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Area postrema, a brain circumventricular organ, is the site of antidipsogenic action of circulating atrial natriuretic peptide in eels

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Summary

Accumulating evidence indicates that circulating atrial natriuretic peptide (ANP) potently reduces excess drinking to ameliorate hypernatremia in seawater (SW) eels. However, the cerebral mechanism underlying the antidipsogenic effect is largely unknown. To localize the ANP target site in the brain, we examined the distribution of ANP receptors (NPR-A) in eel brain immunohistochemically using an antiserum specific for eel NPR-A. The immunoreactive NPR-A was localized in the capillaries of various brain regions. In addition, immunoreactive neurons were observed mostly in the medulla oblongata, including the reticular formation, glossopharyngeal-vagal motor complex, commissural nucleus of Cajal, and area postrema (AP). Trypan Blue, which binds serum albumin and does not cross the blood–brain barrier, was injected peripherally and stained the neurons in the AP but not other NPR-A immunopositive neurons. These histological data indicate that circulating ANP acts on the AP, which was further confirmed by physiological experiments. To this end, the AP in SW eels was topically destroyed by electric cauterization or were by chemical lesion of its neurons by kainic acid, and ANP (100 pmol kg^{-1}) was then injected into the circulation. Both heat-coagulative and chemical lesions to the AP greatly reduced an antidipsogenic effect of ANP, but the ANP effect was retained in sham-operated eels and in those with lesions outside the AP. These results strongly suggest that the AP, a circumventricular organ without a blood–brain barrier, serves as a functional window of access for the circulating ANP to inhibit drinking in eels.

Key words: blood–brain barrier, ANP receptor (NPR-A), fish osmoregulation, drinking behaviour, circumvetricular organ.

Introduction

Atrial natriuretic peptide (ANP) acts on various tissues to regulate body fluid balance and cardiovascular function in fish (Farrell and Olson, 2000; Loretz and Pollina, 2000; Takei and Hirose, 2002; Toop and Donald, 2004). In eels, ANP promotes adaptation to seawater (SW) by reducing excess Na^+ uptake from the environment (Tsukada et al., 2005). The reduced Na^+ uptake is accomplished by decreases in both drinking of environmental SW and subsequent absorption of Na^+ ions by the intestine, while renal and branchial excretion of Na^+ are unchanged by ANP infusion. In fact, removal of endogenous ANP and ventricular natriuretic peptide, which also binds to the type A ANP receptor (NPR-A) with similar affinity, from plasma by immunoneutralization increased plasma Na^+ concentration with a concomitant increase in drinking rate in SW eels (Tsukada and Takei, 2006). These results strongly support the hypo-osmoregulatory roles of ANP in SW. Despite such an important effect of ANP on drinking, the proximate mechanisms and the site of action of ANP in the brain have not been examined in detail.

The mechanisms regulating drinking have been intensively and extensively investigated in mammals, reflecting its important role in body fluid regulation (Fitzsimons, 1998; Balment, 2002). Accumulating evidence has shown that the central nervous system (CNS) controls drinking by integrating neuronal and chemical signals originating in the brain and from the periphery. Circulating hormones are major controllers of drinking through their actions on the brain; these factors include angiotensin II (ANG II), relaxin, bradykinin and ANP (Fitzsimons and Simons, 1968; Katsuura et al., 1986; Fregly and Rowland, 1991; Sinnayah et al., 1999). However, since circulating hormones do not cross the blood–brain barrier (BBB), they must act on the target sites outside the BBB such as circumventricular organs (CVOs) (Ferguson and Bains, 1996; McKinley et al., 1999). In mammals, the CVOs such as the subfornical organ (SFO), area postrema (AP) and organum vasculosum of the lamina terminalis (OVLT) serve as a blood–brain interface for the action of circulating hormones (Ferguson and Bains, 1996). In particular, forebrain CVOs, including the SFO and OVLT, are known to be primary sites for the central regulation of body fluid balance in mammals (Johnson and Gross, 1993; McKinley et al., 1999) and in birds (Simon et al., 1992). For instance, administration of ANP and ANG II into the SFO influences the neuronal activities within the structure (Gutman et al., 1988) and modifies drinking behaviour in mammals and birds (Simpson and Routenberg,

1973; Lin and Hubbard, 1992; Takei, 1977). Further, receptors for ANP (NPR-A type) and ANG II (AT_1 subtype) are densely localised in the SFO of mammals and birds (Saavedra et al., 1986; Quirion, 1989; Gehlert et al., 1990; Simon et al., 1992). Thus, CVOs are the most probable sites of action of circulating hormones in the brain.

In contrast to mammals and birds, fishes are unique in that the hindbrain rather than the forebrain may be involved in the elicitation of drinking, since eels without forebrain and midbrain ('decerebrated' eels) respond to SW transfer (Hirano et al., 1972) and to peripheral injection of ANG II (Takei et al., 1979) by copious drinking. Furthermore, direct injection of ANG II into the third ventricle (Takei, 1992) or fourth ventricle (Kozaka et al., 2003) of eels induced drinking at much lower doses than peripheral injections, suggesting that the ANG II acts on the brain to induce drinking in fishes. Kozaka et al. further showed that ANP injection into the ventricle at the level of medulla oblongata (MO) inhibits drinking in SW eels (Kozaka et al., 2003), but the effect was rather weak compared with the potent inhibition after peripheral injection (Tsuchida and Takei, 1998). Reflecting on all the results obtained thus far, it is likely that the major route of ANP action is from the periphery, and that circulating ANP acts on the brain, probably at the level of MO, in eels. The regulatory motor signals generated from the hindbrain may be transmitted *via* the vagus nerves, since in the eel bilateral vagotomy abolished drinking induced by SW transfer (Hirano et al., 1972) and by peripheral ANG II injection (Takei et al., 1979).

The present study was undertaken to determine the central receptor sites for the antidipsogenic effect of ANP in the eel. Initially, the cellular localization of NPR-A, a specific receptor for biological actions of ANP, was determined in the brain nuclei by immunohistochemistry using an antiserum specific for eel NPR-A. The BBB-deficient structures were identified in the eel brain by peripheral injection of Trypan Blue, which is impermeable to the BBB after binding to albumin (Clasen et al., 1970). Since the AP was the only site possessing ANP receptors and lacks a functional BBB, heat-coagulative lesions or chemical lesions with kainic acid were made topically to the AP, and effects of the lesioning on the antidipsogenic effect of peripherally injected ANP were examined.

Materials and methods

Animals

Cultured, immature eels *Anguilla japonica* Temminck and Schlegel 1847 of either sex were purchased from a local dealer. They were maintained in SW without feeding for at least 2 weeks before use. Water in the tank was continuously circulated, aerated, and regulated at 18°C. All conditions for fish maintenance conformed to the Guideline for Maintenance and Care of Experimental Animals, and the experiments were approved by the Committee for Animal Experiments in the University of Tokyo. Eels weighed 182±2 g (*N*=68) at the time of surgery.

Immunohistochemistry

The eel brain sections were stained to determine the cells possessing NPR-A using the Elite avidin-biotin-peroxidase complex kit (Vector Laboratories, Burlingame, CA, USA). After anesthetizing eels (*N*=7) in 0.1% (w/v) tricaine methanesulfonate (Sigma-Aldrich, St Louis, MO, USA) for 15 min, the skull was opened and the exposed brain was fixed by 4% paraformaldehyde in 0.01 mol l^{-1} phosphate-buffered saline (PBS, pH 7.3) for 1 h *in situ*. The brain was then dissected out of the skull and immersed in the same fixative solution for 48 h at 4°C. The brain was immersed in 30% sucrose in PBS overnight at 4°C for cryoprotection and embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan) at –20°C. Serial cross sections (10 μm thick) were cut and mounted onto gelatin-coated slides and dried overnight at 4°C. For immunohistochemistry, the sections were incubated sequentially with (1) 0.6% H_2O_2 in methanol for 30 min at room temperature, (2) 2% normal goat serum in PBS (NGS-PBS) containing 0.01% sodium azide for 1 h at room temperature, (3) the affinity-purified antiserum for NPR-A diluted at 1:9000 with NGS-PBS for 48 h at 4°C, (4) biotinylated, goat anti-rabbit IgG for 30 min at room temperature, (5) avidin-biotin-peroxidase complex for 45 min at room temperature, and (6) 0.05% diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxidase in 50 mmol l^{-1} Tris buffer (pH 7.2) for 10 min at room temperature. Specificity of immunoreactive signals was confirmed by preabsorption of the antibody with antigen-conjugated CNBr-activated Sepharose 4B (GE Healthcare, Bucks, UK). The specificity of this antiserum in eel tissue was confirmed by western blotting of membrane fractions prepared from the gills as previously reported (Healy et al., 2005). Immunostained sections were counterstained with Haematoxylin and then dehydrated and cleared, and coverslips placed on top for observation with a light microscope (E800, Nikon, Tokyo, Japan). The brain loci were determined in the present study based on the brain atlas of Japanese eel (Mukuda and Ando, 2003a) (Fig. 1).

Trypan Blue vital staining

To identify BBB-deficient structures in the eel brain, we injected 0.5% Trypan Blue diluted with 0.9% NaCl (saline) intraperitoneally (1 ml/day for 4 days) into anesthetized eels (*N*=7). 24 h after the last injection, eels were anesthetized and perfused with 30 ml of saline containing heparin (200 units ml^{-1}) for 30 min to remove blood cells. The brains were fixed with Bouin's solution for 24 h after a brief fixation for 1 h *in situ*. The fixed brain was dehydrated through graded ethanol concentrations, cleared with benzene and then embedded in Paraplast™ (Oxford Labware, St Louis, MO, USA). Serial sections were cut at 10 μm, mounted onto gelatin-coated slides, and observed under a fluorescence microscope (BX 51, Olympus, Tokyo, Japan).

Effect of AP lesioning on ANP-induced antidipsogenesis

Since the AP was identified as a probable target site for circulating ANP (see Results), the involvement of the AP in ANP-induced antidipsogenesis was evaluated *in vivo*. For this purpose, eels (*N*=49) were anesthetized and cannulated with a polyethylene tube (o.d. 0.8 mm) in the ventral aorta for injection of ANP. Then, vinyl tubes (o.d. 1.5 mm) were inserted into the esophagus and stomach of eels for continuous measurement of drinking rate as described previously (Takei et al., 1998).

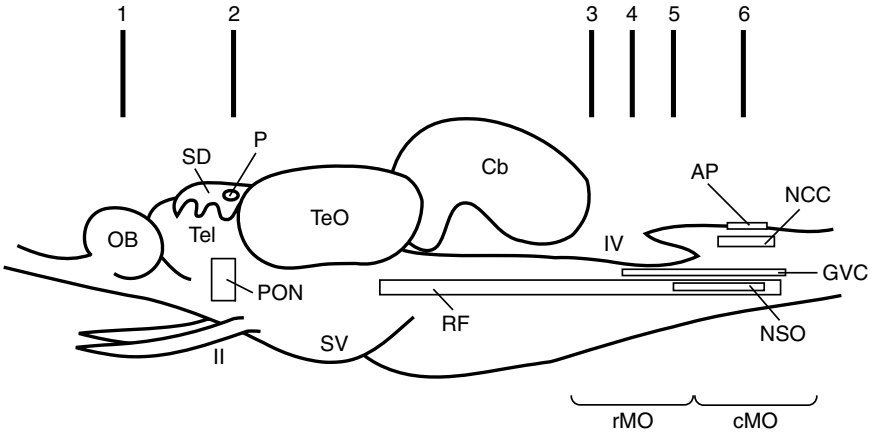

Fig. 1. Diagram showing mid-sagittal plane of eel brain. The numbers on top (1–6) correspond to cross-sectional planes in Figs 2, 4 and 5. OB, olfactory bulb; Tel, telencephalon; TeO, optic tectum; Cb, cerebellum; rMO, rostral part of medulla oblongata; cMO, caudal part of medulla oblongata; P, pineal gland; SD, saccus dorsalis; PON, preoptic nucleus; SV, saccus vasculosus; RF, reticular formation; GVC, glossopharyngeal-vagal motor complex; NSO, spino-occipital motor nucleus; NCC, commissural nucleus of Cajal; AP, area postrema; II, optic nerve; IV, fourth ventricle.

Heat-coagulative lesioning

The skin was incised along the occipital region and skull exposed by removing attached muscles. The skull around the supraoccipital bone was drilled to expose the AP, and the arachnoid membrane was removed carefully so as not to break the blood vessels running mid-sagittally above the AP. The exposed AP was then heat-coagulated by electric cautery (Surgitron EMC, Ellman, Oceanside, NY, USA). After lesioning, the drilled hole was filled with bone wax (Lukens, Albuqueque, NM, USA) and the skin incision was sutured. Ten eels were subjected to the same surgical procedures without heat coagulation and served as sham-operated controls. Eight additional eels were subjected to the lesioning in the caudal part of MO adjacent to the AP and served as lesion controls. All surgical procedures were completed within 40 min/eel. Two to three animals were operated upon in the same day irrespective of the operation (sham control, lesion control or AP-lesion).

After surgery, eels were immediately placed, according to their individual size, in a Plexiglas^{TM} trough through which aerated SW continuously circulated at 18°C to avoid deterioration of physiological conditions. The trough was covered with a black vinyl sheet to minimize visual stress to the animal during the experiments. Beginning at least 18 h after lesioning, each eel received an injection of saline as control *via*

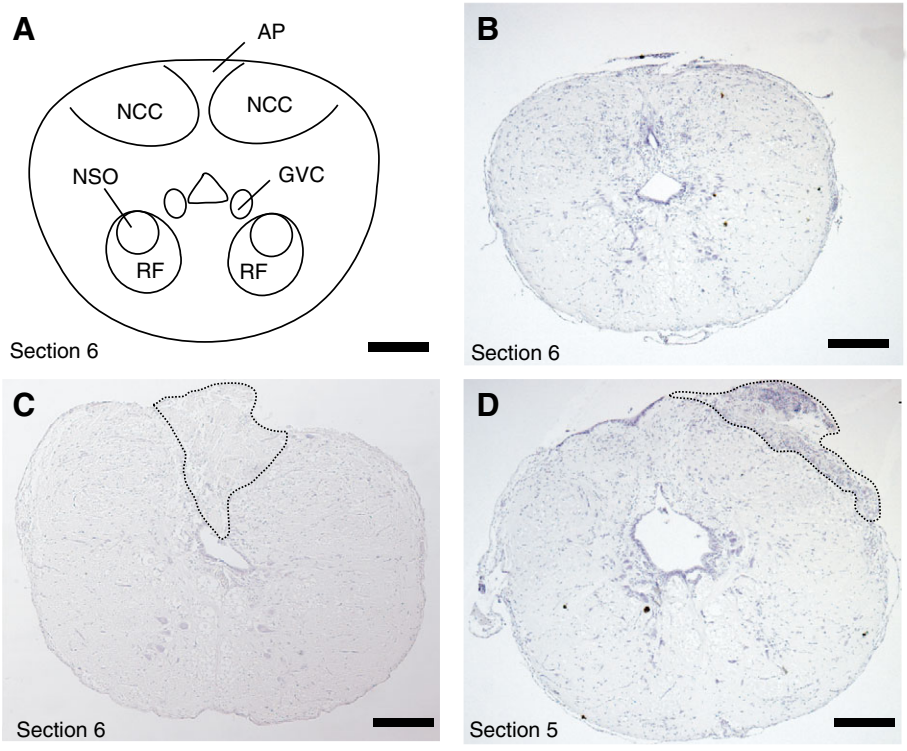

Fig. 2. (A) Localization of major brain nuclei in a cross section taken at the caudal part of medulla oblongata. (B) Sham-operated eel, (C) AP-lesioned eel and (D) eel with lesion outside AP. Dotted areas in C and D indicate areas heat-coagulated by electric cautery. Sections are stained with Haematoxylin. Locations of cross sectional planes are shown in Fig. 1. AP, area postrema; NCC, commissural nucleus of Cajal; GVC, glossopharyngeal-vagal motor complex; NSO, spino-occipital motor nucleus; RF, reticular formation. Scale bar, 200 μm.

aortic cannula in a volume of 50 μl, followed by a flush of cannula with 50 μl saline. Subsequently, 50 μl of ANP containing 100 pmol kg^{-1} eel mass was injected in the same fashion. This dose is sufficient for the antidipsogenic action of ANP (Takei and Tsukada, 2001). The saline and ANP injections were separated by an interval of at least 45 min. Drinking rate was measured throughout the experimental period. After the experiment, eels were anesthetized and decapitated for histological verification of the lesion sites (Fig. 2).

Chemical lesioning to neurons

In addition to heat-coagulative lesioning by electric cautery, chemical lesioning with kainic acid was undertaken to confirm that effect was due to the lesion of AP neurons (Fig. 3). Kainic acid is an excitotoxic glutamate agonist that specifically induces degeneration of neurons (Tomioka et al., 2002). To perform a topical microinjection of kainic acid to the AP, a glass capillary injector was prepared from the glass capillary stock (o.d. 0.9 mm) with a puller and a microgrinder (Narishige, Tokyo, Japan). The capillary injector was connected to a 0.5 μl Hamilton microsyringe *via* polyethylene tubing (o.d. 0.61 mm) (Fig. 3B). After exposure of the AP as described above, anesthetized eels were placed on a stereotaxic apparatus (Narishige), and then the glass capillary filled with kainic acid (4 mmol l^{-1}, Sigma-Aldrich) containing 1% Chicago Sky Blue 6B (Sigma-Aldrich) in PBS was inserted into the AP to a depth of 0.1 mm by the manipulator of the stereotaxic apparatus. Kainic acid solution (50 nl) was injected into the AP (N=7; AP-lesion eels) or into an adjacent area (N=7; lesion control). Control injection was also performed into the AP with 1% Chicago Sky Blue 6B in PBS (N=7; sham control). The injections were accomplished within 1 min, but the capillary remained in place for 3 min to insure complete injection of volume. Beginning at least 18 h post-operatively, the eels were anesthetized and cannulae were inserted into the dorsal aorta for injection of ANP and into the esophagus and stomach for continuous measurement of drinking rate as mentioned above. On the next day of cannulation, ANP was injected into the dorsal aorta and the effect of AP lesioning on the ANP-induced antidipsogenesis was examined. After the experiment, the distribution of kainic acid was confirmed by staining with Chicago Sky Blue 6B (Fig. 3C).

Statistical analyses

The time-dependent changes in drinking rate after ANP injection were expressed in terms of percentage of the value over a 30 min period before ANP injection. Comparisons of the effects of AP lesioning on ANP-inhibited drinking among different groups were made by ANOVA, followed by Steel–Dwass test at each time point. The changes in drinking rate were measured for 20 min before and after ANP injection and compared by paired-sample Student's t-test. Significance was determined at P<0.05. All results were expressed as means ± s.e.m.

Results

Localization of ANP receptors in eel brain

Immunoreactive NPR-A was detected in the capillary endothelial cells in the whole brain areas (Fig. 4A) including the

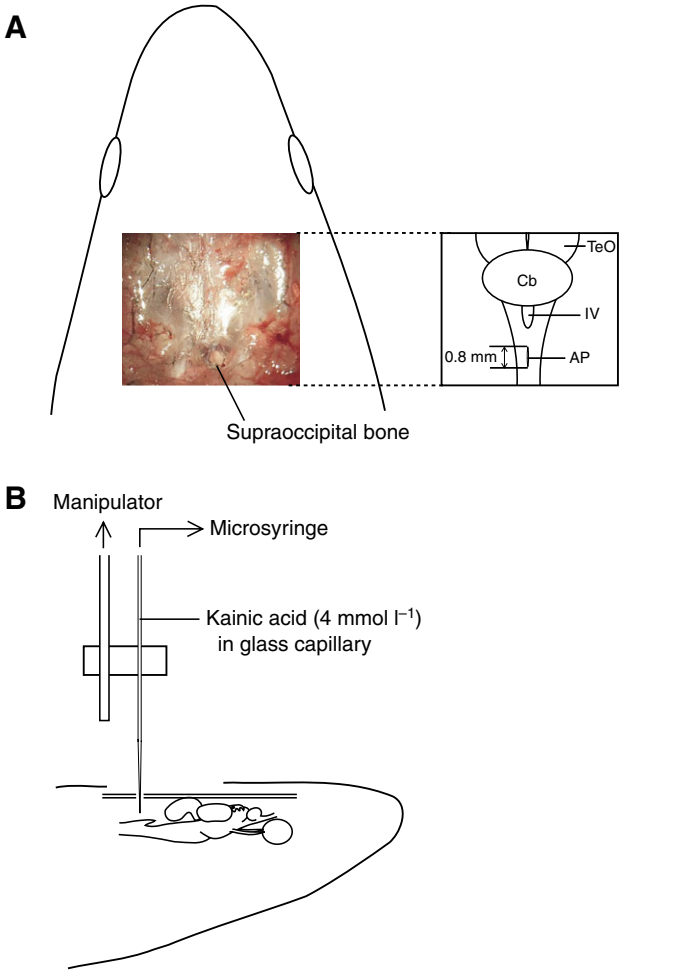

Fig. 3. Surgical procedure for topical microinjection of kainic acid in seawater (SW) eels. (A) Exposed skull of SW eel (left) and corresponding loci of eel brain (right). The drill hole was made around supraoccipital bone to expose the area postrema (AP). Average size of the AP is approximately 0.8 mm. (B) Experimental setup for injection of 50 nl of kainic acid into the AP of SW eels. A glass capillary was connected to the manipulator to facilitate accurate placement of the capillary at the AP. The capillary was also connected to the microsyringe, through which 50 nl of 4 mmol l^{-1} kainic acid containing 1% Sky Blue 6B in PBS is injected. (C) Distribution of Chicago Sky Blue 6B in eel brain (left, AP-lesioned eel; right, lesion control). Area of the AP is circled by broken line. TeO, optic tectum; Cb, cerebellum; IV, fourth ventricle.

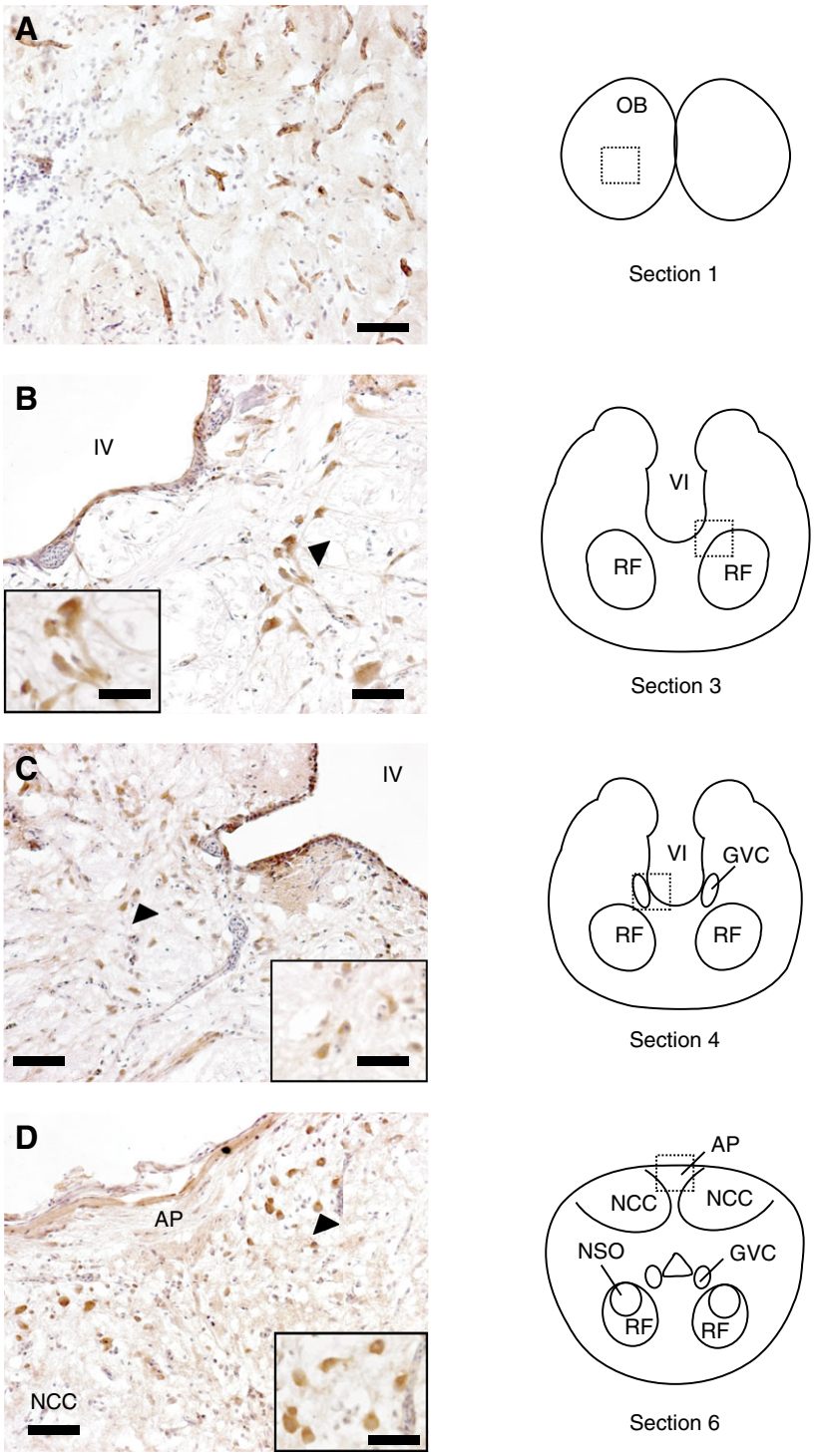

Fig. 4. Immunohistochemical staining of NPR-A in eel brain counterstained with Haematoxylin (left) and corresponding cross-sectional planes (right). (A) Immunoreactive signal in capillaries of whole brain area as represented by the olfactory bulb (OB). (B) Immunoreactive neurons in reticular formation (RF), (C) glossopharyngeal-vagal motor complex (GVC), and (D) area postrema (AP) and commissural nucleus of Cajal (NCC) in the medulla oblongata. Approximate areas photographed are indicated by broken squares drawn in cross sectional planes. Regions indicated by arrowheads are magnified and shown in the boxed areas at the bottom of each photograph. Scale bar, 50 μm (magnified view, 30 μm). Locations of cross sectional planes are shown in Fig. 1. NSO, spino-occipital motor nucleus; IV, fourth ventricle.

vascular-rich saccus vasculosus (SV). Consistent with the immunohistochemical results, preliminary RT-PCR examination showed that the NPR-A gene was expressed minutely and ubiquitously in all regions of the eel brain including olfactory bulb (OB), telencephalon (Tel), optic tectum (TeO), cerebellum (Cb) and MO (data not shown). In addition to the vasculature, NPR-A positive neurons were observed at discrete loci in the MO, such as the reticular formation (RF), glossopharyngeal-vagal motor complex (GVC), commissural nucleus of Cajal (NCC) and AP (Fig. 4B–D). The perikarya of RF neurons were relatively large (ca. 20–30 μm in diameter), while the GVC neurons were ca. 10 μm and ovoid/ellipsoid in shape. The NCC and AP are neighboring nuclei in the caudal MO; NPR-A-immunopositive neurons (ca. 5–10 μm) were located predominantly near the boundary of the two loci. Neuronal staining was not detectable in this study in the more rostral parts, such as the OB, Tel, TeO and Cb (Fig. 1).

Brain loci outside the blood–brain barrier

Intraperitoneally injected Trypan Blue was taken up into the blood stream and stained almost all peripheral tissues. In contrast, no staining was observed in the brain parenchyma except for the pineal gland and surrounding saccus dorsalis (SD), parvocellular and magnocellular part of preoptic nucleus, SV and AP (Fig. 5A–C). In addition to these loci, some RF and GVC neurons were stained, but the signals were much weaker than other Trypan Blue-positive loci (Table 1).

Effect of AP lesioning on ANP-induced antidipsogenesis

As the AP is the sole brain structure that is positive to both NPR-A immunohistochemical staining and vital staining with Trypan Blue (Table 1), it is the most probable target site of circulating ANP. Therefore, the effect of AP lesioning on antidipsogenesis caused by peripheral ANP injection was examined. Effects of the heat-coagulative and chemical lesions to the AP on drinking are summarized as changes in 20 min water intake before and after ANP injection (Table 2), since the effect of 100 pmol kg^{-1} of ANP was usually continued for 20 min. After heat-coagulative lesioning of the AP, ANP injection into the circulation no longer inhibited drinking, although the inhibition was apparent in sham controls and lesion controls. In addition, the AP lesioning also reduced basal drinking in eels (Table 2). Since heat coagulation not only destroys neurons in the AP but also neural circuitry that traverses the AP, chemical lesioning that damages only AP neurons was attempted with kainic acid. Average basal

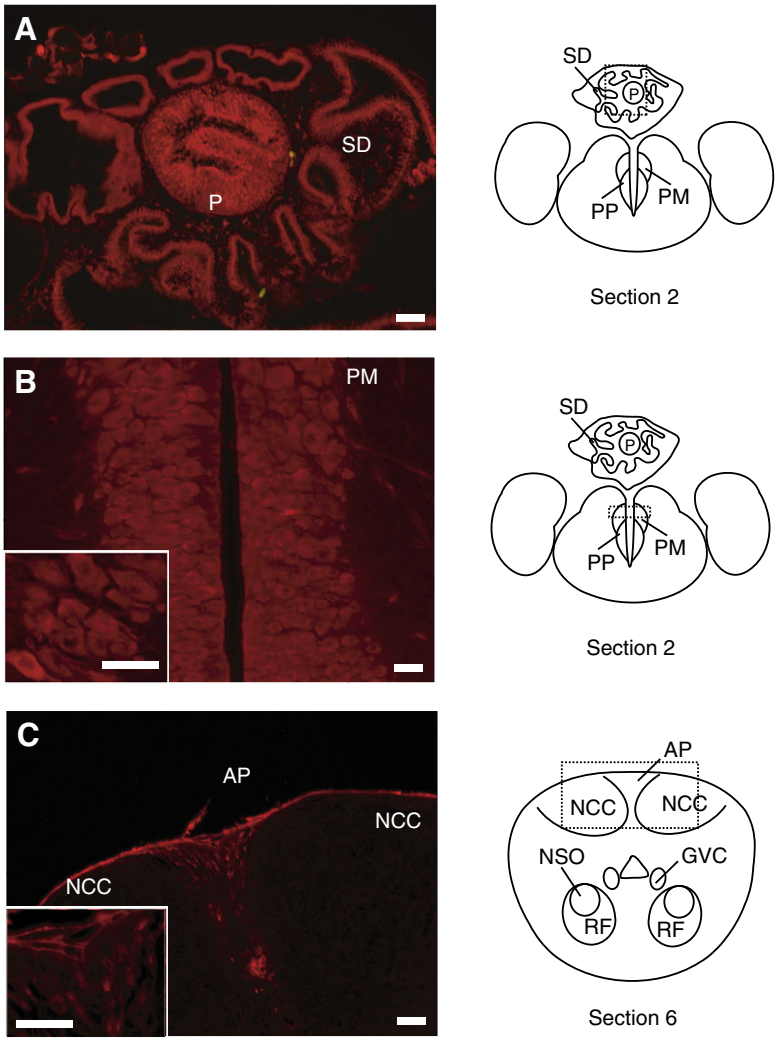

Fig. 5. Distribution of Trypan Blue in the brain of SW eel (left) and corresponding cross-sectional planes (right). Trypan Blue signal (stained strong red color) was detected in (A) the pineal gland (P) and saccus dorsalis (SD); (B) magnocellular part of preoptic nucleus (PM); (C) area postrema (AP) of medulla oblongata. Approximate areas photographed are indicated by broken squares drawn in cross-sectional planes. Magnified views are shown in the boxed areas at left-bottom of B and C. Locations of cross sectional planes are shown in Fig. 1. PP, parvocellular part of preoptic nucleus; NCC, commissural nucleus of Cajal; GVC, glossopharyngeal-vagal motor complex; NSO, spino-occipital motor nucleus; RF, reticular formation. Scale bars, 20 μm.

drinking rate of the AP-lesioned eels with kainic acid was not different from those of sham controls and lesion controls (Table 2). Furthermore, the antidipsogenic effect of ANP was significantly suppressed by kainic acid lesioning of the AP, although slight inhibition of drinking still remained after ANP injection (Table 2). In order to highlight the effect of AP lesions on the ANP effects, the time course of changes in drinking rate was normalized to percentage change in drinking rate after intravascular injection of ANP (Fig. 6). In sham-operated eels and lesion controls with intact AP, drinking was strongly inhibited by ANP within 5 min after injection. However, total destruction of the AP by electric cauterization abolished the antidipsogenic effect of ANP injected into the circulation in SW eels (Fig. 6A). After chemical lesioning with kainic acid, the normalized time-course changes clearly shows that AP-lesioned group significantly attenuated the antidipsogenic effect of ANP compared with sham controls and lesion controls (Fig. 6B).

Discussion

ANP is now established as an antidipsogenic hormone in mammals (Antunes-Rodriguez et al., 1986; Balment, 2002). Although several ANP-binding sites are identified in the mammalian brains (Saavedra et al., 1986; Quirion, 1989), little is known about the mechanisms of how circulating ANP acts on the brain to inhibit drinking compared with the many studies on the cerebral mechanisms for elicitation of drinking by ANG II (Fitzsimons, 1998). In contrast to the relatively weak antidipsogenic effect of ANP in mammals, ANP potently inhibits drinking in the eel, even more potently than the dipsogenic effect of ANG II (Tsuchida and Takei, 1998; Takei, 2002). Furthermore, ANP is highly potent for inhibition of drinking when injected systemically, which is in contrast to the much greater potency of ANG II after intracranial injection compared with peripheral injection. Therefore, the eel is an excellent model animal to investigate the cerebral mechanisms underlying the antidipsogenic effect of circulating ANP. In the present study, we pursued the site of action of peripherally generated ANP in the brain by the two histological methods. The immunohistochemical study using specific antiserum raised against eel NPR-A found immunoreactive neurons only in MO loci, such as the RF, GVC, NCC and AP. Furthermore, the AP was intensively stained by intraperitoneally injected Trypan Blue in eels, as previously reported using Evans Blue (Mukuda et al., 2005). These results show that circulating ANP can act on NPR-A in the BBB-deficient circumventricular structure in the MO. Therefore, the AP appears to be directly involved in the potent antidipsogenic action of ANP delivered from the periphery in the eel.

In addition to the histological approach, the ANP target site was also pursued by physiological studies *in vivo*. Heat-coagulation of the AP by electric cautery completely nullified the antidipsogenic effect of systemic injection of ANP, whereas ANP delivered by the same route strikingly inhibited drinking in sham-operated eels and in eels with lesions outside the AP. These results strongly suggest that the AP is involved in the antidipsogenic effect of circulating ANP in eel. However, heat-coagulative lesions to the AP considerably decreased basal drinking compared with sham controls and with controls experiencing lesions outside the AP. Since heat coagulation by electric cautery destroyed not only AP neurons, but also nerve fibres running through the AP, it is possible that the extensive destruction of the AP may have affected the neural circuitry that is responsible for basal drinking in eels. As an alternative method, therefore, the AP neurons were chemically destroyed by microinjection of kainic acid into the AP. Kainic acid has been successfully used to block the AP neurons in the cat

Table 1. *Brain loci stained by Trypan Blue and NPR-A immunohistochemistry*

	Trypan Blue	NPR-A IHC
Capillaries	–	+++
Saccus dorsalis	+++	ND
Pineal gland	++	ND
Parvocellular part of preoptic nucleus	+++*	–
Magnocellular part of preoptic nucleus	+++*	–
Saccus vasculosus	+++	+++
Reticular formation	+*	+++*
Glossopharyngeal–vagal motor complex	+*	+++*
Commissural nucleus of Cajal	–	+++*
Area postrema	+++*	+++*

+, low density; ++, moderate density; +++, high density; –, no immunoreactive element; ND, not determined; *, neurons were stained.

Table 2. *Effect of lesion to area postrema (AP) on ANP-inhibited drinking in seawater eels*

	AP lesion	*N*	Lesion control	*N*	Sham control	*N*
(A) Heat coagulative lesion						
Before	197.6+39.7[a]	10	302.3+37.6	8	387.4+50.3	10
After	166.4+31.5	10	95.9+36.4*	8	98.8+28.7*	10
(B) Chemical lesion						
Before	505.1+50.9	7	464.3+55.6	7	388.1+56.2	7
After	360.3+40.7*	7	198.7+57.1*	7	130.0+24.6*	7

Values are expressed as water intake measured during a 20-min period before and after injection (see text for details).
Changes in drinking rate are compared by paired-sample Student's *t*-test in the same group (*P<0.05).
[a]Heat-coagulative lesions to the AP significantly decrease basal drinking compared the lesion controls and sham controls.

(Bonigut et al., 1997). With this method, the AP lesion itself did not decrease basal drinking of SW eels. As observed after heat-coagulative lesioning, the chemical lesioning significantly attenuated ANP-induced inhibition of drinking in SW eels. Since injection of kainic acid to other sites in the MO did not affect the ANP effect, the AP neurons are most likely to be involved in ANP-induced antidipsogenesis. Compared with heat-coagulative lesioning, however, the inhibition of the ANP effect by kainic acid was partial in AP-lesioned eels. This partial inhibition is most probably due to the partial destruction of the AP, since Chicago Sky Blue 6B dye did not spread throughout the entire AP (Fig. 3C). Although there are technical limitations with the two methods employed in the present study, these data evaluated by two distinct methods clearly showed that the AP

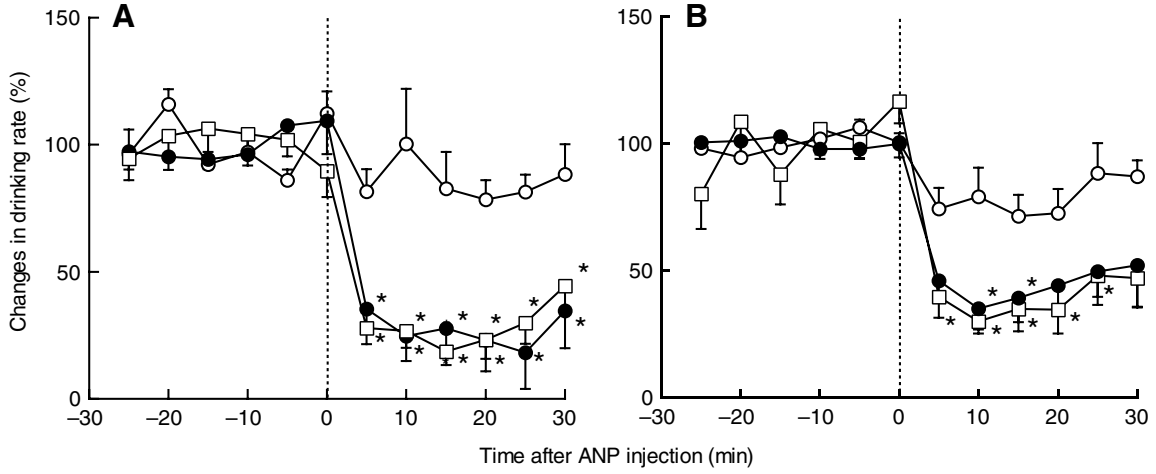

Fig. 6. (A) Effect of heat-coagulative lesioning by electric cautery on antidipsogenic effect of ANP in seawater eels (N=10 for sham-operated eels, open squares; N=10 for AP-lesioned eels, open circles; N=8 for eels with lesion outside AP, filled circles). (B) Effect of chemical lesioning by kainic acid on antidipsogenic effect of ANP in seawater eels (N=7 for sham-operated eels, open squares; N=7 for AP-lesioned eels, open circles; N=7 for eels with lesion outside AP, filled circles). The values are normalized by the averaged drinking rate for 30 min before ANP injection and expressed as percentages (mean ± s.e.m.). Inhibitory effects of ANP on drinking in AP-lesioned eels are compared with those of sham and lesion controls at each time point (*P<0.05; ANOVA followed by Steel–Dwass test).

is the responsible locus for the antidipsogenic action of circulating ANP in eels.

The mechanisms of ANG II-induced drinking in terrestrial species have been well documented. In mammals and birds, the SFO and OVLT are forebrain CVOs important for controlling drinking by systemic hormones (Takei, 1992; Fitzsimons, 1998). The SFO and OVLT are the target sites for circulating ANG II (Kobayashi and Takei, 1996; Ferguson and Bains, 1996; Fitzsimons, 1998), since local injection of femtomolar doses of ANG II into these sites induces drinking, and since local lesioning of these structures eliminates the dipsogenic effects of systemic injection of ANG II (Simpson and Routenberg, 1973; Takei, 1977; Ehrlich and Fitts, 1990; Schmid and Simon, 1992; Li and Ferguson, 1993). Further, the ANG II subtype 1 receptor (AT1) is localized in the SFO (Song et al., 1992) and ANG II enhances neuronal activities of SFO (Gutman et al., 1988; Hattori et al., 1988) in the rat. Since autoradiographic study showed that ANP-binding neurons are located in the SFO in the rat (Saavedra et al., 1986), a similar cerebral mechanism may be applicable to the antidipsogenic action of ANP in terrestrial vertebrates (Antunes-Rodorigues et al., 1986), although the exact site of action of ANP has not been identified in mammals. In fishes, on the other hand, the site of action of ANG II is thought to be in the hindbrain, since 'decerebration' of the entire forebrain does not influence ANG II-induced drinking in eels (Takei et al., 1979). ANP and ANG II injection into the ventricle near the AP inhibited and stimulated drinking, respectively, in a dose-related manner in eels (Kozaka et al., 2003). However, the exact target sites for circulating ANP and ANG II have not been determined yet for fishes. Since the site of action of ANP in the brain has also not been determined in mammals, the present study on eel is the first report to show the cerebral target site for the antidipsogenic action of circulating ANP in vertebrates, which is localized in the AP, a hindbrain CVO.

Although the SFO and OVLT have not been identified in eels, the magnocellular preoptic nuclei (PON) in the forebrain appears to be outside the BBB as its neurons were stained by Trypan Blue in the present study and by Evans Blue in a previous study (Mukuda et al., 2005). However, the NPR-A antibody did not stain neurons in the PON and thus this nucleus may not be a target site for circulating ANP. We show in the present study that the GVC and RF, as well as AP, were stained by Trypan Blue vital staining in the eel brain, although the intensity of staining was weaker and the number of positive neurons was smaller in the RF and GVC than those of the AP. It is possible that the RF and GVC neurons have taken up the dye from the peripheral tissues. Indeed, when Evans Blue, a retrograde tracer of neurons and structural isomer of Trypan Blue, is injected into the esophageal muscles, some neurons in the GVC and spino-occipital motor nucleus (NSO) within the RF are stained (Mukuda and Ando, 2003b).

The functional role of the AP in drinking regulation in fishes remains largely unknown at present. However, previous histological studies showed that the sinus capillaries in the AP are fenestrated, and the dendrites of AP neurons extend dorsally to reach the basal lamina of the capillaries in goldfish (Morita and Finger, 1987), suggesting that teleost AP serves as an avenue for endocrine signals such as ANP and ANG II to communicate with the CNS. Furthermore, it has been shown that the GVC neurons project directly to the striated muscles of the opercular membrane, pharynx, upper esophageal sphincter and esophageal body, all of which are implicated in swallowing (Mukuda and Ando, 2003b). It was also shown that the AP neurons project ventrally to the GVC (Ito et al., 2006). Although further experiments are required to identify precise neuronal networks that are involved in ANP-induced inhibition of drinking, it is possible that the AP acts as a center for integrating the information of dipsogenic and antidipsogenic hormones from the periphery and that it regulates the coordinated behaviour of swallowing through the vagus nerves in eels.

List of abbreviations

ANG II	angiotensin II
ANP	atrial natriuretic peptide
AP	area postrema
BBB	blood-brain barrier
Cb	cerebellum
CNS	central nervous system
CVO	circumventricular organ
GVC	glossopharyngeal-vagal motor complex
MO	medulla oblongata
NCC	commissural nucleus of Cajal
NPR-A	type A ANP receptor
NSO	spino-occipital motor nucleus
OB	olfactory bulb
OVLT	organum vasculosum of the lamina terminalis
PBS	phosphate-buffered saline
PON	magnocellular preoptic nuclei
RF	reticular formation
SD	saccus dorsalis
SFO	subfornical organ
SV	saccus vasculosus
SW	seawater
Tel	telencephalon
TeO	optic tectum

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