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Central effects of various ligands on drinking behavior in eels acclimated to seawater

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Summary

Intracranial injection of eel angiotensin II (eANG II, 5×10^{-13}–5×10^{-8} mol), acetylcholine (ACh, 5×10^{-12}–5×10^{-9} mol), substance P (5×10^{-10} mol) and isoproterenol (a β-adrenoceptor agonist, 5×10^{-11}–5×10^{-9} mol) enhanced water intake in the seawater eel. The effects of eANG II, ACh and isoproterenol were dose-dependent. By contrast, water intake was inhibited by intracranial injection of eel atrial natriuretic peptide (eANP, 5×10^{-13}–5×10^{-10} mol), serotonin (5-HT, 5×10^{-12}–5×10^{-8} mol), ghrelin (5×10^{-12}–5×10^{-10} mol), γ-amino butyric acid (GABA, 5×10^{-11}–5×10^{-8} mol), prolactin (PRL, 5×10^{-10}–5×10^{-9} mol), arginine vasotocin (AVT, 5×10^{-12} mol), vasoactive intestinal peptide (VIP, 5×10^{-11} mol), noradrenaline (5×10^{-9} mol l^{-1}) and phenylephrine (α-adrenoceptor agonist, 5×10^{-11}–5×10^{-9} mol). The inhibitory effects of eANP, 5-HT, ghrelin, GABA, PRL and phenylephrine were dose-dependent. The intracranial stimulatory effect of eANG II was relatively long-lasting compared with the intravenous effect. The stimulatory effect of intravenous eANG II disappeared immediately, and was followed by an inhibition, which could be well explained by an increase in eANP secretion from the atrium.

Key words: seawater eel, *Anguilla japonica*, drinking behavior, intracranial administration, intravenous administration, angiotensin II, atrial natriuretic peptide, circumventricular organ.

Introduction

Maintenance of blood homeostasis is essential for vertebrate life. In particular, drinking behavior is of vital importance for terrestrial vertebrates and seawater teleost fish. However, the mechanisms controlling drinking behavior are not understood even in mammals (Bourque et al., 1994; Fitzsimons, 1998), possibly because of the complexity of their drinking behavior. On perception of thirst, mammals must first seek water, which is then ingested and finally swallowed. Furthermore, the central neuronal networks of osmo- and thermoregulation seem to overlap in mammals (Takahashi et al., 2001). By contrast, fish (poikilothermic animals) can swallow immediately following thirst perception, since they live in water and water is constantly held in the mouth for respiration. Therefore, the neuronal circuitry controlling drinking behavior in fish may be less complex.

Among fish, the drinking behavior of the euryhaline eel has been extensively studied using an esophageal cannulation technique developed by Hirano (1974). Furthermore, various regulators that affect water intake are already known in the eel (Takei et al., 1979; Hirano and Hasegawa, 1984; Ando et al., 2000a; Takei, 2000). In particular, endogeneous angiotensin II (eANG II) and atrial natriuretic peptide (eANP) are considered a potent dipsogen and antidipsogen, respectively (Takei, 2000). Application of these regulators has, however, been restricted to the systemic circulation, thus limiting our understanding of their effects on central locations such as brain. It is well known in mammals that circulating ANG II, a most potent dipsogen, acts on the subfornical organ (SFO), a circumventricular organ (CVO) that lacks the blood–brain barrier (BBB) (Kobayashi and Takei, 1996; Fitzsimons, 1998; Takei, 2000), and it is likely that these systemic regulators may also act on the CVOs in the eel brain. If these regulators do act on the CVOs, the effects of central administration of these regulators must be identical to those observed on systemic application.

The aim of the present study was to identify the ligands affecting drinking behavior in the brain of the eel. Various ligands were injected intracranially *via* a cerebral cannula, and the effects compared with previous results obtained after intravenous injection (Ando et al., 2000a). Similarities and differences between these two applications are discussed in relation to brain morphology.

Materials and methods

Cultured Japanese eels *Anguilla japonica* Temminck & Schlegel, weighing about 200 g, were obtained from a commercial source. They were kept unfed in seawater aquaria at 20°C for more than 1 week before use. After anesthesia using 0.1% methane tricaine sulfonate (MS-222, Sigma, St. Louis, USA) in seawater, an incision (2 cm long) was made

longitudinally in the abdominal wall along the posterior half of the liver. A vinyl tube (o.d. 2.0 mm) was inserted into the esophagus to measure the water intake, as described previously (Ando and Nagashima, 1996; Ando et al., 2000a). The cannula was connected to a drop counter (PG-602, Keyence, Osaka, Japan) for continuous recording of the drinking rate. Each drop (21 µl) was recorded as a spike on a chart recorder (EPR-121A, TOA, Tokyo, Japan). Another tube (o.d. 1.1 mm) was inserted into the intestine for application of 0.17 mol l^{-1} NaCl solution, determined from the concentration measured at the gastrointestinal junction in seawater eels (Ando and Nagashima, 1996). Perfusion of this cannula was done *via* a peristaltic pump (MS-1 Reglo 160, Ismatic, Zurich, Switzerland) controlled by an electric stimulator (SEN-3201, Nihon Koden, Tokyo), which was triggered by the drop counter. With this system, the swallowed seawater can be reintroduced into the intestine. Details of the experimental system are as described previously (Ando et al., 2000b).

To insert a cerebral cannula, the skin of the occipital region was incised and the muscle removed. After exposing the skull, the surface was flattened with a grinder (No. 28511, Kiso Power Tool, Osaka), then drilled (approx. 1 mm) at the front of the supraoccipital bone (Fig. 1A). A needle (approx. 0.5 mm) was inserted through the dura into the fourth ventricle, and the cerebral cannula (No. 9571, BAS, Tokyo) filled with 0.9% NaCl solution was inserted into the pore, 1.4 mm below the skull (Fig. 1B,C). The cannula was fixed to the skull with dental cement (1-1PKG, GC Corporation, Tokyo).

After the operation, the incision was closed using silk suture and all cannulae were sutured to the body. Eels were then transferred to a plastic trough of the same size. Well aerated seawater was circulated continuously through the trough at room temperature (20–23°C). Experiments were started on the following day, when the drinking rate was relatively constant. Only eels responsive to intracranial eANG II (5×10^{-10} mol l^{-1}, 5 µl) but not to 0.9% NaCl (10 µl) were used for the following experiments. To avoid habituation to a ligand, each ligand was injected at intervals of more than 5 h, and different ligands were used on the same day. The sequence of ligand application was random. If the response obtained was unexpected, responsiveness to eANG II and 0.9% NaCl as controls was checked again. Under such experimental conditions, eels survived for more than 1 week. After the experiments, 1% Methylene Blue (5 µl) was injected through the cerebral cannula, and the fourth ventricle and a part of the third ventricle were stained with dye.

Reagents were all dissolved in 0.9% NaCl solution (vehicle) and injected intracranially with a syringe pump (MF-9090, Bioanalytical Systems, IN, USA) at a rate of 1.25 µl min^{-1} for 4 min (total 5 µl). Since the dead volume of the cerebral cannula was 5 µl, a further 5 µl of vehicle was injected into the cannula, making the total volume of injectate 10 µl.

Acetylcholine chloride (ACh), carbamylcholine chloride (CCh), histamine dihydrochloride (HA), 5-hydroxytriptamine creatine sulphate (5-HT), phenylephrine HCl, sheep prolactin (PRL) and propranolol HCl were purchased from Sigma. Arginine vasotocin (AVT), eel angiotensin II ([Asn^1]eANG II), cholecystokinin (26–33) (CCK-8), human ghrelin, substance P (SP), saralasin and vasoactive intestinal polypeptide (VIP) were purchased from Peptide Institute, Osaka, Japan; dopamine HCl (DA), γ-amino butyric acid (GABA), noradrenaline HCl (NA) and heparin sodium from Katayama Chemical, Osaka, Japan; eel atrial natriuretic peptide (eANP) from Peninsula Laboratories, CA, USA; PD 123319 ditrifluoroacetate and CGP 42112 from Research Biochemicals International, Natick, USA. Losartan potassium (Banyu, Tokyo, Japan), Exp 3174 (Dupont, Wilmington, USA)

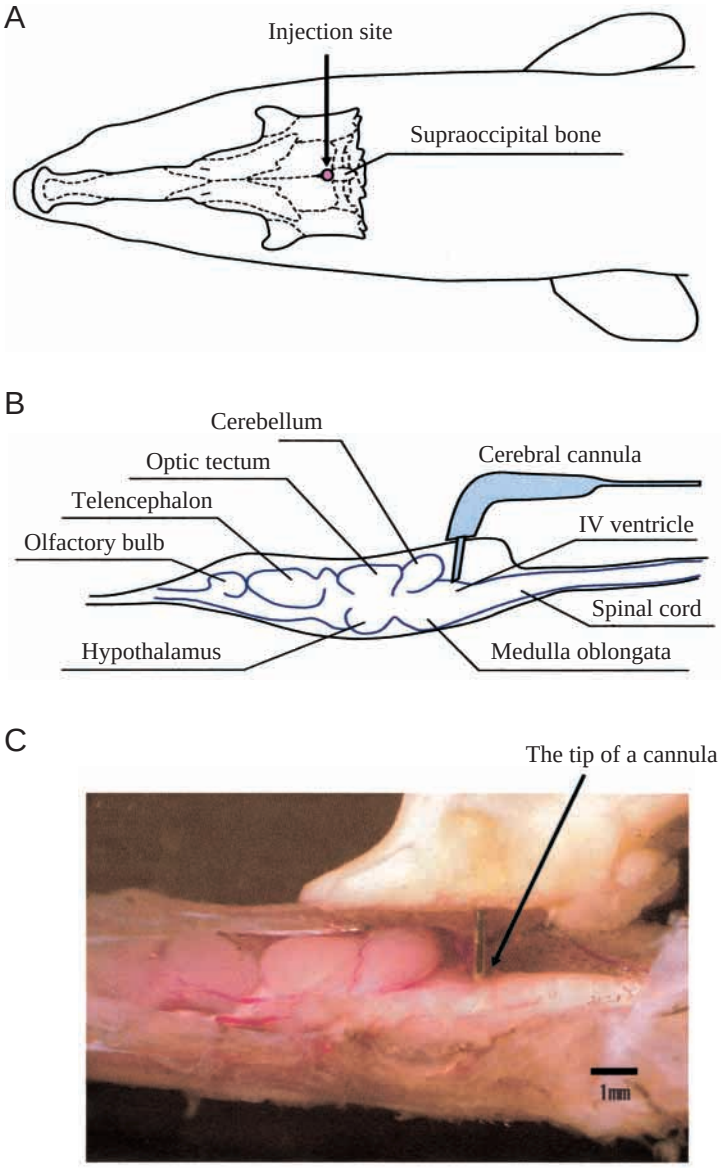

Fig. 1. Position of the cerebral cannula. At the front of the supraoccipital bone, the skull was drilled (A). The position of the cannula is shown schematically (B) and photographically (C) from the lateral side of the brain.

and CV 11974 (Takeda Chemical Industries, Tokyo, Japan) were kind gifts from these companies.

For intravenous injection, a venous cannula (SP-10, Natume, Tokyo, Japan) filled with heparinized saline (100 i.u. ml^{-1}) was inserted into the posterior cardinal vein as described previously (Ando et al., 2000a).

The effects of ligands were evaluated by comparing the drinking rates for a 20 min period before and after application of the ligand. Statistical analyses of the results were performed using a paired *t*-test. Results are given as means ± S.E.M. and were considered significant at $P<0.05$.

Results

Substances accelerating drinking rate

As a control, 13 µl of 0.9% NaCl solution (vehicle) was injected intracranially into the fourth ventricle. Vehicle injection alone had no significant effect on drinking rate (274±35 *versus* 248±27 µl 20 min^{-1}, $N=11$). When eel angiotensin II (eANG II, 5×10^{-11} mol) was injected into the fourth ventricle, the drinking rate was enhanced after a few minutes, then gradually decreased. The effect of eANG II was dose-dependent, with a threshold at 5×10^{-13} mol, and was maximal at 5×10^{-10} mol (Fig. 2). However, at higher doses (5×10^{-8} mol), the enhancement was reduced. The enhancement by eANG II was not inhibited by pretreatment with standard angiotensin receptor antagonists such as saralasin, losartan, Exp 3174, CV 11974, PD 123319 and CGP 42112 (data not shown).

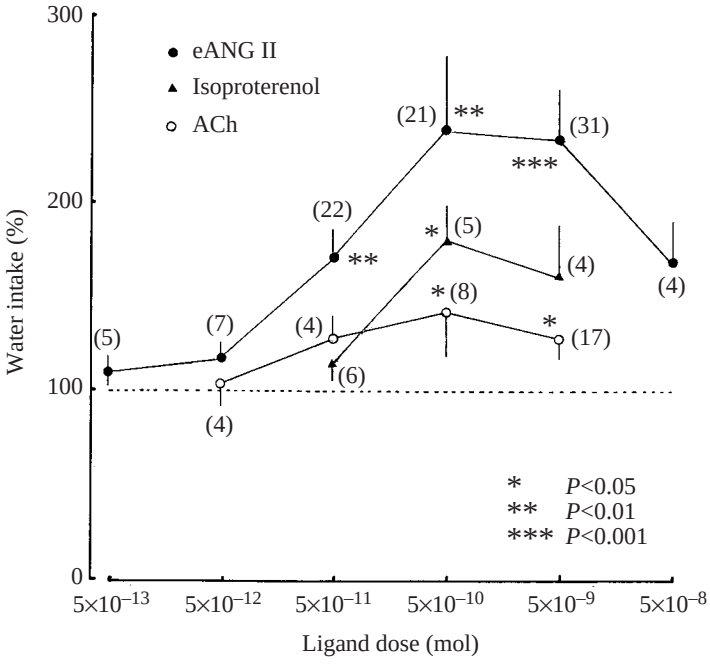

Fig. 2. Dose–response curves of the effect of eel angiotensin II (eANG II; filled circles), isoproterenol (filled triangles) and acetyl choline (Ach; open circles) on water intake by seawater eels. Water intake was measured during a 20 min period after administration of the ligand, and is expressed as % of the control rate measured during a 20 min period before administration (100%). The injection rate was 1.25 µl min^{-1} for 4 min. Values are means ± S.E.M. Number of experiments is indicated in parentheses. *$P<0.05$, **$P<0.01$, ***$P<0.001$, compared with the control value (paired *t*-test).

The drinking rate was also enhanced by acetylcholine (ACh) in a dose-dependent manner (Fig. 2), but the effect was smaller than that of eANG II, with a threshold at 5×10^{-12} mol and a 1.4-fold increase at 5×10^{-10} mol. An acetylcholine agonist, carbachol (CCh), enhanced the water intake to 266±71% ($N=6$) at 5×10^{-10} mol (not shown). Isoproterenol, a β-adrenoceptor agonist that accelerates drinking in mammals upon systemic administration (Fitzsimons, 1998), also enhanced water intake in a dose-dependent manner, with a threshold at 5×10^{-11} mol, and maximal effect at 5×10^{-10} mol (Fig. 2). Substance P (SP) increased the drinking rate to 197±51% ($N=5$) at 5×10^{-10} mol (data not shown). In some preparations, cholecystokinin (CCK-8, 5×10^{-13}–5×10^{-11} mol) and dopamine (DA, 5×10^{-12}–5×10^{-8} mol) increased the drinking rate, but the reproducibility and dose-dependency were low (not shown).

Substances inhibiting drinking rate

When eel atrial natriuretic peptide (eANP, 5×10^{-11} mol) was injected intracranially, basal drinking rate was decreased after a few minutes and then returned to the initial level after 1 h. The inhibitory effect of eANP was dose-dependent, with a threshold at 5×10^{-13} mol. Fig. 3 shows dose–response curves for various inhibitors, including ANP, ghrelin, serotonin (5-HT), phenylephrine (an α-adrenoceptor agonist), prolactin (PRL), and γ-amino butyric acid (GABA, a general inhibitory neurotransmitter in the brain). Ghrelin, a 28-amino-acid peptide isolated originally from rat stomach and exhibiting an orexigenic effect (Kojima et al., 1999), was the most potent among these inhibitors examined. Water intake was also inhibited by arginine vasotocin (AVT, 50±7%, $N=3$, at 5×10^{-12} mol), vasoactive intestinal peptide (VIP, 80±7%, $N=6$, at 5×10^{-11} mol), noradrenaline (NA, 51±11%, $N=5$, at 5×10^{-9} mol) and somatostatin (SS-14, 66±13%, $N=5$, at 5×10^{-12} mol) (not shown). Although β-endorphin (5×10^{-11} mol), an opioid peptide that affects drinking in mammals (Fitzsimons, 1998), reduced the drinking rate in some preparations, the reproducibility was low (not shown).

When glutamate (a general excitatory neurotransmitter in the brain; 5×10^{-11}–5×10^{-7} mol), histamine (5×10^{-11}–5×10^{-8} mol), glycine (an inhibitory neurotransmitter; 5×10^{-9}–5×10^{-8} mol) and eel intestinal pentapeptide (EIPP; 5×10^{-13}–5×10^{-12} mol) were applied intracranially, the water intake was not altered (not shown).

Comparison between intracranial and intravenous administrations of ANG II

Fig. 4 shows the time course after the intracranial and intravenous administration of eANG II, as well as that after intracranial administration of eANP. The dose of eANG II was chosen to induce a comparable effect, i.e. 5×10^{-11} mol for intracranial and 10^{-7} mol for intravenous application.

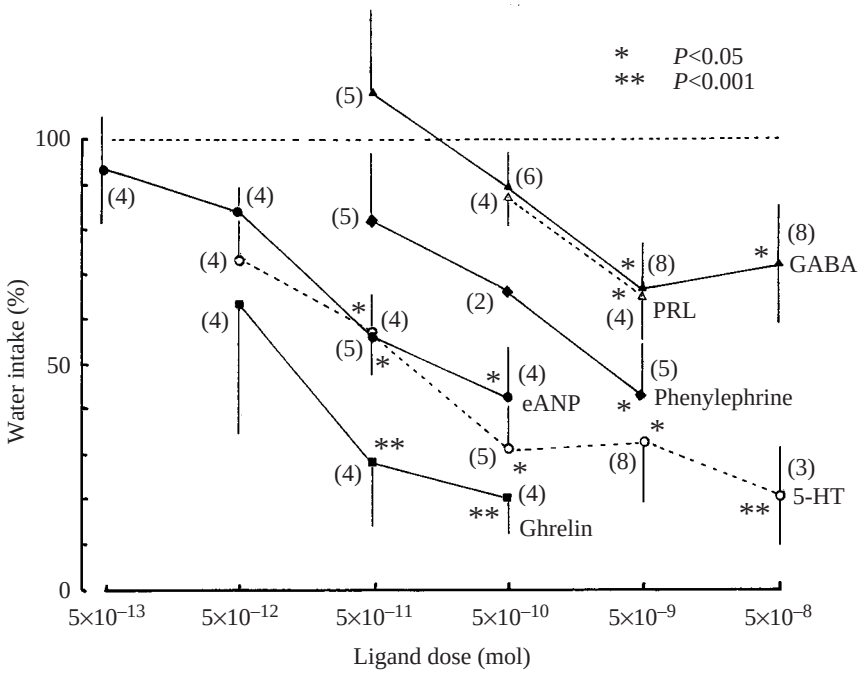

Fig. 3. Dose–response curves of various inhibitors on water intake by seawater eels. Water intake was measured during a 20 min period after administration of the inhibitor, and is expressed as % of control rate measured during a 20 min period before administration (100%). Ghrelin (filled squares), eel atrial natriuretic peptide (eANP; filled circles), serotonin (5-HT; open circles), phenylephrine (filled diamonds), prolactin (PRL; open triangles) and γ-amino butyric acid (GABA; filled triangles) were injected intracranially at a rate of 1.25 μl min^{-1} for 4 min. Values are means ± S.E.M. Number of experiments is indicated in parentheses. *P<0.05, **P<0.001, compared with the control value (paired t-test).

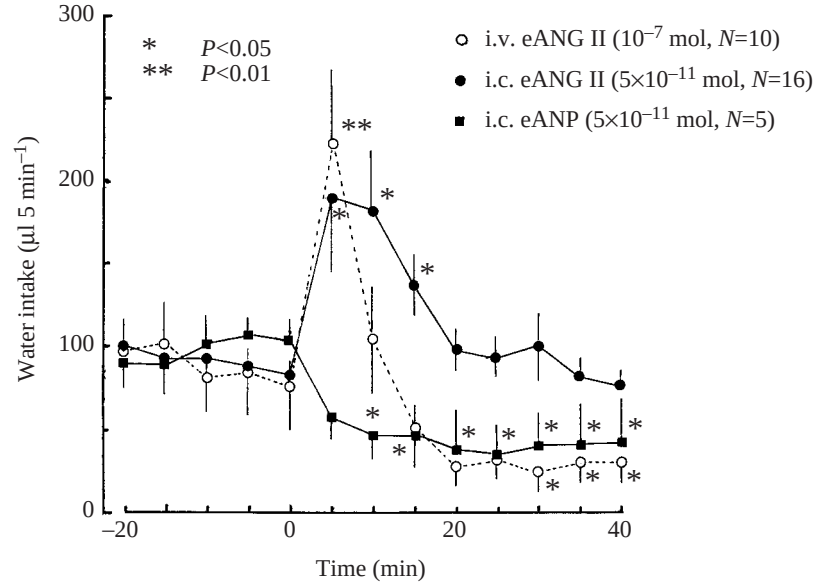

Fig. 4. Time course of the effect of intravenous (i.v., open circles) and intracranial (i.c., filled circles) administration of eel angiotensin II (eANG II). The dose of eANG II, 10^{-7} mol i.v. and 5×10^{-11} mol i.c., was chosen to obtain similar enhancement of water intake. For comparison, the time course of the effect of intracranial eel atrial natriuretic peptide (eANP, filled squares) is also shown. Water intake was measured every 5 min, and expressed as mean ± S.E.M. *P<0.05, **P<0.01, compared with the value before administration at time zero (paired t-test).

Both applications of eANG II enhanced the water intake transiently, with similar peaks after 5 min. However, the effect of intracranial ANG II lasted longer than that of the intravenous ANG II. eANP (5×10^{-11} mol), however, decreased water intake, and the inhibitory effect was maintained for more than 40 min. The drinking rate 15 min after intravenous administration of eANG II was almost identical to that after eANP.

Discussion

The present study demonstrates that various ligands injected into the fourth ventricle affect the drinking behavior of the seawater eel. To our knowledge, this is the first study of effects of intracranial substance injection on drinking in fish. Angiotensin II (ANG II), acetylcholine (ACh), substance P (SP) and isoproterenol (a β-adrenoceptor agonist) accelerated the drinking rate, while atrial natriuretic peptide (ANP), arginine vasotocin (AVT), vasoactive intestinal peptide (VIP), serotonin (5-HT), prolactin (PRL), ghrelin, γ-amino butyric acid (GABA), noradrenaline (NA) and phenylephrine (an α-adrenoceptor agonist) inhibited the water intake (Table 1). These results indicate that the eel brain contains specific sites responsive to these ligands.

With the exception of 5-HT, PRL, HA and EIPP, all ligands examined in the present study had qualitatively similar effects, irrespective of the site of administration. Recently, intravenous ghrelin (10^{-10} mol) was demonstrated to inhibit drinking robustly (Y. Watanabe and M. Ando, unpublished observation). The most basic explanation for this similarity is that central and peripheral ligands act on identical sites concerned with regulating drinking behavior, and these sites are most probably situated in a central location within the brain. However, as the brain is for the most part isolated from the systemic circulation by the blood–brain barrier (BBB), ligands administrated intravenously would only have access to the specific control sites where there is no BBB. Such specific regions are called circumventricular organs (CVOs) (see Fitzsimons, 1998; Takei, 2000). Recently, we demonstrated that the magnocellular preoptic nucleus (PM), the anterior tuberal nucleus (ATN) and the area

Table 1. *Effects on water intake of various ligands administrated intracranially in the eel*

Ligand	Eel (i.c.)	Eel (i.v.)	Mammals (i.c.)
Angiotensin II (eANG II)	+	+	+
Acetylcholine (ACh, CCh)	+	+	+
Isoproterenol	+	+	ND
Substance P (SP)	+	+	+ (rabbit, sheep) – (rat)
Atrial natriuretic peptide (eANP)	–	–	–
Arginine vasotocin (AVT)	–	–	+(AVP)
Vasoactive intestinal peptide (VIP)	–	–	+
Phenylephrine	–	–	–
Serotonin (5-HT)	–	+	+ (monkey) – (rat)
Prolactin (PRL)	–	+	ND
Ghrelin	–	ND	ND
γ-Amino butyric acid (GABA)	–	ND	–
Noradrenaline (NA)	–	ND	+ (cat, rat) – (rat)
Histamine (HA)	NE	+	+
Eel intestinal pentapeptide (EIPP)	NE	–	ND

i.c., intracranial; i.v., intravenous.

Results from i.v. administration in the eel (Ando et al., 2000a) and from i.c. administration in mammals (Fitzsimons, 1998) are also shown for comparison.

+, stimulatory effect; –, inhibitory effect; ND, not determined; NE, no effect.

postrema (AP) are analogous CVOs in the brain of the eel (T. Mukuda and M. Ando, unpublished observation). The PM and the ATN are situated in the hypothalamus and the AP is in the medulla oblongata. Since the eel brain possesses CVOs, where neurons can respond similarly both to the cerebrospinal fluid and to the systemic circulation, intravenous ligands can act on the CVOs and control drinking behavior directly.

The intracranial effects of 5-HT, PRL, HA and EIPP were different from the intravenous effects (Table 1). Intravenous 5-HT, PRL, HA and EIPP may not directly act on the CVOs. Indeed, the intravenous effects of 5-HT and HA were completely blocked by intravenous pretreatment with captopril, an inhibitor of angiotensin converting enzyme, suggesting that intravenous 5-HT and HA stimulate ANG II synthesis and ANG II acts directly on the CVOs to enhance the water intake (Ando et al., 2000a). PRL and EIPP might act indirectly on the CVOs *via* other mediators that are synthesized peripherally but not yet identified.

Although eANG II showed a qualitatively similar effect with both intracranial and intravenous administration, the time courses differed for the two treatments (Fig. 4). The stimulatory effect lasted for 10 min after intracranial administration, whereas it was reduced to the original level after 10 min following intravenous application and significantly inhibited after 30 min. Interestingly, the low level was almost the same as that seen after intracranial eANP administration. These results could be explained by a dual effect of intravenous eANG II. Circulating eANG II may act on the CVOs directly to stimulate drinking, and simultaneously on the atrium to stimulate ANP (an antidipsogen) secretion. Indeed, a 1.6-fold increase in plasma ANP levels was induced following intra-arterial administration of 10^{-10} mol ANG II in the Japanese eel (Tsuchida and Takei, 1999). Such enhancement of plasma ANP may result in an inhibition of drinking, since the antidipsogenic effect of systemic eANP was 100 times more potent than the dipsogenic effect of eANG II (Takei, 2000).

Although a higher dose of eANG II is required to give the same peak effect when injected intravenously than intracranially, this phenomenon can be explained by difference in the volumes of blood and cerebrospinal fluid (CSF) that flow from the third ventricle to the fourth ventricle. In addition, intravenous ANG II may be inactivated by peptidases before arriving at the CVOs.

Angiotensin receptor in the eel seems to be different from mammalian types, since mammalian AT_1- and AT_2-receptor antagonists did not inhibit eANG II action. Similar lack of effect has been observed in non-mammalian species (Ji et al., 1993; Murphy et al., 1993; Tierney et al., 1997).

Comparing the present results with those obtained in mammals, the effects of AVT (AVP in the case of mammals) and VIP have opposite effects, whereas intracranial ANG II, ACh, ANP and GABA show similar effects in both vertebrate groups (Table 1). In mammals, it is well known that blood hyperosmolarity stimulates AVP secretion (Brimble et al., 1977) and drinking rate (Gilman, 1937; Olsson, 1972; Thrasher et al., 1980a,b; Kadekaro et al., 1995). On the other hand, in the seawater eel, blood hyperosmolarity decreases water intake (Ando et al., 2000a; Takei, 2000). Therefore, the different effects of antidiuretic hormone (ADH) seen in mammals and eels may be attributable to their different drinking behavior responses to blood hyperosmolarity. The difference in the effect of VIP may be also due to a different response to ADH, as it is known that intracerebroventricular administration of VIP releases AVP in rats (Bardrum et al., 1988; Murase et al., 1993; Nagai et al., 1996). Except for ADH, most ligands act in a similar way in both eel and mammalian brain. Isoproterenol is known to be a potent thirst stimulus upon systemic administration (Fitzsimons, 1998), and to stimulate water intake after injection into the SFO in the brain of rats (Menani et al., 1984). Therefore, the eel may be a suitable model for analyzing drinking behavior in vertebrates.

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