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Original Article

MC4R expression in pedunculopontine nucleus involved in the modulation of midbrain dopamine system

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Abstract: Background and objective: Separate studies have implicated the pedunculopontine tegmental nucleus (PPTg) in processing aversive stimuli to dopamine systems, and melanocortin-4 receptor (MC4R) are broadly expressed by the neurons in the PPTg, but the exact neurosubstrate underlying the regulation of dopamine systems by the central melanocortin pathway is poorly understood. Methods: In this study, the PPTg of 6 adult mice expressing green fluorescent protein (GFP) under the control of the MC4R promoter was detected by fluorescence immunohistochemistry. Results: A large number of GFP-positive neurons in the dissipated parts of PPTg (dpPPTg) were found, and approximately 50% of MC4R-GFP- positive neurons in the dpPPTg coexpressed tyrosine hydroxylase, a marker of dopamine neurons, indicating that they were dopaminergic. Conclusions: Our findings support the hypothesis that MC4R signaling in the dpPPTg may involve in the modulation of midbrain dopamine systems.

Keywords: Melanocortin-4 receptor, dopamine, the pedunculopontine tegmental nucleus

Introduction

It is well known that midbrain dopamine neurons play an important role in Parkinson's disease [1]. The pedunculopontine tegmental nucleus (PPTg), a part of the mesencephalic locomotor region, is involved in the gait disturbance that characterized parkinsonian syndromes [2, 3]. Neurons in the PPTg exhibit the sophisticated neurochemical properties, including cholinergic, serotonergic, catecholaminergic, GABAergic and glutamatergic-containing neurons [4-9]. Previous studies in rat and mouse documented that a subpopulation of PPTg neurons expresses the melanocortin-4 receptor (MC4R), a G protein-coupled, seven-transmembrane receptor expressed in the brain [10-13]. Otherwise, there is evidence that the melanocortins can act on mesolimbic dopamine pathways [14]. These data suggest that there exist a tight link between MC4R and dopaminergic system in the PPTg. We speculate that MC4R in the PPTg may primarily involve in the modulation of midbrain dopamine systems.

Although it is now widely recognized that dopaminergic activity are tightly interconnected via central melanocortinergic pathways involving the MC4R [15-17], the exact neurosubstrate underlying the regulation of dopamine systems by the central melanocortin circuit is poorly understood. Many studies have shown that tyrosine hydroxylase (TH) is the marker of midbrain dopamine neurons [1, 18-21]. The main objective of this study is to provide direct neuroanatomical evidence for the central melanocortin-dopaminergic circuits in the PPTg using fluorescence immunohistochemical detection.

Materials and methods

Animal care

Generation of MC4R-GFP mice was described previously [22, 23]. Male mice between 7 and

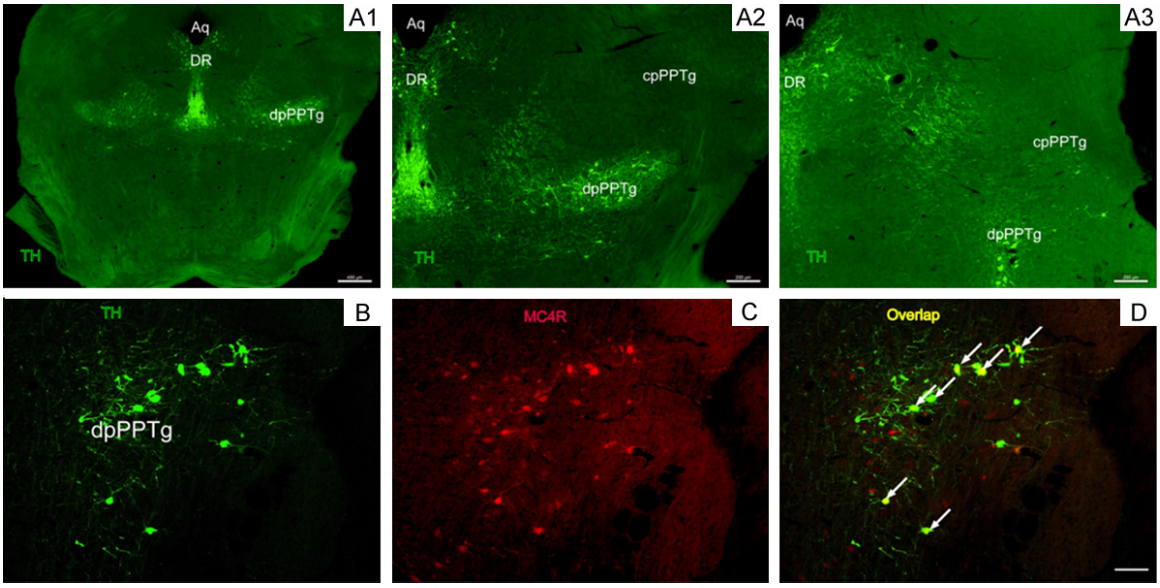

Figure 1. Colocalization of TH in subsets of MC4R-GFP positive neurons within PPTg areas. (A1-A3 and B) show TH-positive neurons (green); (A2) indicates split channel image of the corresponding PPTg area in panel (A1) during high magnification. (C) shows MC4R-GFP -positive neuron (red); (D) shows overlaid images of (B) plus (C). Arrows indicate double-labeled neurons (yellow). TH and MC4R-GFP are broadly expressed by the neurons in the dpPPTg. In contrast to the dpPPTg, we did not detect dual labeled neurons in the compact (cp) parts of PPTg (cpPPTg). Aq, aqueduct; DR, doral raphe; dpPPTg, the dissipated parts of the PPTg; cpPPTg, the compact parts of the PPTg. Some drawings were taken from HB Xiang (Brain 2013, Med Hypotheses 2011, Epilepsy & Behavior 2013). Scale bar: 400 μm for (A1), 200 μm for (A2 and A3), 100 μm for (B-D).

12 weeks old were used for the experiments. All the mice were housed under a 12 h light/dark cycle with food and water provided *ad libitum*. All experiments were performed in accordance with the guidelines of the NIH and the International Association for the Study of Pain and were approved by the Institutional Animal Care and Use Committees at Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology University.

Immunohistochemistry

Standard procedures were used as described previously [22, 24]. Briefly, mice were anesthetized with the mixture of ketamine (10 mg/ml) and xylazine (0.5 mg/ml) by intraperitoneal injection, and fixed by intracardiac perfusion of cold 0.01 M PBS (pH 7.4) and 4% paraformaldehyde. Brain tissues were immediately removed, post-fixed in the same fixative overnight at 4°C, and cryoprotected in 30% sucrose PBS solution.

Brain tissues were frozen and sectioned at 25 μm thickness on a cryostat. Free-floating sections were blocked in a solution containing 2% donkey serum and 0.3% Triton X-100 in PBS for 1 h at room temperature. The sections were incubated with primary antibodies overnight at 4°C followed by secondary antibodies. The following primary antibodies were used at the specified dilutions: a chicken polyclonal antibody against GFP (ab13970, 1:1,000; Abcam), rabbit anti-TH (1:2000; Chemicon International, Temecula, CA). The secondary antibodies included Cy3- or FITC-conjugated donkey anti-rabbit or anti-mouse IgG (1:1,000, Invitrogen, Molecular Probes, Eugene, OR). Finally, the sections were washed in 0.1 M PBS, mounted on gelatin-coated slides, dried, and observed via the fluorescence microscope (Leica DM2500) .When taking pictures, we defined the FITC as the red while the Cy3 as the green.

Results

Specific expression of TH and MC4R-GFP in the PPTg

TH-positive neurons were observed in the dissipated parts of PPTg (dpPPTg) but TH-positive fibers expressed in the compact parts of PPTg (cpPPTg) (**Figure 1A1-A3**). We assayed GFP

expression in the MC4R-GFP reporter mouse and observed a large number of GFP-positive neurons in the dpPPTg (**Figure 1C**) and the cpPPTg.

MC4R-GFP and TH co-expression in the PPTg

We found that double-labeled MC4R-GFP/TH neurons were mainly located in the dpPPTg, and approximately 50% of MC4R-GFP-positive neurons coexpressed TH-immunoreactive cells in the dpPPTg (**Figure 1D**). In contrast to the dpPPTg, we did not detect dual labeled neurons in the cpPPTg.

Discussion

In this study, we used a unique MC4R-green-fluorescent protein transgenic mouse model to demonstrate the distribution of MC4R-GFP and TH in the caudal parts of PPTg. Three major findings have emerged from this investigation: 1) a large number of GFP-positive neurons were located in the dpPPTg and cpPPTg; 2) approximately 50% of the MC4R-GFP-positive neurons were TH-immunoreactive cells in the dpPPTg; and 3) MC4R-GFP-positive neurons in the cpPPTg were not coexpressed with TH.

Some research has identified that the MC4R interacts with dopaminergic systems involved in the regulation of physiological and behavioral processes [25, 26]. Lindblom et al reported that alpha- melanocyte stimulating hormone (alpha-MSH) administered into the ventral tegmental area induced a significant increase in dopamine and DOPAC levels in the nucleus accumbens, and this increase was completely blocked by pre-treatment with the MC4R selective antagonist HS131, indicating that the MC4R may mediate the effects of alpha-MSH on dopamine transmission [25]. Lute et al reported that the hypometabolic/hypothermic effect of the nonselective melanocortin agonist MTII was prevented by dopamine antagonists, and MTII selectively activated arcuate nucleus dopaminergic neurons [27]. Cuit et al reported that the development of locomotor sensitization to repeated administration of cocaine was blunted in MC4R-null mice and normalized in MC4R/dopamine 1 receptor (D1R) mice, suggesting that the effects of MC4R signaling within D1R neurons may be involved in the long-term regulation of energy balance and behavioral responses to cocaine [28]. These data highlight that the MC4R contributes to a regulated response occurring at dopaminergic neurons, potentially beneficial during extreme physiologic stress. We found that approximately 50% of the MC4R-GFP-positive neurons were TH-immunoreactive cells in the caudal parts of PPTg, this result was consistent with the concept that melanocortins may act on mesolimbic dopamine pathways [14], indicating that MC4R expression in the PPTg is hypothesized to be involved in the modulation of midbrain dopamine systems.

Early studies of PPTg neurons contained at least six neuronal phenotypes: dopaminergic, cholinergic, serotonergic, catecholaminergic, glutamatergic, and GABAergic cells, but our work had emphasized its MC4R-positive neurons. It has been established that the PPTg areas are crucial for motor processes and behavioral state control [29]. There is growing evidence that the participation of midbrain melanocortinergic systems in diverse clinical contexts suggests these systems are highly complex, including energy balance, glucose homeostasis, and nociception [12, 14, 22, 30-32]. It has been shown previously that there is a physiological role of MC4R-mediated signaling, for example, MC4R enhances adenylyl cyclase activity by coupling to the stimulatory G protein (Gs), and leads to increased cyclic AMP (cAMP) production that subsequently increases the activity of protein kinase A (PKA) [33-35]. The present results showed that MC4R-positive neurons within midbrain dopamine regions may play important roles in motor processes and behavioral state control.

In summary, the results of the present study demonstrated that double-labeled MC4R-GFP/TH neurons were mainly located in the dpPPTg, suggesting that melanocortin-4 receptor expression in the PPTg may be involved in the modulation of midbrain dopamine systems. Further work on elucidating the organization, function and modification of midbrain melanocortin-dopaminergic circuits will importantly contribute to understanding the importance of central melanocortinergic system in mediating the pathophysiology of midbrain motivational systems.

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Disclosure of conflict of interest

None.

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