function of the dopamine receptors depends on their cellular location. Dopamine receptors can be expressed on the soma and induce changes in neuronal excitability (Surmeier et al. 1992; Neve et al. 2004). Dopamine receptors have also been detected on axon terminals and on postsynaptic sites where they can modulate synaptic transmission and synaptic plasticity (Tritsch and Sabatini 2012). In the striatum, dopamine modulates cortical input to SPNs of the indirect pathway via receptors expressed on dendritic spines of SPNs or on presynaptic cortical terminals (Pickel et al. 1981; Bamford et al. 2004). Postsynaptic expression of dopamine receptors on striatal SPNs and interneurons also allows dopamine to directly modulate the excitability of these neurons (Surmeier et al. 1992; Straub et al. 2014). Thus, discovering the expression pattern and specific sites of expression of the different dopamine receptors is crucial to our understanding of the role of dopamine in the integration of basal ganglia pathways. Here, we show that dopamine receptors are expressed both on presynaptic GABAergic synapses as well as postsynaptically by EP neurons. These findings strongly imply that the activity of EP neurons is modulated directly by dopamine via postsynaptic receptors, as well as indirectly via modulation of GABAergic synaptic transmission, thus supporting the working hypothesis that dopamine plays an important role in synaptic integration in the EP.

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Compliance with ethical standards

Conflict of interest The authors declare they have no conflict of interest.

Ethical approval All experimental procedures were approved and supervised by the Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Bar-Ilan University Guidelines for the Use and Care of Laboratory Animals in Research. This study was approved by the Israel National Committee for Experiments in Laboratory Animals at the Ministry of Health. This article does not contain any studies with human participants performed by any of the authors.

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