学 位 論 文 の 要 旨

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学 位 論 文 名	Neuroanatomical and Neurochemical Organization of Projections From the Central Amygdaloid Nucleus to the Nucleus Retroambiguus via the Periaqueductal Gray in the Rat
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論 文 内 容 の 要 旨

INTRODUCTION

The periaqueductal gray (PAG) has been known to be involved in the control of vocalization and reproductive behavior. The PAG sends projection fibers to the nucleus retroambiguus (NRA), which is a small nucleus in the caudal ventrolateral medulla and contains premotor neurons related to vocalization and mating behavior, and receives projection fibers from the central amygdaloid neucleus (CeA), which is one of the main output nuclei of the amygdaloid complex. Judging from the above, it seems quite probable that the PAG-NRA pathway is under the direct influence of the CeA in the control of reproductive behavior as well as of vocalization. It remains, however, an unsettled question whether or not the PAG neurons that send their axons to the NRA receive monosynaptic inputs from the CeA.

Projection neurons in the CeA or PAG have been revealed to employ gamma-aminobutyric acid (GABA) or glutamate, respectively, as a main neurotransmitter. However, there have been no morphological studies to clarify not only whether the CeA-PAG projection is GABAergic, but also whether the PAG-NRA projection is glutamatergic.

In this study, we first provide morphological evidence for the existence of a disynaptic pathway from the CeA to the NRA via the PAG by using anterograde tracing with biotinylated dextran amine (BDA) and retrograde tracing with cholera toxin B subunit (CTb), and then examine whether or not NRA-projecting PAG neurons are glutamatergic, as well as whether or not PAG-projecting CeA neurons are GABAergic, using a combined retrograde tracing with Fluoro-Gold (FG) and *in situ* hybridization for vesicular glutamate transporter 2 (VGLUT2) mRNA and glutamic acid decarboxylase (GAD67) mRNA.

MATERIALS AND METHODS

The experiments were performed on male Wistar rats, which were anesthetized by chloral hydrate. Ipsilateral injections of BDA into the CeA and CTb into the NRA were made in 14 rats. After 7-10 days survival, the rats were perfused transcardially with saline, followed by a solution composed of 4% paraformaldehyde (PA) and 0.2% glutaraldehyde in 0.1M phosphate buffer (PB; pH 7.4) and then with a solution composed of 4% PA and 5% glycerol in 0.1M PB. The brains were postfixed in a solution of 4% PA and 10% glycerol in 0.1M PB and saturated with 20% sucrose in 0.1M PB. Subsequently, serial transverse sections were cut 40 μm thick on a freezing microtome. BDA-labeled axons were visualized with avidin-biotin-peroxidase complex (ABC) and stained dark blue by using diaminobenzidine (DAB) and nickel ammonium sulfate as a chromogen. CTb-labeled neurons were detected immunohistochemically and stained brown by using DAB as a chromogen. In the electron microscopic experiments, BDA-labeled terminals were detected with ABC, and then silver-gold intensification of DAB reaction product of BDA was performed. CTb-labeled neurons were detected as mentioned above. The specimens were cut out from the lateral/ventrolateral PAG region, postfixed with osmium tetroxide, stained with uranyl acetate, dehydrated, cleared in propylene oxide, and then embedded flat in Epon. Subsequently, ultrathin sections were cut, stained with lead acetate, and then examined under the electron microscope.

CTb or BDA was injected into the lateral/ventrolateral PAG, where good overlapping distribution of CeA fibers and NRA-projecting neurons had been found, in 9 or 8 rats, respectively. Frontal sections of the brains were obtained, and then immunohistochemical visualization of CTb-labeled neuronal cell bodies or histochemical visualization of BDA-labeled fibers and terminals was performed.

In the combined retrograde tracing and *in situ* hybridization experiments, FG was injected into the lateral/ventrolateral PAG in 10 rats or into the NRA region in 12 rats. After 7-10 days survival, the rats were perfused transcardially with saline, followed by a solution composed of 4% PA which contained 0.2% picric acid. The brains were cut into frontal sections of 30 μm thickness on a freezing microtome. The sections were incubated in hybridization buffer containing antisense digoxigenin (DIG)-labeled VGLUT2 riboprobe (0.5 μg/μl) or GAD67 riboprobe (0.5 μg/μl) for 16-48 h at 50 °C. Subsequently, the sections were washed in standard saline citrate (SSC) with 50% formamide, treated with RNase, washed in SSC with 50% formamide, and incubated overnight in Tris-buffered saline containing sheep anti-DIG antibody conjugated to peroxidase, and then reacted with tyramide-conjugated Cy3. After *in situ* hybridization, the sections were processed to detect FG immunohistochemically; the sections were treated with rabbit anti-FG antibody, and then treated with Alexa488-conjugated anti-rabbit IgG. Finally, the sections were observed under an epifluorescent microscope as well as a confocal laser scanning microscope.

RESULTS AND DISCUSSION

In the rats that received combined injections of BDA into the CeA and CTb into the NRA, CTb-labeled neurons were embedded in the plexus of BDA-labeled axon terminals in the lateral/ventrolateral PAG at the caudal level of the PAG, and additionally in the lateral PAG at the midlevel of the PAG. In these areas, bouton-like varicosities labeled with BDA were often observed to be in contiguity with somata or dendrites of the CTb-labeled neurons. When the lateral/ventrolateral part of the caudal PAG was observed under the electron microscope, BDA-labeled axon terminals contained not only large numbers of pleomorphic clear vesicles but also small numbers of large dense cored vesicles, and made symmetrical synaptic contacts predominantly with dendrites and additionally with somata of the CTb-labeled neurons; the symmetrical synapses are usually thought to be inhibitory.

In the rats injected with CTb into the lateral/ventrolateral part of the middle or caudal PAG, large numbers of CTb-labeled neurons were observed bilaterally with an ipsilateral predominance in the amygdaloid complex. The highest number of labeled neurons was found in the CeA; they were distributed predominantly in the medial division, and additionally in the lateral division.

After BDA injection into the lateral/ventrolateral part of the middle or caudal PAG, BDA-labeled axons were observed bilaterally with an ipsilateral predominance in the medulla oblongata. A column of plexus of labeled fibers was found throughout the entire rostrocaudal extent of the ventrolateral medulla; rostrally, terminal labeling was seen in and around the nucleus ambiguus, and caudally in and around the NRA.

In the rats injected with FG into the lateral part of the middle PAG, large numbers of FG-labeled neurons expressing GAD67 mRNA were found in the medial division of the CeA; 556 (69%) out of 812 CeA neurons labeled with FG expressed GAD67 mRNA. On the other hand, after FG injection into the NRA region, large numbers of FG-labeled neurons expressing VGLUT2 mRNA were found in the lateral PAG throughout the rostrocaudal extent of the PAG; 381 (85%) out of 446 lateral PAG neurons labeled with FG expressed VGLUT2 mRNA. Judging from these data, it seems quite likely that the CeA-PAG pathway is GABAergic, and the PAG-NRA pathway is glutamatergic.

CONCLUSION

The present study demonstrated that there exists a disynaptic pathway from the CeA to the NRA via the PAG and that PAG-projecting CeA neurons express GAD67 mRNA and NRA-projecting PAG neurons express VGLUT2 mRNA, suggesting that the glutamatergic PAG-NRA pathway is under the inhibitory influence of the GABAergic CeA neurons in the control of vocalization and reproductive behavior.