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RESEARCH ARTICLE

Circulating isotocin, not angiotensin II, is the major dipsogenic hormone in eels

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ABSTRACT

Angiotensin II (AngII) is generally known as the most important dipsogenic hormone throughout vertebrates, while two other neurohypophysial hormones, vasopressin and oxytocin, are not dipsogenic in mammals. In this study, we found that systemic isotocin, but not vasotocin, is the potent dipsogenic hormone in eels. When injected intra-arterially into conscious eels, isotocin, vasotocin and AngII equally increased ventral aortic pressure dose dependently at 0.03–1.0 nmol kg^{-1}, but only isotocin induced copious drinking. The dipsogenic effect was dose dependent and occurred significantly at as low as 0.1 nmol kg^{-1}. By contrast, a sustained inhibition of drinking occurred after AngII injection, probably due to baroreflexogenic inhibition. No such inhibition was observed after isotocin injection despite similar concurrent hypertension. The baroreceptor may exist distal to the gill circulation because the vasopressor effect occurred at both ventral and dorsal aorta after AngII but only at ventral aorta after isotocin. By contrast, intra-cerebroventricular (i.c.v.) injection of isotocin had no effect on drinking or blood pressure, but AngII increased drinking and aortic pressure dose dependently at 0.03–0.3 nmol per eel. Lesioning of the area postrema (AP), a sensory circumventricular organ, abolished drinking induced by peripheral isotocin, but not i.c.v. AngII. Collectively, isotocin seems to be a major circulating hormone that induces swallowing through its action on the AP, while AngII may be an intrinsic brain peptide that induces drinking through its action on a different circumventricular site, possibly a recently identified blood–brain barrier-deficient structure in the antero-ventral third ventricle of eels, as shown in birds and mammals.

KEY WORDS: Swallowing, Thirst, Area postrema, Neurohypophysial hormone

INTRODUCTION

Body fluid homeostasis is maintained by balancing the gain and loss of water and ions across body surfaces (Schmidt-Neilsen, 1997). In terrestrial vertebrates, water is lost through evaporation from the skin and respiration, and in the urine and faeces. In order to compensate for the loss, water is ingested through thirst-motivated drinking behaviour, which is followed by intestinal absorption (Fitzsimons, 1998). The most potent dipsogenic hormone thus far known is angiotensin II (AngII). In addition, renal loss is minimized by active reabsorption of water from the primary urine (>95%) by renal tubules. Antidiuretic hormone, of both vasopressin and vasotocin species, is responsible for the renal reabsorption in terrestrial organisms (Bentley, 2002). Vasotocin also plays an essential role for water absorption by the skin and urinary bladder of amphibians. In contrast to the typical water balance response of tetrapods, water regulation in aquatic fishes differs greatly depending on the environmental salinity. In teleosts whose plasma ion concentration is ca. one-third that of seawater (SW), the patterns of water regulation are different when they are in freshwater (FW) and SW. In FW, oral drinking is usually suppressed and the excess water that enters the body osmotically across the gills is excreted by the kidney as dilute urine (Marshall and Grosell, 2006). In SW, however, water is lost osmotically across the gills, and fish drink a copious amount of environmental SW to compensate for the loss. Thus, oral drinking is essential for marine teleosts to acquire water as in terrestrial vertebrates (Takei and Balment, 2009; Nobata et al., 2013).

In mammals and birds, copious drinking is induced by peripheral injection of AngII, which acts on the subfornical organ (SFO) and organum vasculosum of the lamina terminalis (OVLT), sensory circumventricular organs that are devoid of a blood–brain barrier (BBB) in the forebrain (Simpson and Routtenberg, 1973; Takei, 1977; McKinley et al., 1982; Johnson and Gross, 1993). In terrestrial species, AngII-induced thirst motivates a series of drinking behaviours: water searching, intake of water into the mouth and swallowing. In fishes, however, drinking can occur simply by reflex swallowing as water is always in their buccal cavity for respiration (Hirano et al., 1972). The site of action of circulating hormones for the regulation of drinking appears to be the area postrema (AP), another sensory circumventricular organ without a BBB in the hindbrain (Morita and Finger, 1986; Mukuda et al., 2005), as lesioning of the AP abolished the dipsogenic action of AngII and antidipsogenic actions of arterial natriuretic peptide and ghrelin after peripheral injection into eels (Tsukada et al., 2007; Nobata and Takei, 2011). Consistent with the AP location of the regulatory site, AngII induced drinking in the eel in which the whole forebrain was removed (Takei et al., 1979). AngII is the most potent dipsogenic hormone in all vertebrate classes thus far examined (Perrott et al., 1992; Kobayashi and Takei, 1996; Johnson and Thunhorst, 1997; Fitzsimons, 1998; McKinley et al., 2003a,b). However, even though AngII is the only dipsogenic hormone in teleosts, the effect is much less potent and only transient in eels compared with the effect in terrestrial species (Takei, 2002). It is likely that a more reliable and potent dipsogenic hormone may exist in teleost fishes to support SW acclimation.

One of the candidates is vasopressin/vasotocin as it is the most important water-retaining hormone as a result of its antidiuretic action in the mammalian, avian and reptilian kidney (Nishimura and Fan, 2002; Dantzler and Braun, 1980; Braun and Dantzler, 1997; Nielsen et al., 2002) and hydrosmotic action in the amphibian skin and urinary bladder (Bentley, 2002; Hillyard and Willumsen, 2011). In addition, the vasotocin/aquaporin axis involved in water retention is expressed in the kidney of the lungfish only in the terrestrial

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condition (Konno et al., 2009; 2010a). Thus, vasopressin/vasotocin seems to be a key hormone that enabled animals to survive in the water-deficient terrestrial environment. In teleosts, however, the effect of vasotocin on water retention is conflicting. Plasma vasotocin concentration increased transiently after hyperosmotic challenge in euryhaline flounder (Warne and Balment, 1995; Warne et al., 2005), but gene expression in the preoptic nucleus was reduced in the rainbow trout (Hyodo and Urano, 1991). Peripheral injection of vasotocin usually induces pressure diuresis, but it induced glomerular antidiuresis in the perfused trunk preparation where arterial pressure was maintained constant (Amer and Brown, 1995). The expression of the V2 receptor gene was confirmed in two teleosts (Konno et al., 2010b). Another neurohypophysial hormone, oxytocin, has been implicated in sodium homeostasis in mammals. Specifically, it induces natriuresis and inhibits sodium appetite in rats (Balment et al., 1980; Conrad et al., 1993; Blackburn et al., 1993). In teleosts, isotocin, an orthologue of oxytocin, stimulates Cl^- secretion from the gill pavement cells more potently than does vasotocin (Guibbolini and Avella, 2003). Taken together, neurohypophysial hormones are involved in water retention and ion excretion in most vertebrate species thus far examined, so it is possible that they are beneficial for SW adaptation and thus elicitation of drinking in teleost species (Balment et al., 2006). In the amphibious mudskipper goby, indeed, mRNA expression of vasotocin and isotocin precursors in the brain increases under terrestrial conditions, and intra-cerebroventricular (i.c.v.) injection of both peptides promotes migration to a seawater pool from land (Sakamoto et al., 2015).

In this study, we examined the possible involvement of the neurohypophysial hormones vasotocin and isotocin in the regulation of drinking in eels. An earlier report showed that vasopressin and oxytocin inhibited drinking with a simultaneous increase in arterial pressure in eels (Hirano and Hasegawa, 1984). More recent data showed that isotocin relaxed the upper oesophageal sphincter (UOS) muscle to increase drinking but vasotocin constricted the UOS to decrease drinking in eels (Watanabe et al., 2007). However, it is not known whether these hormones act on the brain to regulate drinking. In this study, therefore, we extensively examined the effects of vasotocin, isotocin and AngII on drinking and arterial pressure after peripheral and central injection, using conscious eels with cannulas in the dorsal and ventral aorta for injections and measurement of arterial pressure, in the oesophagus and stomach for measurement of drinking rate, and in the third ventricle for central injections.

MATERIALS AND METHODS

Animals

Immature cultured eels, *Anguilla japonica* Temminck and Schlegel 1846, were purchased from a local supplier. They were acclimated in SW (natural SW; ∼33 p.s.u.) tanks without feeding for more than 2 weeks before use. Water in the tanks was filtered, aerated and maintained at 18°C. All animal experiments described in this paper were approved by the Animal Experiment Committee of the University of Tokyo and performed in accordance with the Manual for Animal Experiments prepared by the committee.

Peptide synthesis

Vasotocin, and [Asn^1, Val^5]-angiotensin II (AngII) and oxytocin were purchased from Peptide Institute Inc. (Osaka, Japan), and isotocin from Bachem (Bubendorf, Switzerland). For injections, peptides were dissolved with distilled water or 1 mol l^{-1} acetic acid (for isotocin) at a concentration of more than 10^{-4} mol l^{-1}, and subsequently diluted with isotonic 0.9% NaCl (saline) as necessary before injection.

Effects of intra-arterial (i.a.) injection

SW-acclimated eels (mean±s.e.m. 170.4±4.8 g, n=8) were anaesthetized in 0.1% (w/v) tricaine methanesulfonate (Sigma, St Louis, MO, USA) for 15 min, and polyethylene tubes (o.d. 0.8 mm) were surgically inserted into the ventral and dorsal aortae for blood pressure measurement and injections. Vinyl tubes (o.d. 2.0 mm) were surgically inserted through the body wall into the oesophagus and stomach for continuous measurement of drinking rate. These surgical procedures have been described in detail previously (Takei et al., 1998; Nobata and Takei, 2011). After surgery, eels were placed in a plastic trough of their own size, through which aerated water circulated at 18°C. The outflow of the oesophagus cannula was connected to a drop counter to measure ingested water, and the stomach cannula was connected to a pulse injector to reintroduce half-strength SW in synchrony with a drop counter. The arterial cannula was connected via a three-way stopcock to pressure transducers (DX-300, Nihon Koden, Tokyo, Japan) for continuous monitoring of ventral and dorsal aortic pressure (P_{VA} and P_{DA}, respectively). The signal was amplified by a carrier amplifier (7903, NEC San-Ei, Tokyo, Japan). Drinking rate and blood pressure were recorded by a water balance monitoring system (MTS00658, Medical Try System, Tokyo, Japan).

Peptide injections were started 3 days following surgery at 0.03, 0.1, 0.3 and 1.0 nmol kg^{-1} for isotocin, vasotocin and AngII, and 1.0 nmol kg^{-1} for oxytocin in saline into the dorsal aorta. Each injection was immediately followed by flushing of the cannula with 50 µl of saline (cannula dead space was 30 µl). Injection intervals were more than 1 h to ensure reproducible responses. Saline alone was used as a control.

Effects of i.c.v. injection

SW-acclimated eels (184.9±8.6 g, n=8) were anaesthetized, and cannulas were inserted as above with an additional one into the cerebral ventricle according to published protocols (Nobata et al., 2011). Briefly, a stainless steel guide cannula (i.d. 0.35 mm, o.d. 0.6 mm) was implanted into the third ventricle to a depth of 0.7 mm from the surface of the brain. An injector (o.d. 0.3 mm) was connected to the polyethylene tube (i.d. 0.28 mm, o.d. 0.61 mm). For i.c.v. injection of 0.5 µl of a peptide solution, the injector was inserted into the guide cannula, the tip of which was 1 mm below that of the guide cannula. Injection was made 50 min after insertion to obviate any effects of cannula insertion on measured parameters. The doses given were 0.03, 0.1 and 0.3 nmol per eel. The same volume of saline injections served as controls.

Effect of AP ablation

The AP lesioning was performed according to the method reported previously (Nobata and Takei, 2011). Briefly, after exposure of the skull, a hole (∼.5 mm diameter) was made in the skull by a drill (Nakanishi, Tochigi, Japan) and then in the exposed part of the arachnoid membrane by sharp-pointed tweezers, carefully, so as not to break the blood vessels. The AP was surgically lesioned using electric cautery (Surgitron EMC, Ellman, Oceanside, NY, USA). After lesioning, the hole was filled with Spongel (Yamanouchi, Tokyo, Japan) and sealed with Bone Wax (Lukens, Albuquerque, NM, USA). Eels subjected to the same surgical procedures without the lesioning served as sham controls. Subsequently, a guide cannula for i.c.v. injection was implanted into the third ventricle and cannulas were inserted into the ventral and dorsal aorta, and into the oesophagus and

stomach as mentioned above. Each peptide was injected into sham controls (175.9±11.2 g, n=7) and the AP-lesioned (APx) eels (197.2 ±4.7 g, n=7) as mentioned above at a dose of 1.0 nmol kg^{-1} for i.a. injection and 0.3 nmol per eel for i.c.v. injection.

Histological analysis

To confirm the AP lesioning, the brain was taken out after the experiment and fixed in Bouin's solution. After more than 24 h, the medulla oblongata was isolated and embedded in Paraplast Plus (McCormick Scientific, St Louis, MO, USA). Serial cross-sections were cut at 10 µm and stained with haematoxylin–eosin.

Statistical analyses

Changes in drinking rate after injection of each peptide were compared with controls by Steel's test or Wilcoxon's signed rank sum test. The differences of the effects among peptides at each dose were compared by the Steel–Dwass test. Changes in P_{VA} and P_{DA} after peptide injection were compared with controls by Dunnet' test and among peptides by Tukey's test. Changes after AP lesioning were compared with those of sham controls by Student's t-test. Significance was determined at P<0.05. All results were expressed as means±s.e.m.

RESULTS

Effects of i.a. peptide injection

After i.a. injection of isotocin, drinking rate increased within 10 min and remained at an elevated level for 60 min (Fig. 1A). The increase was dose dependent and reached a maximum of 130% at 0.3 nmol kg^{-1} (Fig. 1C). Oxytocin had a similar effect, but vasotocin slightly decreased drinking rate for some time at all doses (Fig. 1A,C). At the highest dose of vasotocin, however, drinking rate gradually increased more than 30 min after injection. In contrast to isotocin, AngII had no effect on drinking at doses lower than 0.3 nmol kg^{-1}, and reduced it for more than 1 h at 1.0 nmol kg^{-1} (Fig. 1A,C).

All peptides examined in this study increased P_{VA} similarly at 1.0 nmol kg^{-1}, and the effect of isotocin and vasotocin continued longer than that of AngII (Fig. 1B). However, the vasopressor effects of neurohypophysial hormones were much smaller at the dorsal aorta (P_{DA}), particularly that of isotocin (and oxytocin), compared with that of AngII (Fig. 1B). The effects of all hormones on P_{VA} were dose dependent and the effect of vasotocin was smaller than those of isotocin and AngII (Fig. 1D). The effect of AngII on P_{DA} was dose dependent, but isotocin failed to increase P_{DA} even at the highest dose (Fig. 1E). Vasotocin increased P_{DA} only slightly compared with AngII, but the effect was dose dependent at 0.1–1.0 nmol kg^{-1}. All hormones increased heart rate in a dose-dependent manner, and isotocin (and oxytocin) exhibited the greatest effect (Fig. S1A).

Effect of i.c.v. peptide injection

In contrast to i.a. injections, the i.c.v. injection of isotocin had no effect on drinking rate, but AngII increased it for more than 1 h after injection (Fig. 2A). The stimulatory effect of AngII was dose dependent at 0.03–0.3 nmol per eel (Fig. 2C). Vasotocin was inhibitory for drinking in a dose-dependent manner (Fig. 2A,C).

The i.c.v. injection of isotocin had no effect on P_{VA} and P_{DA}, but AngII equally increased both parameters in a dose-dependent manner (Fig. 2B,D,E). Vasotocin and oxytocin increased P_{VA} at 0.3 nmol per eel, but had no effects on P_{DA}. In contrast to i.a. injections, i.c.v. injection of hormones had little effect on heart rate except for an increase after the highest dose of isotocin (Fig. S1B).

Effect of AP ablation

The dipsogenic effect of i.a. isotocin was completely abolished by AP lesioning (APx eels) compared with sham controls (Fig. 3A, Table 1). The i.a. injection of AngII decreased drinking rate in sham controls and the initial decrease became more prominent in APx eels, suggesting the disappearance of the initial transient increase after AP ablation (Fig. 3B, Table 2). Heart rate, P_{VA} and P_{DA} were not influenced by AP lesioning (Table 2). The i.a. injection of vasotocin induced a delayed increase in drinking rate in sham controls as in intact fish, but this effect disappeared in APx eels (Fig. 3C, Table 2).

In contrast to i.a. injection of isotocin, the dipsogenic effect of i.c.v. AngII was not influenced by AP lesioning (Fig. 3D, Table 1) and nor were AngII effects on heart rate, P_{VA} and P_{DA} (Table 1). The AP ablation was histologically confirmed after experiments (Fig. 4).

DISCUSSION

With respect to water regulation, marine teleosts are similar to terrestrial animals because both live in dehydrating environments. Thus, they must compensate for water loss by oral drinking, which is essential for water homeostasis in both groups. It is generally accepted that AngII is the primary hormone that induces drinking for water acquisition throughout vertebrate species (Fitzsimons, 1998; Kobayashi and Takei, 1996). However, there are obvious differences in the accessibility to water between the two groups: marine teleosts can obtain water whenever needed simply by reflex swallowing of environmental SW, whereas terrestrial animals must seek out water, motivated by thirst, before drinking. Therefore, the mechanisms inducing thirst are primary for terrestrial animals, while inhibitory mechanisms appear to have developed in fish to avoid over-drinking (Takei, 2002, 2015). Consistently, the dipsogenic potency of AngII is much greater in terrestrial animals than in fish. In fact, only brief drinking was induced at doses higher than 1.0 nmol kg^{-1} (the highest dose used in this study), and this transient drinking was followed by a long inhibition in this study (see also Takei et al., 1979). Another interesting difference in body fluid regulation is that marine teleosts have developed ion-extruding mechanisms to cope with excess ion uptake by drinking SW, while terrestrial animals have developed mechanisms to decrease water loss by the kidney. Vasopressin/vasotocin is the most important hormone to decrease renal water loss through terrestrial animals' kidneys (Nishimura and Fan, 2002; Dantzler and Braun, 1980; Braun and Dantzler, 1997; Nielsen et al., 2002) and through the lungfish kidney under terrestrial conditions (Konno et al., 2009; 2010a). Oxytocin/isotocin is known as an ion-extruding hormone in mammals (Balment et al., 1980; Conrad et al., 1993) and in teleost fishes (Guibbolini and Avella, 2003). In addition, amphibian teleosts, such as the mudskipper, drink water stored in the buccal and opercular cavities and migrate to water pools after i.c.v. injection of neurohypophysial hormones (Sakamoto et al., 2015; Katayama et al., 2018). Thus, neurohypophysial hormones are strong candidates for supporting fully aquatic teleost life in SW, but the data thus far obtained are somewhat conflicting (Takei and McCormick, 2013).

Effect on drinking

In the present study, we found that isotocin induced copious drinking for more than 1 h after injection at a dose as low as 0.1 nmol kg^{-1}, demonstrating that it is the most potent dipsogenic hormone identified thus far. In addition, vasotocin, a major water-retaining hormone in mammals, was rather antidipsogenic. To the best of our knowledge, this is the first study to identify a dipsogenic

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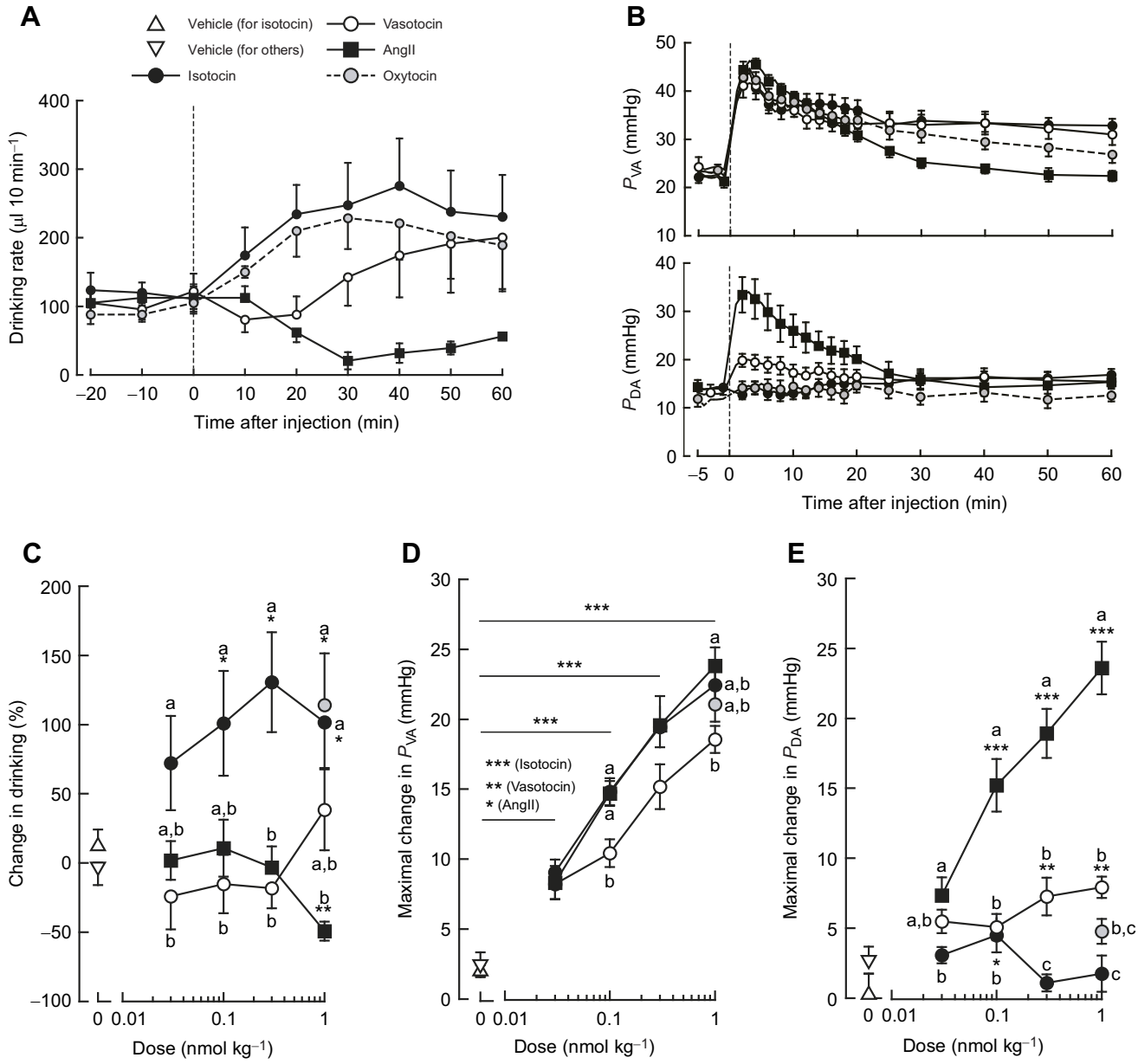

Fig. 1. Effects of intra-arterial (i.a.) injection of isotocin, vasotocin, angiotensin II (AngII) and oxytocin on drinking and cardiovascular function in conscious eels. (A,B) Time course of changes in drinking rate (A), ventral aortic pressure (P_{VA}) and dorsal aortic pressure (P_{DA}) (B) after injection of each peptide at 1.0 nmol kg^{-1} (n=8). (C–E) Dose–response relationship of drinking rate (C), P_{VA} (D) and P_{DA} (E) after injection of each peptide in which changes in drinking were expressed as the percentage change after injection and maximal changes were used for P_{VA} and P_{DA}. Asterisks indicate a significant difference between vehicle (control) and peptides (*P<0.05, **P<0.01, ***P<0.001). Different letters indicate a significant difference among peptides at each dose.

hormone that is more potent and efficacious than AngII in vertebrates.

The dipsogenic effect of isotocin was abolished by AP lesioning, indicating that circulating isotocin acts on the AP and probably causes reflex swallowing to initiate drinking. In the reflex swallowing system of fish, the glossopharyngeal–vagal motor complex (GVC) nuclei innervate drinking-related muscles in the pharynx and oesophagus, including the UOS muscle in eels (Mukuda and Ando, 2003). The catecholaminergic neurons in the AP send their axons to the GVC to suppress its activity (Ito et al., 2006), and the cholinergic neurons in the GVC innervate the UOS muscle to constrict it (Mukuda and Ando, 2003; Ando et al., 2013). Thus, it is possible that isotocin stimulates the AP neurons to inhibit GVC neurons, resulting in relaxation of the UOS muscle for water ingestion. Although the presence of isotocin receptors has not yet been demonstrated in the AP neurons, this study confirms that, in teleosts, the AP is an important component in the neural network for regulation of drinking (Nobata and Takei, 2011). The action of peripheral oxytocin on the AP has been consistently suggested in rats and mice (Morton et al., 2012; Maejima et al., 2015; Jurek and Neumann, 2018).

In contrast to peripheral injection, i.c.v. injection of isotocin into the third ventricle did not enhance drinking in eels. As the cerebrospinal fluid (CSF) is constantly stirred and transported caudally by cilia of the ventricular surface of ependymal cells (Weindl and Joynt, 1972; McKinley et al., 2003a,b), we anticipated that isotocin injected into the CSF would induce swallowing through action at the AP located near the fourth ventricle. The

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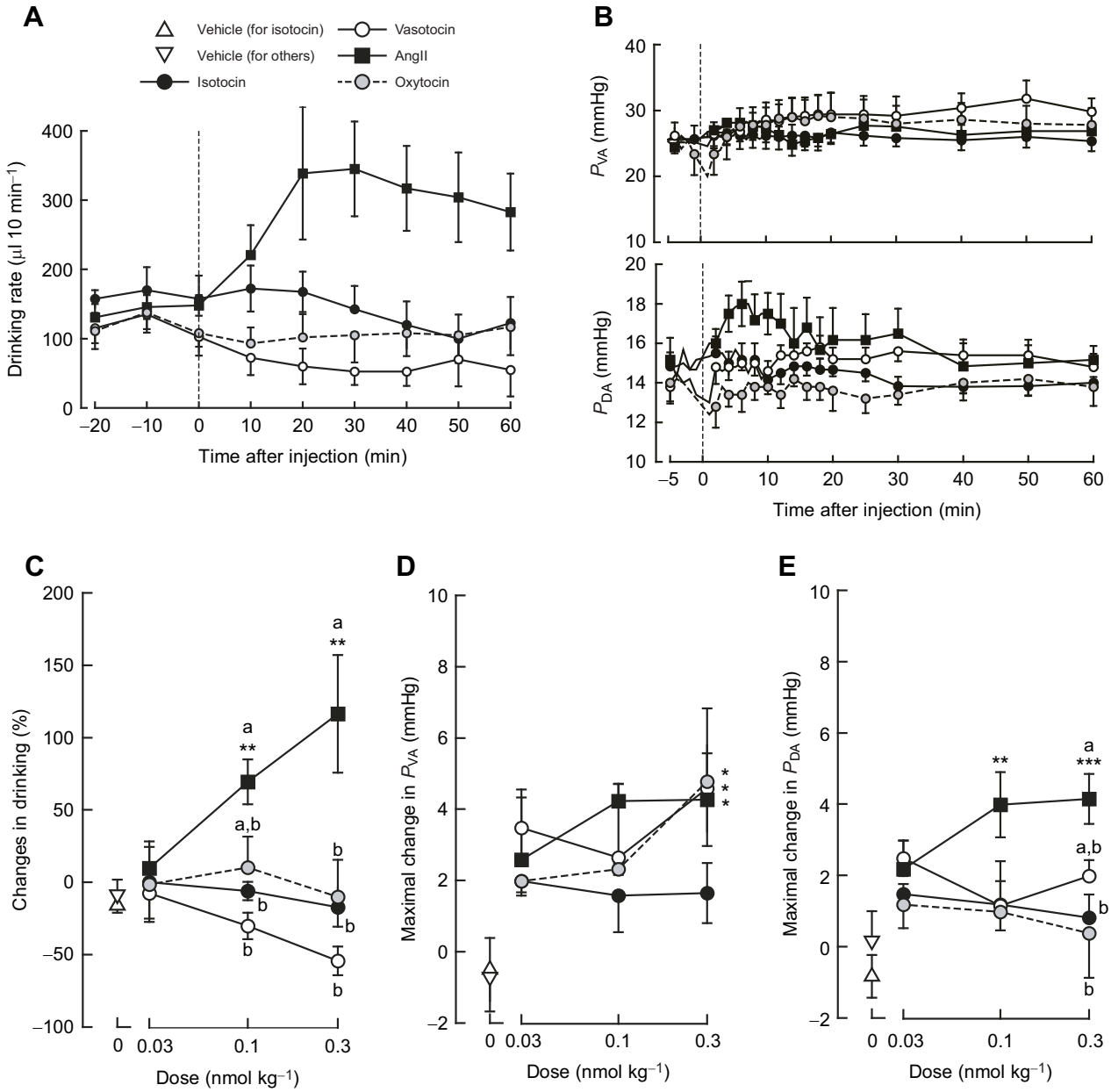

Fig. 2. Effects of intra-cerebroventricular (i.c.v.) injection of isotocin, vasotocin, AngII and oxytocin on drinking and cardiovascular function in conscious eels. (A,B) Time course of changes in drinking rate (A), P_{VA} and P_{DA} (B) after injection of each peptide at 0.3 nmol per eel (n=8). (C–E) Dose–response relationship of drinking rate (C), P_{VA} (D) and P_{DA} (E) after injection of each peptide as in Fig. 1. Asterisks indicate a significant difference between saline and peptides (*P<0.05, **P<0.01, ***P<0.001). Different letters indicate a significant difference among peptides at each dose.

absence of this anticipated action may be a result of the inability of isotocin to cross the CSF–brain barrier via ependymal cells of the AP. In support of this, circulating AngII is dipsogenic through the AP (Nobata and Takei, 2011) but AngII injected into the forth ventricle is not dipsogenic (Ogoshi et al., 2008). Further, the current study showed that drinking induced by AngII injected into the third ventricle was not affected by AP lesioning. Thus, AngII seems not to cross the CSF–brain barrier of the AP to induce swallowing. As vasotocin/isotocin fibres from the preoptic area project widely in the brain of trout in addition to the neurohypophysis (Saito et al., 2004), intrinsic isotocin in the brain may also be involved in the regulation of drinking in teleost fishes.

In this study, transient stimulation of drinking by peripheral AngII was undetectable at 1.0 nmol kg^{-1} because of simultaneous baroreflexogenic inhibition of drinking. As the vasopressor action of AngII is mediated in part through adrenergic activation in American eels (Oudit and Butler, 1995b), it is possible that α-adrenergic blockade attenuates the baroreflexogenic inhibition of drinking. In a preliminary study, however, phentolamine failed to diminish the vasopressor effect and antidipsogenic effect of AngII (S.N., unpublished data). The result is consistent with the report that AngII did not increase plasma catecholamine in American eels (Bernier et al., 1999). Our previous study showed that 5.0 nmol kg^{-1} of AngII is required to elicit transient stimulation of drinking and that stimulation is mediated by the AP in eels (Nobata and Takei, 2011). As the inhibition by 1.0 nmol kg^{-1} of AngII was exaggerated after AP lesioning (Fig. 3B), it is apparent that transient stimulation occurred at this dose but it was too small to override the inhibition.

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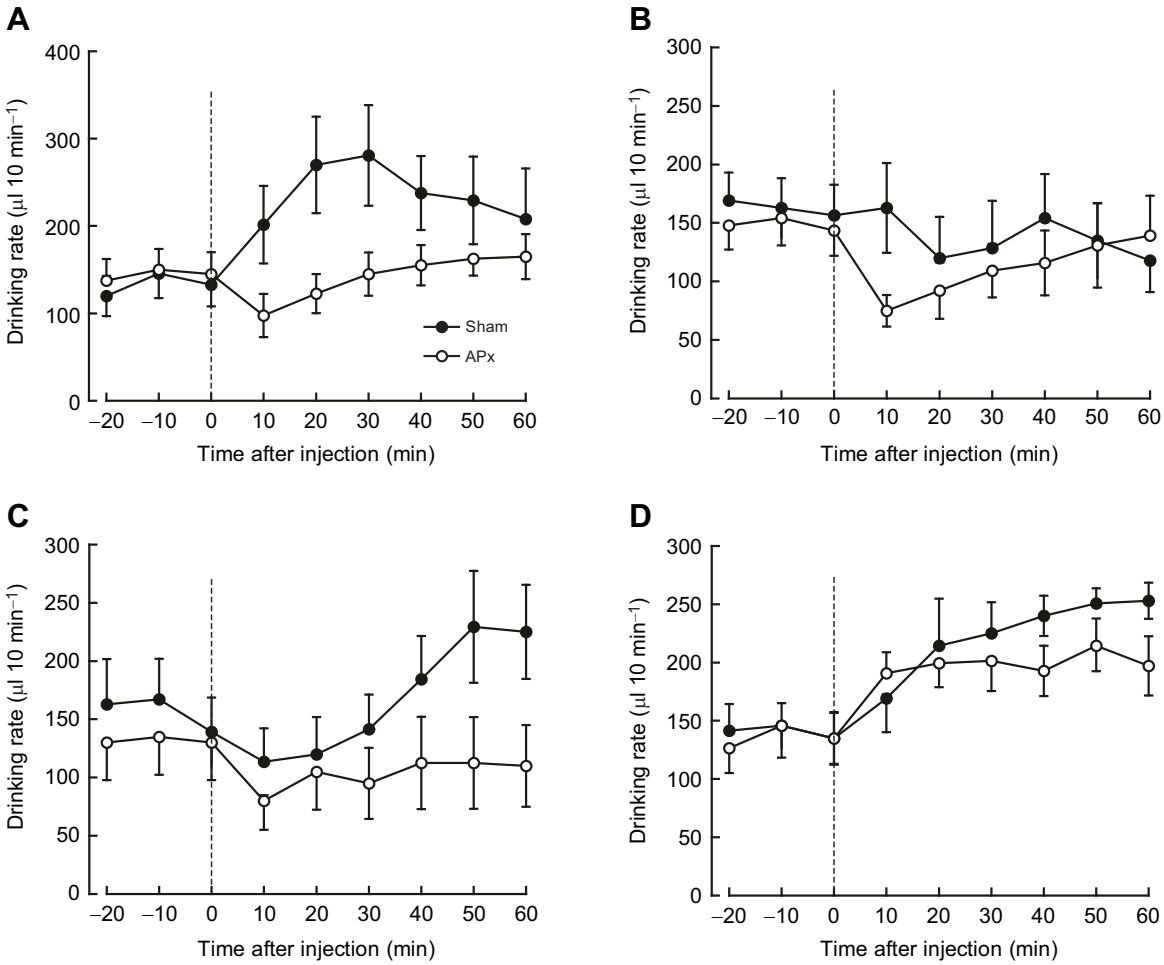

Fig. 3. Effects of area postrema (AP) ablation on hormone actions in conscious eels. Time course of changes in drinking rate after i.a. injection of isotocin (A), AngII (B) and vasotocin (C) at 1.0 nmol kg^{-1} and i.c.v. injection of AngII (D) at 0.3 nmol per eel into sham controls and AP-lesioned (APx) eels (n=7 in each group).

In contrast to the results seen with peripheral injection, AP lesioning failed to block the dipsogenic action of i.c.v. AngII. The site of action of AngII is not known, but it is possible that it acts on the OVLT-like structure recently identified in the AV3V region of the eel brain (Kozaka et al., 2003; Mukuda et al., 2013). AngII acts on the OVLT and/or the median preoptic nucleus (MnPO) in mammals and birds (Johnson and Thunhorst, 1997; Kobayashi and Takei, 1996). Consistently, AngII injected into the fourth ventricle did not enhance drinking in eels (Ogoshi et al., 2008). In mammals, the MnPO angiotensinergic neurons project their fibres to the supraoptic nucleus and paraventricular nucleus, and AngII can stimulate vasopressin secretion from these nuclei (Mckinley et al., 2003a,b). However, it is unlikely that i.c.v. AngII enhanced drinking through isotocin secretion because APx eels still displayed the dipsogenic response to i.c.v. AngII. The AP is responsible for enhanced drinking in dehydrated SW eels (Nobata and Takei, 2011), but not for i.c.v. AngII-induced drinking in this study. Therefore, CSF-borne AngII is not involved in the upstream signals that are transmitted to the AP in the signal pathway of drinking to compensate for dehydration in SW. In the amphibian goby, i.c.v. injection of AngII induced swallowing and then indirectly stimulated local thirst in terrestrial conditions, but did not upregulate expression in the OVLT of the immediate early gene *c-fos* (Katayama et al., 2018). Although the nuclei activated by i.c.v. AngII remain unknown, the local thirst induced by i.c.v. AngII in the terrestrial goby may be induced through a different pathway

Table 1. Changes in drinking rate, heart rate and ventral and dorsal aortic pressure (P_{VA} and P_{DA}) after intra-arterial (i.a.) injection of isotocin and intra-cerebroventracular (i.c.v.) injection of angiotensin II in sham controls (n=7) and area postrema-lesioned (APx) eels (n=7)

Peptide	Group	Drinking rate (%)	Heart rate (beats min^{-1})	P_{VA} (mmHg)	P_{DA} (mmHg)
Isotocin (1.0 nmol kg^{-1} i.a.)	Sham	73.6±21.5	11.5±1.5	17.7±1.4	−1.1±1.5
	APx	2.3±10.4*	7.5±3.5	22.8±2.2	1.0±1.3
Angiotensin II (0.3 nmol per eel i.c.v.)	Sham	77.8±22.7	0.1±3.8	5.4±3.7	2.4±0.6
	APx	71.0±29.4	−1.3±2.2	5.0±1.3	3.0±1.3

Data are means±s.e.m. Changes in average drinking rate (μl per 30 min) for the 60 min after injection are presented as a percentage of the drinking rate over the 30 min before injection. Drinking rate was compared between sham controls and APx eels by Mann–Whitney U-test (*P<0.01). Heart rate, P_{VA} and P_{DA} are represented as the maximum change, and were compared between sham controls and APx eels by Student's t-test.

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Table 2. Changes in drinking rate, heart rate, P_{VA} and P_{DA} after i.a. injection of angiotensin II and vasotocin in sham controls (n=7) and APx eels (n=7)

Peptide	Group	Drinking rate (%) 0–30 min	Drinking rate (%) 30–60 min	Heart rate (beats min^{-1})	P_{VA} (mmHg)	P_{DA} (mmHg)
Vasotocin (1.0 nmol kg^{-1} i.a.)	Sham	−14.3±13.4	40.6±10.5	4.0±9.0	12.9±1.8	4.7±1.0
	APx	−27.3±10.7	−11.5±17.3*	11.0±1.0	18.0±2.1	6.5±1.4
Angiotensin II 1.0 nmol kg^{-1} i.a.)	Sham	−26.8±20.8	−25.1±13.1	9.5±2.5	15.4±3.2	17.4±3.1
	APx	−40.2±10.2	−18.2±11.5	6.0±2.0	18.3±1.7	18.2±1.8

Changes in drinking rate 0–30 min and 30–60 min after injection are presented as a percentage of that for the 30 min before injection. Drinking rate was compared between sham controls and APx eels by Mann–Whitney U-test (*P<0.05). Heart rate, P_{VA} and P_{DA} are presented as the maximum change and were compared between sham controls and APx eels by Student's t-test (*P<0.05).

from that for the reflex swallowing induced by i.c.v. AngII in fully aquatic eels.

Vasotocin decreased drinking for some time after injection but increased it thereafter at high doses. The initial inhibition may be due to the greater increase in P_{DA} after i.a. vasotocin than with i.a. isotocin. The delayed enhancement of drinking was abolished by AP ablation. It seems that vasotocin is basically antidipsogenic but mimics the isotocin effect at high doses. Ligand selectivity of fish vasotocin and isotocin receptors is generally low compared with that of mammalian receptors (Morel et al., 1992; Kimura et al., 1992; Birnbaumer et al., 1992; Lema, 2010; Yamaguchi et al., 2012). However, as vasotocin concentration in plasma does not increase even in extreme dehydration in eels (S.N., unpublished data), vasotocin-induced drinking may not be significant in the natural state to enhanced drinking in dehydrated SW eels (Nobata and Takei, 2011).

In drinking of SW teleosts, it is likely that the AP is a key nucleus because dehydrated SW-acclimated eels fail to drink water after AP lesioning (Nobata and Takei, 2011). Among potent dipsogens in eels, namely peripheral isotocin and central AngII, isotocin induced drinking through the AP but AngII did not. Also, isotocin relaxes the UOS by directly acting there, resulting in stimulation of drinking, and this action is inhibited by the oxytocin receptor antagonist H-9405 (Watanabe et al., 2007). As the dipsogenic effect

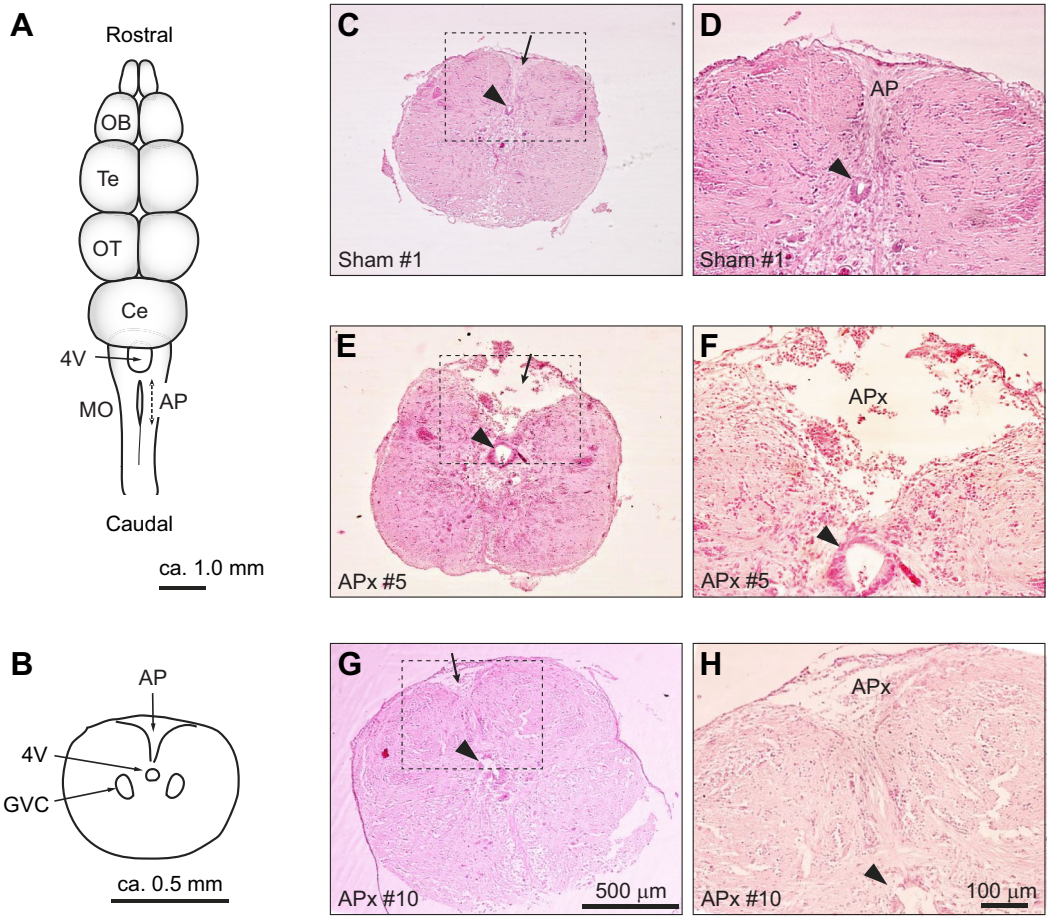

Fig. 4. Typical cross-sections of the medulla oblongata (MO) in sham controls and APx eels. (A,B) Schematic representation of eel whole brain from the dorsal side (A) and cross-section at the MO (B). (C,D) Cross-section of the MO of sham control (C) and an enlarged view of the boxed region in C (D). (E–H) Cross-sections of the MO of APx eels (E,G) and corresponding enlarged views of the boxed regions (F,H). OB, olfactory bulb; Te, telencephalon; OT, optic tectum; Ce, cerebellum; AP, area postrema; 4V, forth ventricle; GVC, glossopharyngeal–vagal motor complex.

of isotocin was completely diminished by AP lesioning in this study but not inhibited by the antagonist (S.N., unpublished data), the direct action on the UOS is likely to be minor in the dipsogenic effect of circulating isotocin. These findings suggest that isotocin is stronger as a candidate hormone for the regulation of drinking than is AngII, which is evidently different from the pattern seen in terrestrial animals. Probably, this is due to the difference in accessibility to water and the necessity for thirst arousal between two groups.

Cardiovascular effects

AngII increased P_{VA} and P_{DA} equally, but isotocin elevated only P_{VA} in this study. As the circulatory system in teleosts consists of the heart, ventral aorta, gill circulation, dorsal aorta and systemic circulation connected in series, the increase of only P_{VA} illustrates that isotocin selectively constricted the resistant vessels in the gill circulation. The increase in arterial pressure usually decreases drinking rate via a baroreceptor-mediated reflex in eels (Hirano and Hasegawa, 1984). Indeed, urotensin II, a potent vasopressor hormone for both P_{VA} and P_{DA}, strongly inhibits drinking in eels and the inhibition was not abolished by AP lesioning (Nobata et al., 2011; Nobata and Takei, 2011). Similar increases in both P_{VA} and P_{DA} after AngII also accompanied inhibition of drinking. These observations indicate that the baroreceptor for inhibition of drinking is located distal to the gill circulation. Consistently, i.c.v. AngII enhanced drinking without apparent inhibition of drinking, which induced only a slight increase in P_{DA} compared with the profound increase after i.a. injection.

The gill circulation consists of an arterio-arteriolar pathway with high pressure and flow rate for respiration, and an arterio-venous pathway with low pressure and flow rate for ionoregulation and acid–base regulation, with the pressure and flow for these pathways being modulated mainly by a sphincter in the arterio-arteriolar pathway (Laurent and Dunel, 1980; Evans et al., 2005). Isotocin increased only P_{VA} by constricting the resistance vessels in the gill circulation. Thus, isotocin seems to restrict the arterio-arteriolar pathway and increase blood flow to the arterio-venous pathway, as does vasotocin (Oudit and Butler, 1995a), with possibly a resultant increase in Na^+ and Cl^- excretion by SW-type ionocytes (Evans et al., 2005). Isotocin may be able to bind the ionocytes in the same way as vasotocin in eels (Guibbolini et al., 1988), and to stimulate Cl^- excretion by gill respiratory cells with higher potency than vasotocin does in the sea bass (Guibbolini and Avella, 2003). Together with the enhancement of drinking, the increased ion excretion across the gills is beneficial for SW acclimation of teleosts.

Conclusion

Circulating isotocin is involved not only in ion excretion but also in water acquisition through enhanced drinking. Thus, it is a candidate for a dipsogenic hormone that is essential for body fluid homeostasis in SW teleosts. The unique dipsogenic potency of isotocin in teleosts may be a result of their aquatic life, as isotocin acts specifically on the induction of reflex swallowing. Plasma levels, storage in the pituitary and mRNA levels in the hypothalamus are almost the same in sea bream under hyper- and hypo-osmotic conditions (Kleszczyńska et al., 2006; Martos-Sitcha et al., 2013). AP lesioning abolishes the effects of several factors, such as hormones and osmotic stimuli, on drinking, while APx eels can increase drinking volume after SW transfer to almost the same levels as controls (Nobata and Takei, 2011). Transient receptor potential vanilloid 4 (TRPV4) modulates ion balance through the isotocinergic neurons in the hypothalamus in zebrafish (Liu et al., 2020). Thus, isotocin may be transiently secreted through the pathway originated from sensing by osmosensors, such as TRPV4, and fine-tune drinking volume through the AP. Consistently, drinking volumes are a little bit lower in SW-acclimated APx eels than in sham controls, resulting in higher osmolality in the APx eels (Nobata and Takei, 2011). Our results highlight the important roles that neurohypophysial hormones (vasopressin/oxytocin lineage) play in body fluid regulation throughout vertebrate species.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.N., Y.T.; Methodology: S.N.; Validation: S.N.; Formal analysis: S.N.; Investigation: S.N.; Resources: S.N.; Data curation: S.N.; Writing - original draft: S.N.; Supervision: Y.T.; Project administration: Y.T.; Funding acquisition: Y.T.

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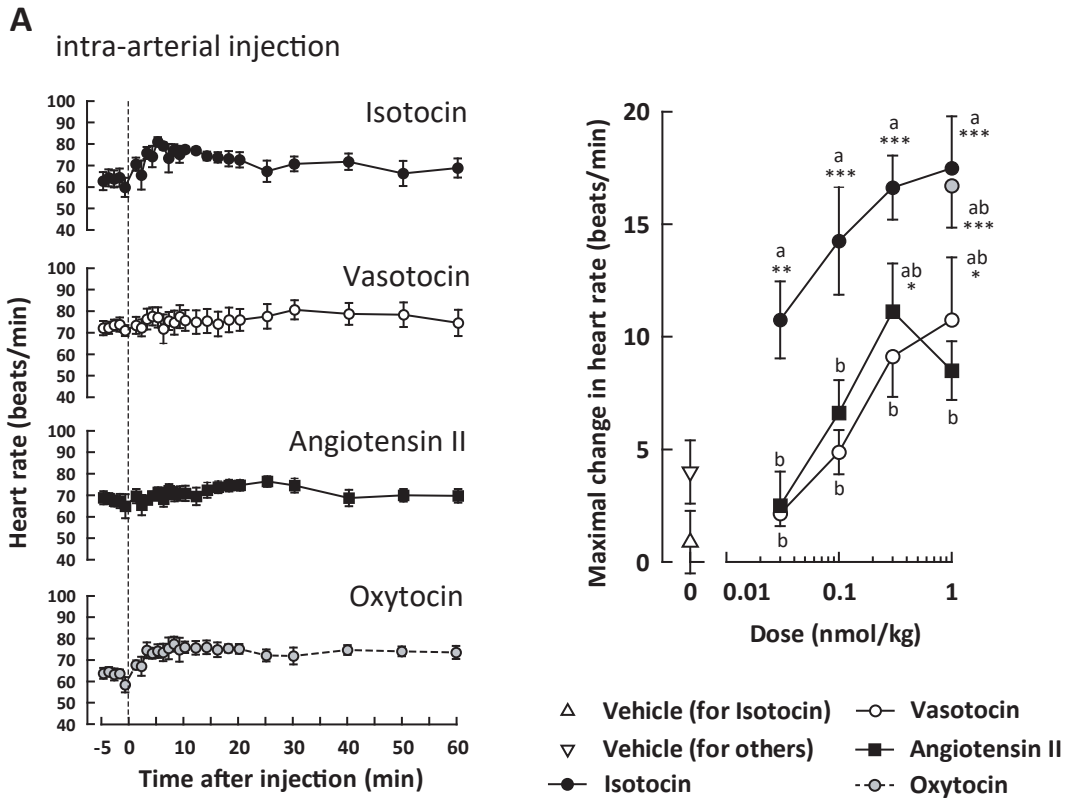

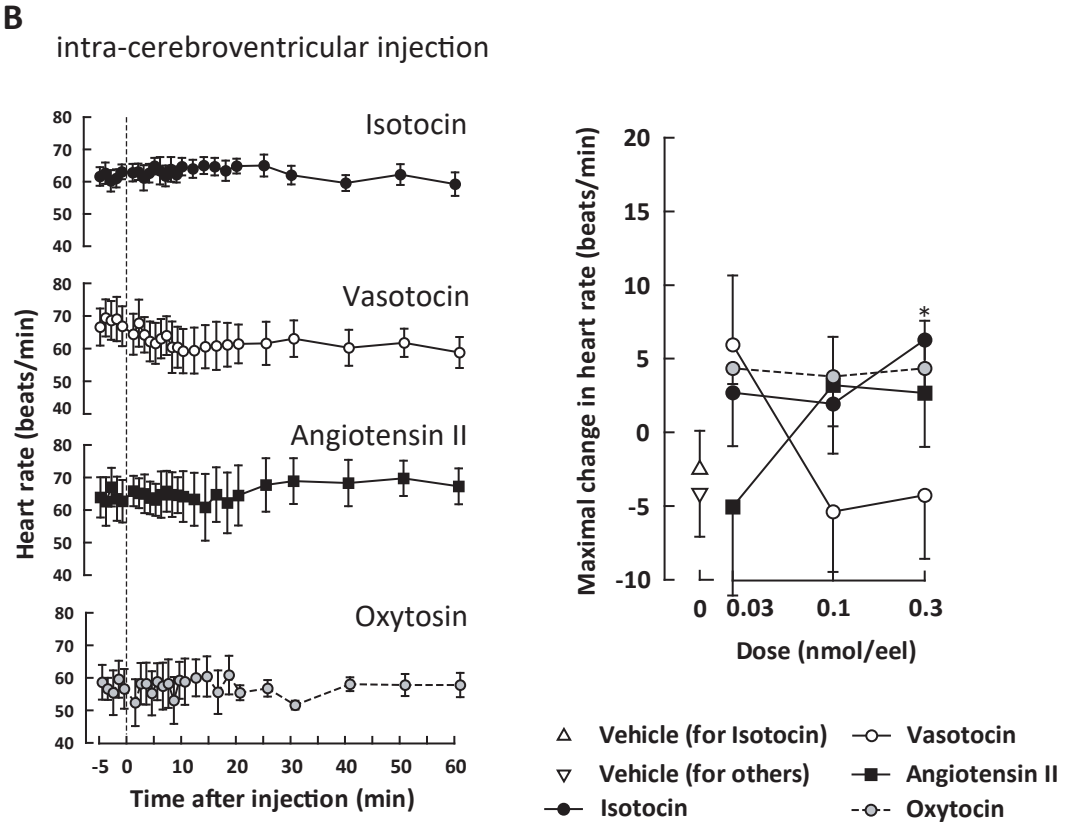

Fig. S1. Effects of isotocin, vasotocin, angiotensin II (AngII) and oxytocin injected intra-arterially (i.a.) (A) and intra-cerebroventricularly (i.c.v.) (B) on heart rate in conscious eels. (A) Time course changes in heart rate after i.a. injection of each peptide at 1.0 nmol/kg (n=8) (left) and the dose-response relationship at 0.03-1.0 nmol/kg (n=8) (right). (B) Time course changes in heart rate after i.c.v. injection of each peptide at 0.3 nmol/eel (n=8) (left) and the dose-response relationship at 0.03-0.3 nmol/eel (n=8) (right). Difference between saline and peptides was represented by asterisk ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Difference among peptides at each dose was represented by letters, which mark sample groups that are not significantly different from one another.