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Neural connection supporting endogenous 5-hydroxytryptamine influence on autonomic activity in medial prefrontal cortex

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ABSTRACT

5-hydroxytryptamine (5-HT) transmission in the medial prefrontal cortex (mPFC) enhances or suppresses signal outflow to influence emotion-/cognition-based function performances and, putatively, the autonomic responses. The top-down cortical modulation of autonomic activities may be mediated in part through projections from mPFC to brain stem dorsal vagal complex (DVC). The abundant and heterogeneous densities of 5-HT fibers across laminae in mPFC suggest serotonergic innervation of mPFC-DVC projection neurons whereby endogenous 5-HT acts to regulate autonomic activities. The present study investigated the physical relationship between 5-HT fibers and the autonomic-related mPFC neurons by examining and quantitatively characterizing the 5-HT contacts upon retrogradely labeled mPFC-DVC projection neurons in pre- and infra-limbic cortices (PrL/IL) with light and electron microscopies combined with immunocytochemistry for 5-HT and presynaptic vesicle marker synaptophysin (Syn). 5-HT varicosities were observed, under confocal microscope, to form close appositions to or, at ultrastructural level, to form asymmetric axodendritic synapses and direct contacts upon the target neurons. About 16% of the entire 5-HTergic varicosities in lamina V of PrL/IL coexpressed Syn and about 24% of the peri-somatic 5-HTergic swellings demonstrated Syn-immunoreactivity (ir), suggesting a low frequency of putative synapses estimated at optical level. Ultrastructurally, examination of thirty-seven serially cut thin 5-HT boutons closely apposed to the labeled dendritic profiles demonstrated that only three contacts presented with identifiable asymmetric, synaptic membrane specializations. These data provide the first and direct morphological evidence supporting that endogenous 5-HT may be released mainly via direct contacts bearing no identifiable synaptic specializations as well as synapses, targeting autonomic-related mPFC neurons for autonomic regulation.

1. Introduction

The medial prefrontal cortex (mPFC) is characterized by heterogeneity in cytoarchitecture, neuron type and connectivity across its mediolateral areas and subserves diverse functions including regulation of emotion- and cognition-based tasks (Bush et al., 2000; Heidbreder and Groenewegen, 2003; Vogt et al., 2004; Dalley et al., 2004; Hoover and Vertes, 2007; Vogt and Paxinos, 2014). In addition, mPFC is actively involved in autonomic regulation, as is evidenced by the decreased gastric motility, hypotension and bradycardia following stimulation of the prelimbic (PrL) and infralimbic cortices (IL) (Hurley-Gius and Neafsey, 1986; Heidbreder and Groenewegen, 2003; Sevoz-Couche et al., 2006; Hassan et al., 2013), the two important subdivisions of mPFC. Previous studies indicated that this autonomic effect might be mediated through divergent pathways including mPFC projections to brain stem dorsal vagal complex (DVC), which is composed of nucleus of solitary tract (Sol), the dorsal motor nucleus of vagus (DMV) and area postrema, either directly or indirectly via relay of such structures as amygdala and hypothalamus (Saper, 2004; Gabbott et al., 2005; Sevoz-Couche et al., 2006; Mussa and Verberne, 2013). Thus, DVC plays a pivotal role in mediating descending control of autonomic activities.

Serotonin or 5-hydroxytryptamine (5-HT), an important neuromodulator, can exert excitatory and/or inhibitory effects on mPFC neurons via activating pre- and/or post-synaptic 5-HT receptors associated with glutamate/GABA transmissions (Aghajanian and Marek 1997 and 1999; Marek and Aghajanian, 1998; Arvanov et al., 1999; Martín-Ruiz et al., 2001; Cai et al., 2002; de Groote et al., 2002;

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Yuen et al. 2005 and 2008; Zhong et al., 2008). Its overall effects are contingent on the 5-HT receptor subtype(s) activated, the milieu 5-HT concentration and the cell type of specific mPFC neurons (Arvanov et al., 1999; Cai et al., 2002; Puig et al., 2003; Yuen et al. 2005 and 2008; Gui et al., 2010; Llado-Pelfort et al., 2012). Data shows that dysfunction and/or manipulation of 5-HT signaling system within mPFC is causally associated with the pathogenesis and treatment of psychological disorders (e.g., depression, anxiety and schizophrenia) (Arvanov and Wang, 1998; Marek and Aghajanian, 1998; Yuen et al., 2008; Solati et al., 2011). An important characteristic of these psychiatric disorders is the comorbidity of autonomic disturbance (Van Oudenhove et al., 2007 and 2010). In addition, 5-HT injection into ventral PrL and/or IL directly induced depressor response (Hassan et al., 2015). All these findings converge to uphold the critical, autonomic role of 5-HT in mPFC; however, the research work addressing a rather fundamental issue, the morphological relationship between 5-HT inputs and mPFC neurons involved in autonomic regulation, is thus far unavailable.

5-HT fibers are widely distributed in mPFC, demonstrating distinct heterogeneity across laminae in fiber density (e.g., highest in laminae I and V/VI) and in proportion of component fiber types (large vs fine caliber) that originate respectively from different raphe nuclei, thus implying possible compartmentalized innervations in mPFC (DeFelipe et al., 1991; Smiley and Goldman-Rakic, 1996; Linley et al., 2013). Since the functional and cytoarchitectural pleomorphism of mPFC implicates distinct fiber connectivities with different mPFC neuron types, the possibility exists that PrL/IL neurons projecting to the DVC (mPFC-DVC neurons), whose activities may influence the autonomic performance, contact directly, in a specific connection mode, with 5-HT fibers. Therefore, the present study sought to address this issue by using immunofluorescence and electron microscopy combined with tract-tracing approaches and by quantitatively evaluating the proportion of synapse upon mPFC-DVC neurons versus the direct contact with no obvious membrane specializations.

2. Materials and methods

2.1. Animals

Fifteen adult male Sprague-Dawley rats, weighing 200–250 g, were divided into three groups ($n = 5$, per group) and injected with tracers followed by multiple immunofluorescence and immunoelectron microscopies, respectively (Table 1). The surgery protocol was in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and followed the ethical guidelines at the Committee of Animal Use for Research and Education of the Fourth Military Medical University (P.R. China). All the animals were housed in cages, with food and water ad libitum, under a 12/12 h light/dark cycle (06:00 light on and 18:00 light off) at 22 °C with controlled humidity before killing.

Table 1
Experiment groups, scheme and fluorophores used for visualization.

Groups Tracer: approach	Scheme	Fluorophore excitation/emission (nm)
1. FG: electrophoresis	a. FG mapping	FG: 330– 385/≥395
	b. Double labeling for FG/5-HT	Alexa 488 (FG): 490/520– 530 Alexa 594 (5-HT): 590/600– 630
2. TMR: injection	TMR labeling	TMR: 520– 560/570– 620 Alexa 488 (Syn): id. Alexa 594 (5-HT): id.
	a. Double labeling for 5-HT/Syn.	
	b. Triple labeling for 5-HT/Syn/TMR	Alexa 488 (Syn): id. Alexa 594 (TMR): id. Alexa 647 (5-HT): 650/664– 668
3. WGA-HRP: injection	Immunocytochemistry for 5-HT (electron microscopy)	

2.2. Retrograde tract tracing and tissue preparation

To map the cortical neurons that project to the DVC, retrograde tracer was deposited at unilateral Sol of DVC (Bregma: −14.28 to −14.50) of pentobarbital sodium anesthetized (40 mg/kg body weight, i.p.), stereotaxically fixed rats, according to stereotaxic atlas of Paxinos and Watson (2005). Briefly, 4% Fluoro-Gold (FG) (Biotium), 0.05 μl of 10% tetramethylrhodamine-dextran amine (TMR, Simga) or 0.05 μl of 2% wheat germ agglutinin-horseradish peroxidase (WGA-HRP) (Abcam) was administered electrophoretically (current: 5 μA; 7 s on-off cycle for 5–10 min) or via pressure through a glass micropipette (internal tip diameter 15– 25 μm) attached to a Hamilton syringe (Table 1). Following surgery, the skin was sutured antiseptically and the animals were allowed a 3–5-day or 48–60-h survival before light or electronic microscopic (EM) studies, respectively. Since TMR could label cortical neurons in the same pattern as FG but, unlike FG, yields visible fluorescence with confocal microscopy without additional section staining. Thus, for a quick examination under confocal microscope of the retrograde labeling before immunofluorescence, TMR was used in the batch of triple labeling.

Animals for bright-field or fluorescence microscopic experiments were fixed with 4% paraformaldehyde in phosphate buffer (PB, pH 7.2– 7.4) and the brains were cut serially in a cryostat (CM 1900, Leica, Germany) into coronal sections in 25 μm thickness and divided into four series of every four sections. One series containing DVC and mPFC was used for examination of tracer deposition and the labeling with an epifluorescence microscope (BX-60, Olympus, Japan) under appropriate filters for FG or TMR (Table 1) and the labeling was then imaged through a computer-interfaced CCD imaging system for mapping work. Other series were processed for multiple immunofluorescence for 5-HT, Synaptophysin (Syn) and/or FG/TMR, respectively (Table 1). Substituting normal rabbit serum for the primary antibodies yielded no immunoreactivities.

For EM experiment, animal brains were removed following perfusion with ice-cold fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde in PB, and brain segments containing DVC and mPFC were serially cut into coronal sections of 60 μm thick on a vibratome.

2.3. Light microscopy of immunocytochemistry

2.3.1. Single label immunocytochemistry for FG

For visualization of FG labeling under bright field, sections were incubated sequentially with rabbit polyclonal antibodies against FG (1:200; Abcam), biotinylated goat anti-rabbit IgG (1:200; Vector) and avidin-biotin complex (ABC) (1:200; Vector). Color reaction was performed with 3, 3′-diaminobenzidine tertrahydrochloride (DAB) as chromogen. Nissl's staining was performed when necessary to facilitate the laminar localization of the retrograde labeling.

2.3.2. Immunofluorescence labeling

For double immunofluorescence for 5-HT/FG and 5-HT/Syn, sections were incubated sequentially through (1) a mixture of goat anti-5-HT (1:200, Immunostar) and rabbit anti- FG (1:200; Abcam) or mouse anti-Syn monoclonal antibodies (1:500; Cell Signaling) and (2) a mixture of Alexa 594 and Alexa 488 conjugated antibodies raised in donkey against goat and rabbit or mouse (1:500, Invitrogen), respectively. For TMR/5-HT/Syn triple labeling, the same primary antibodies with an additional rabbit *anti*-TMR IgG (1:100; Abcam) were used, followed by incubation with a mixture of Alexa594-, Alexa647- and Alexa488-conjugated immunoglobulins raised in donkey against rabbit, goat and mouse IgG, respectively (1:500, all from Invitrogen). Sections were finally observed under epifluorescence microscope (BX-60, Olympus, Japan) with appropriate filters for FG and Alexa 488 or under laser

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