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ORIGINAL

Dopamine Receptor Presence in the Rat Area Postrema Identified by RT-PCR, Immunohistochemistry, and *In Situ* Hybridization

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Abstract：Dopamine (DA) is a major central nervous system (CNS) neurotransmitter with many important physiological activities. Investigations into the neuroanatomy and neurologic functions of the dopaminergic neural systems have generated much debate. Regarding neuroanatomy, physiological and pharmacological criteria have divided DA receptors into D1 and D2 subtypes. The genes encoding these subtypes have been cloned and classified into a D1 subfamily encompassing D1 and D5 receptors and a D2 subfamily with D2, D3, and D4. Based on the sequences of the cloned receptors, we prepared antibodies and riboprobes to elucidate the expression of the corresponding proteins and mRNAs in the rat area postrema (AP) by immunohistochemistry and *in situ* hybridization (ISH). The AP was obtained from adult male Sprague-Dawley rats undergoing brain surgery, and tissue samples were used for RT-PCR, immunohistochemistry, and ISH. The results showed that D2 and D5 receptors and their mRNAs exist in the rat AP. On the other hand, D1, D3, and D4 receptors and their mRNAs were not detected.

Introduction

Dopamine (DA) exerts many important physiological activities, such as cognitive, emotive, motor, and endocrine functions. Animal studies have clarified that DA receptors are widely expressed[1,2)] and the dopaminergic system plays an important role in autonomic modulation[3,4)]. The area postrema (AP) is one of the circumventricular organs located at the caudal end of the fourth ventricle in the dorsal medulla, and functions as a regulatory site for food intake[5,6)], body fluid homeostasis[7)], cardiovascular regulation[4,8,9)], and

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nausea and vomiting[10—14] and as an interface with the immune system[15,16]. Disturbances in dopaminergic neurotransmission are thought to disrupt AP functions, and drugs that interact with DA receptors in the AP are used to restore these functions. Little has been elucidated, however, about the existence of DA receptor subtypes associated with the autonomic functions[17] within the AP[18]. Therefore, we investigated the existence of DA receptors and their mRNAs associated with autonomic functions within the rat AP using RT-PCR, immunohistochemistry, and *in situ* hybridization (ISH).

Materials and Methods

1. RT-PCR

Male Sprague-Dawley albino rats weighing 200—250 g (Charles River, Japan) were anesthetized with halothane and then decapitated. Each brain was rapidly removed from the skull and kept for 1 min in ice-cold, oxygenated, and pre-sterilized PBS solution. The cerebellum was removed and the AP was surgically resected with sterilized incision tools under a microscope. Immediately after resection, it was put into a sterilized tube containing ISOGEN (Nippon Gene, Japan) to extract total RNA. For reverse transcription, RNA was processed and PCR was performed using a TaKaRa RNA PCR Kit (TaKaRa, Japan) according to the manufacturer's recommendations. The reaction was amplified in a PROGRAM TEMP CONTROL SYSTEM PC-707 (ASTEC, Japan) at optimum conditions. The annealing temperature was 62℃ and the number of cycles was 35 for D1. For D2 and D5, the amplification conditions were 58℃ and 35 cycles, for D3 they were 65℃ and 35 cycles, and for D4 they were 67℃ and 35 cycles.

The oligonucleotide primers used were as follows：

D1：sense primer, 5'-GACAACTGTGACACAAGGTTGAGC-3'.

D1：antisense primer, 5'-ATTACAGTCCTTGGAGATGGAGCC-3'.

D2：sense primer, 5'-GCAGTCGAGCTTTCAGAGCC-3'.

D2：antisense primer, 5'-TCTGCGGCTCATCGTCTTAAG-3'.

D3：sense primer, 5'-AGAACAGCCAGTGCATCAGTATCAGACC-3'.

D3：antisense primer, 5'-TGCGGTCTCTTCTCCTCCTTCAGC-3'.

D4：sense primer, 5'-GTGCCACTGCGCTACAACCAG-3'.

D4：antisense primer, 5'-AAGCAGTAGCATGAGCGGACAG-3'.

D5：sense primer, 5'-AGTCGTGGAGCCTATGAACCTGAC-3'.

D5：antisense primer, 5'-GCGTCGTTGGAGAGATTTGAGACA-3'.

The expected sizes of the amplicons were 609 base pairs (bp) for D1, 317 and 404 bp for D2, 680 bp for D3, 212 bp for D4, and 517 bp for D5. PCR amplicons of the samples were visualized on ethidium bromide-containing 2% TAE agarose gels using a trans-illuminator.

2. Immunohistochemistry of the DA receptor

1) Tissue preparation

For immunohistochemical experiments, adult male Sprague-Dawley albino rats were anesthetized with halothane and then decapitated. Each brain was then rapidly removed from the skull and kept for 1 min in ice-cold, oxygenated, and pre-sterilized in PBS solution. The brain was cut coronally at the brainstem including the area postrema into 5 mm-thick samples using a razor blade. The slice block sample was embedded in Tissue-Tek® O. C. T. compound (Miles, USA), then quickly frozen with Spot Freeze S (FC-302, Finechemical Japan, Japan), and stored in a freezer at −20℃ until required. For sectioning, the sample was cut at a 12-μm thickness with a cryostat microtome and thaw-mounted on silane-coated glass slides.

2) Immunohistochemistry

Sections were probed with rabbit polyclonal antibody against rat DA receptor subtypes D1, 2, 3, 4, and 5. The probed sections were subsequently detected using the avidin-biotin complex (ABC；Vector Laboratories, USA) and diaminobenzidine as a substrate. They were then air-dried, dehydrated in a graded series of ethanol solutions, cleared in 3

changes of xylene, and coverslipped with Entellan New (Merck, Germany). Counterstaining was performed with eosin and hematoxylin solution, and the sections were examined by light microscopy under brightfield illumination. The specificity of the immunostaining was controlled by omitting primary antisera or secondary antibody. No immunoreactivity was noted in either case.

3. *In situ* hybridization (ISH)

1) Probe preparation

Probes for ISH were synthesized as follows: Complementary DNA (cDNA) from the rat AP was used to prepare riboprobes for D2 and D5 receptors. DNA fragments were amplified from total RNA from the rat AP by RT-PCR, and the templates were then ligated to the pCR® -II TOPO vector (Invitrogen, California, USA) and cloned in TOP 10 competent cells using a TOPO TA Cloning® Kit (Invitrogen). Single-stranded digoxigenin-labeled antisense and sense RNA probes for D2 and D5 receptors were generated by *in vitro* transcription with SP6 or T7 RNA polymerase from template plasmid DNA constructs linearized with *Bam*HI or *Eco*RV using a TOPO TA Cloning® Kit (Invitrogen) and DIG RNA Labelling Kit (Roche Diagnostics Gmbh, Germany). The riboprobes were aliquoted and stored at −20℃ until use.

2) ISH

For ISH, as with immunohistochemistry, the brainstem was quickly dissected from the brain and sectioned in the frontal plane at 12 μm on a cryostat. The sections were then thaw-mounted on silane-coated slides and stored at −20℃. Prior to hybridization, sections were fixed in 4% PFA/PBS for 5 min at room temperature (RT), followed by 3 μg/mL proteinase K treatment in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA for 5 min at 37℃, and fixed with 4% PFA in 0.1 M PB (pH 7.4) for 10 min at RT. After this, the sections were washed with 0.1 M PB, acidified with 0.2 M HCl for 10 min, and washed again with 0.1 M PB at RT, followed by acetylation with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min to reduce non-specific binding of the probes to D2 and D5 DA receptor mRNAs. Sections were hybridized in buffer containing 50% deionized formamide, 300 mM NaCl, 1×Denhardt's solution, 20 mM Tris-HCl (pH 7.6), 2 mM EDTA, 10% dextran sulphate, 0.25 mg/mL yeast tRNA, 0.25 mg/mL denatured herring sperm DNA, and 200 ng/mL of antisense or sense D2 and D5 riboprobes. Hybridization was performed overnight at 55℃ in a chamber humidified with 50% formamide/2×SSC. After RNase A treatment (100 μg/mL) at 37℃, non-specifically hybridized residual probe was washed away through several post-hybridization steps in a shaking water bath, starting with 2×SSC and ending with a high-stringency washing in 0.1×SSC at 65℃. A final wash in 0.1×SSC was performed at RT. The DiG-labeled hybrids were immunologically detected using a DIG Nucleic Acid Detection Kit (Boehringer Mannheim, Germany), as recommended by the manufacturer. Briefly, the slides were incubated with anti-digoxigenin-AP, Fab fragment (1:500), and then stained with a freshly prepared solution of 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP). Subsequently, developed sections were rinsed with distilled water, counterstained with 2% methyl green, and coverslipped with Pristine Mount (Falma, Japan). Slides were examined on an Olympus BX40 microscope (OLYMPUS, Japan), and images were stored on an Apple Macintosh G4 computer. The experimental protocol was approved by the Ethical Committee for Animal Experimentation of Okayama University.

Results

1. Detection of DA receptor mRNA in the rat AP by RT-PCR

Using the oligonucleotide primers described above, products with expected sizes of 317 and 404 bp for D2, and 517 bp for D5 were observed when total RNA from the rat AP was used as a template. The photograph of electrophoresis on the 2% ethidium bromide-stained agarose gel shows the DA receptor subtype mRNAs in the rat AP by RT-PCR. Lane 3 shows the PCR amplicon from DA D2 receptor mRNA and two alternative splicing isoforms. Lane 6 shows the PCR amplicon from DA D5 receptor mRNA, and lanes 2, 4, and 5 show no PCR amplicon

signals. These results indicate that D2 and D5 mRNAs exist in the rat AP. Lane 1 is the 100-bp DNA marker. Lanes 2, 3, 4, 5, and 6（D1, 2, 3, 4, and 5）are for each expected DA receptor subtype 1, 2, 3, 4, and 5 amplicon（Fig. 1）.

2．Detection of the DA receptor by immunohistochemistry

To investigate the existence of the DA receptor in the rat, we performed immunohistochemistry. Immunoreactivity against the D2 receptor in the rat AP was significant, as indicated by the arrowheads（Fig. 2a）, and immunoreactivity against the D5 receptor in the rat AP was also significant, also highlighted by arrowheads（Fig. 2b）. Furthermore, D2 immunoreactivity was similarly found diffusely in the peripheral tissue on the same section. On the other hand, D5 immunoreactivity was specifically strong in the AP compared to the peripheral tissue. D2 immunoreactivity was similarly expressed in other regions of the medulla oblongata including the AP, but D5 immunoreactivity was expressed particularly strongly in the AP.

3．Probe preparation（RNA probe synthesis using *in vitro* transcription）

Figure 3 shows the results of the electrophoretogram to verify the synthesis of single-stranded digoxigenin-labeled RNA probes transcribed from the linearized template using the restriction enzyme *Bam*HI or *Eco*RV. The far left lane is the DNA marker, the second lane is a positive control, and the third lane is a negative control in which no RNA polymerase was used. The fourth lane verified that synthesized RNA probe was transcribed from the linearized plasmid DNA template with restriction enzyme *Bam*HI and T7 RNA polymerase. The plasmid DNA was purified from the No. 214 colony and was used as a D2 antisense probe. In contrast, the fifth lane was used to verify the synthesis of the RNA probe transcribed from the linearized plasmid DNA template with *Eco*RV and SP6 RNA polymerase. This plamid DNA was purified from the No. 214 colony and was used as a D2 sense probe. The sixth and seventh lanes were used to verify transcription of the synthetic probe from the No. 524 colony. 524 *Bam*HI

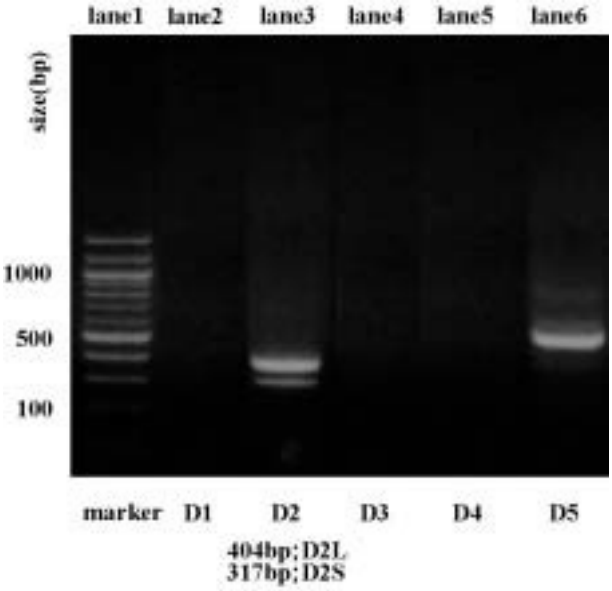
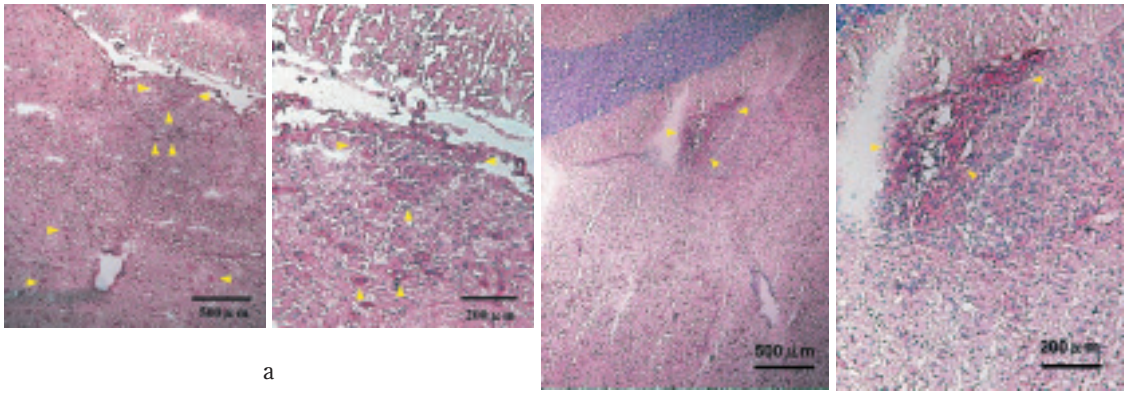


Fig. 1 Identification of DA receptor subtype mRNA in the rat AP by RT-PCR
This is the photograph of an 2% ethidium bromide-stained agarose gel in which PCR amplicons of the DA receptor subtype mRNA from the rat AP have been separated by electrophoresis. It demonstrates subtype D2 and D5 receptor mRNA expression in the rat AP and that subtype D2 receptor mRNA has two alternative splicing isoforms. Lane 1：100-bp DNA marker. Lanes 2, 3, 4, 5, and 6（D1, 2, 3, 4, and 5）：DA receptor subtypes 1, 2, 3, 4, and 5, respectively.

（T7）was used as a D5 sense probe and 524 *Eco*RV（SP6）was used as a D5 antisense probe（Fig. 3）.

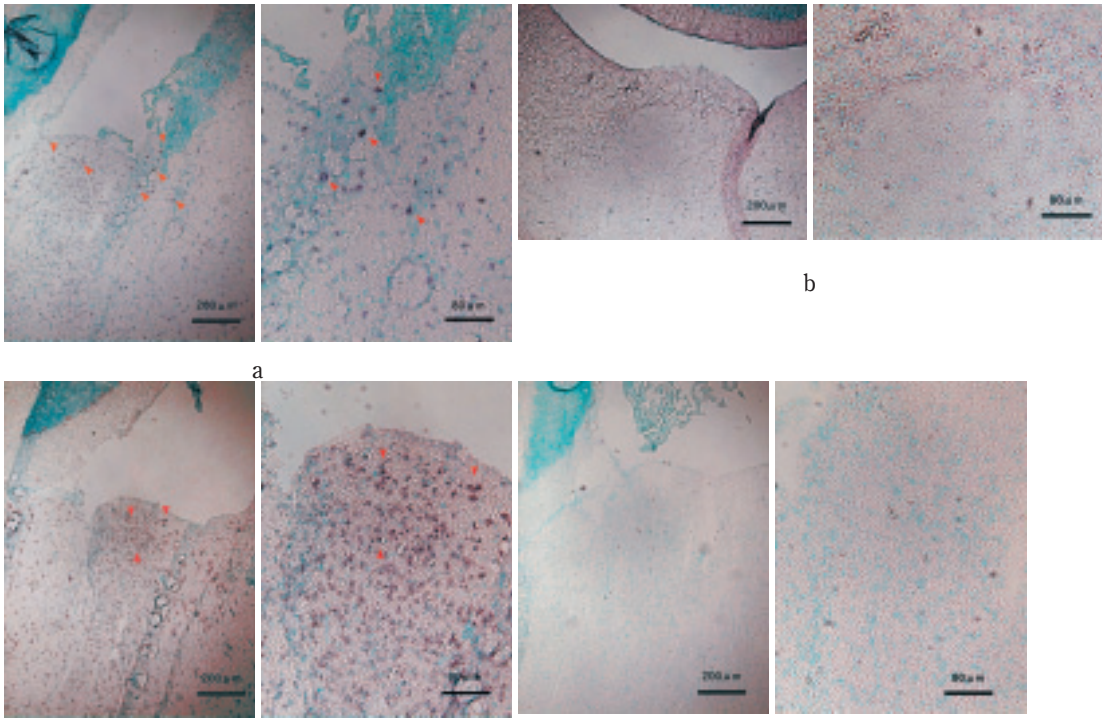
4．ISH

To investigate the existence of DA receptor mRNA in the rat AP, we performed ISH. The expressions of D2 and D5 receptor mRNAs in the rat AP were significant, as indicated by arrowheads in Fig. 4a, c. The distribution of D2 mRNA was similar to that of D2 receptor immunoreactivity. D2 mRNA signals were similarly found in the peripheral tissue. Therefore, D2 mRNA was diffusely present in the medulla oblongata including the AP on the same section. On the other hand, D5 mRNA signals were specifically strong in the AP compared to the peripheral tissue on



a b

Fig. 2 a：Distribution of D2 receptor protein immunoreactivity in the rat AP. Note immunoreactivity for D2 in the AP（arrowheads）. Scale bars：200 and 500 μm. b：Distribution of D5 receptor protein immunoreactivity in the rat AP. Note the strong immunoreactivity for D5（arrowheads）. Scale bars：200 and 500 μm.



a b c d

Fig. 4 a：ISH of the rat AP with the digoxigenin-labeled rat antisense D2 receptor mRNA riboprobe. Expression of D2 dopamine receptor mRNAs in the rat AP（arrowheads）. Signals are present in the AP（arrowheads）. Scale bars：80 and 200 μm. b：ISH of the rat AP with the digoxigenin-labeled rat D2 dopamine receptor sense riboprobe. No signals were seen in the AP. Scale bars：80 and 200 μm. c：ISH of the rat AP with the digoxigenin-labeled rat D5 receptor antisense riboprobe. Expression of D5 receptor mRNAs in the AP（arrowheads）. Intense signals were seen in the AP（arrowheads）. Scale bars：80 and 200 μm. d：ISH of the AP with the digoxigenin-labeled rat D5 receptor sense riboprobe. No signals were seen in the AP. Scale bars：80 and 200 μm.

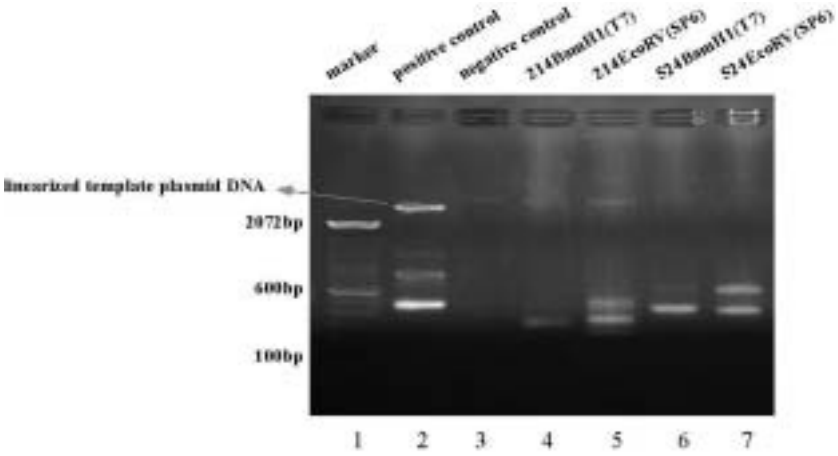


Fig. 3 RNA probe synthesis using *in vitro* transcription
The far left lane is the DNA marker, the second lane is a positive control, and the third lane is a negative control in which no RNA polymerase was used. The fourth lane verified that the synthesized RNA probe was transcribed from the linearized plasmid DNA template with *Bam*HI and T7 RNA polymerase. The plasmid DNA was purified from the No. 214 colony and was used as a D2 antisense probe. In contrast, the fifth lane was used to verify synthesis of the RNA probe transcribed from the linearized plasmid DNA template with *Eco*RV and SP6 RNA polymerase. This plamid DNA was purified from the No. 524 colony and was used as a D2 sense probe. The sixth and seventh lanes were used to verify the transcription of synthetic probe from the No. 524 colony. 524 *Bam*HI(T7) was used as a sense probe and 524 *Eco*RV(SP6) was used as an antisense probe.

the same section. For the control, no signals were seen in the AP using digoxigenin-labeled rat D2 or D5 DA receptor sense riboprobe (Fig. 4b, d).

Discussion

In the present study, we examined all DA receptor subtypes and identified types D2 and D5 within the rat AP by immunohistochemical and molecular biological means.

Studies have indicated that the AP is involved in emesis[19—22] and clarified the existence of the D2 receptor within the canine AP pharmacologically using *in vitro* autoradiography[23]. Data on the DA receptor subtype obtained using a pharmacological approach are consistent with our results. However, Stafanini and Clement-Cormier did not refer to the existence of other DA receptor subtypes. According to Schalling *et al*., who recorded autoradiographs of the rat medulla oblongata and cerebellum after ISH with oligonucleotide probes complementary to DARPP-32(a)(a marker for D1-receptive neurons) and the D2 receptor, there are markedly different hybridization patterns with DARPP-32 mRNA in Purkinje cells and the choroid plexus and D2 receptor mRNA is spread diffusely in the medulla oblongata[24]. We scrutinized the paper and concluded that the region markedly expressing DARPP-32 mRNA includes not only the choroid plexus in the fourth ventricle but also the AP, and D2 receptor mRNA is widespread in the medulla oblongata including the AP. These findings are in good agreement with ours. Concerning methods of detecting the D3 receptor, Yoshikawa *et al*. and Hyde *et al*. used R(+)-7-OH-DPAT as a specific antagonist of the D3 receptor, and Yoshikawa *et al*. elucidated that the D3 receptors in the ferret AP play an important role in R(+)-7-OH-DPAT-induced emesis[25]. In contrast, Osinski *et al*. used R(+)-7-OH-DPAT as a D2/D3 agonist and demonstrated that, in ferrets, D2 and/or D3 receptors are involved in emesis and D1, D4, and D5 receptors are not[26]. Osinski *et al*. contradict Yoshikawa *et al*. in

that their results strongly suggest that stimulation of the D2 receptor likely contributes to the emetic activity of agents that have been incorrectly classified as dopamine "D3-selective" agonists, and so a definitive conclusion on the emetogenicity of dopamine D3 receptor stimulation awaits the development of truly selective dopamine D3 receptor agonists. In general, the usage of R(+)-7-OH-DPAT as a D2/D3 agonist is well accepted. Judging from these facts, the existence of D2 receptors in the rat AP in the present study is reasonable. Furthermore, the histochemical response of the D2 receptor in peripheral tissue in the present study was consistent with that reported by Hyde *et al*., but the histochemical responses of the D3 and D4 receptors were not[27]. Concerning the D3 receptor's response, Hyde *et al*. used R(+)-7-OH-DPAT as a selective D3 receptor agonist in the same way as Yoshikawa *et al*. Concerning the D4 receptor's response, differences may depend on the detection methods and/or species used. That is, Hyde *et al*. detected the distribution of DA receptor subtypes in the human dorsal vagal complex based on pharmacological subtype-specific affinity, whereas we detected subtypes in the rat using molecular-biological methods. Bouthenet *et al*. demonstrated the expression of D2 and D3 mRNA within the rat nucleus of the solitary tract (NTS) and the expression of D2 but not D3 mRNA within the dorsal motor nucleus of the vagus[28]. However, they did not examine the AP. Concerning the D5 receptor or D1-like receptors, D1 and D5 receptors are located in the hypothalamus of primates and their activation acutely influences SCN, SON, and PVN activity[29]. According to Ciliax *et al*., the distribution of D5 in the brain is clearly different from that of other subtypes, including D1, the other member of the D1-like receptor subfamily. In this study, immunoreactivity for the D5 receptor was expressed in the rat and monkey cerebellar cortex. The expression of D5 receptors within the cerebellum is consistent with the report of Schalling *et al*. Khan *et al*. showed that D5 receptors are located in the substantia nigra pars compacta, hypothalamus, striatum, cerebral cortex, nucleus accumbens, and olfactory tubercle[30]. Judging from these reports, there has been no distinct finding of D5 receptors in the AP. Therefore, identification of the D5 receptor within the rat AP in the present study is novel.

The physiological effects of DA are mediated by at least five G protein-coupled receptor subtypes and based on their affinity for classical D1 and D2 ligands. D1-like receptors (D1 subfamily) stimulate adenylate cyclase, while D2-like receptors (D2 subfamily) inhibit adenylate cyclase when expressed in appropriate cell lines. Given the differences in signal transduction and distribution, individual molecular subtypes probably have specific roles in dopaminergic neurotransmission[3,31—36]. According to one popular theory, DA is a major neurotransmitter that modulates neuroendocrinal, locomotory, emotional, and nociceptive functions. Numerous DA receptors exist in the hypothalamus, which regulates homeostasis and has regulatory areas for thirst, hunger, body temperature, water balance, and blood pressure and links the nervous to the endocrine system. It is also the main center of the autonomic nervous system[37]. The medulla oblongata and hypothalamus contain the circuits for the specific homeostatic regulation in which the sympathetic nervous system is involved. On the other hand, the mesencephalon and hypothalamus contain the neuronal programmes for the defensive mechanisms which are switched on when an animal is in pain and under stress[38]. Research over the past 20 years has shown that the immune/nervous/endocrine systems are closely linked and communicate with each other. The central nervous system (CNS) signals the immune system *via* hormonal and neuronal pathways, and the immune system signals the CNS through similar routes *via* immune mediators and cytokines. The immune and nervous systems maintain extensive communication with each other *via* the 'hardwiring' of sympathetic and parasympathetic nerves to lymphoid organs[39,40], including the AP[41,42].

In the present study, D2S and D2L receptor isoforms were identified in the rat AP. The D2 receptor has six introns, and two isoforms are produced by alternative splicing. D2S receptors are pre-synaptic, having modulatory functions (called autoreceptors, they regulate neurotransmission *via* feed-back mechanisms, *i.e*., the synthesis, storage, and release into the synaptic cleft of DA) and regulate neurotran-

smission. Autoreceptors have autonomic functions and can respond immediately to marked environmental changes. In contrast, D2L receptors may have the classic function of a post-synaptic receptor, *i.e.*, maintaining the neurotransmission (excitatory or inhibitory) blocked by a receptor antagonist or stimulated by the endogenous neurotransmitter itself or a synthetic full or partial agonist[43,44]. In most tissues, more D2L receptors are expressed than D2S receptors and the expression level depends on the tissue site. There is no pharmacological difference between the D2S and D2L receptors, but the D2S receptors are expressed more in the brainstem. From these findings, it is suggested that D2S receptors principally function as an autoreceptor and play a critical self-regulatory or self-inhibitory role. Considering that autoreceptors respond to marked changes in the environment, the AP requires autoreceptors because it regulates cardiovascular control, body fluid balance, and feeding in autonomic control mechanisms. These discussions provide insight into the neuro-anatomical basis of the differential effects of therapeutic agents that act on D2 (D2S and/or D2L) or D5 receptors within the AP.

Conclusion

The present study showed that the D2 and D5 receptors and their mRNAs are expressed in the rat AP. This is the first immunohistochemical and molecular-biological study of DA receptors in the rat AP.

Abbreviations

DA : dopamine, CNS : central nervous system, ISH : *in situ* hybridization, AP : area postrema, NTS : nucleus of the solitary tract.

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