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, but only isotocin induced copious drinking. The dipsogenic effect was dose-dependent and occurred significantly at as low as 0.1 nmol/kg. By contrast, a sustained inhibition of drinking occurred after AngII, probably due to baroreflexogenic inhibition. No such inhibition was observed after isotocin despite similar

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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/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-Nielsen, 1997). In terrestrial vertebrates, water is lost through evaporation from the skin and respiration, and in the urine and feces. 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

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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, 2008). 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 is devoid of blood-brain barrier (BBB) in the forebrain (Simpson and Routenberg, 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, water-taking into the mouth and swallowing. In fishes, however, drinking can occur simply by reflex swallowing since water is always in their buccal cavity for respiration (Hirano et al., 1972). The site of action of circulating hormone for regulation of drinking appears to be the area postrema (AP), another sensory circumventricular without BBB in the hindbrain (Morita et al., 1986; Mukuda et al., 2005), as lesioning of the AP abolished 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 (Kobayashi and Takei 1996; Johnson and Thunhorst, 1997; Fitzsimons, 1998; McKinley et al., 2003). However, even

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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 by its antidiuretic action in the mammalian, avian and reptilian kidney (Nishimura, 2002; Danzler 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 retaintion is expressed in the kidney of the lungfish only in the terrestrial condition (Konno et al., 2009, 2010a). Thus, vasopressin/vasotocin seems to be a key hormone that enabled the animals to survive in water-deficient terrestrial environment. In teleosts, however, the effect of vasotocin on water retention is conflicting. Plasma vasotocin concentration increased transiently after hyperosmotic challenges 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 induced pressure diuresis, but it induces glomerular antidiuresis in the perfused trunk preparation where arterial pressure was maintained constant (Amer and Brown, 1995). The expression of 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., 1995). In teleosts, isotocin, an ortholog 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

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adaptation and thus elicitation of drinking in teleost species. In the amphibious mudskipper goby, indeed, mRNA expression of vasotocin and isotocin precursors in the brain increase under terrestrial conditions, and intra-cerebroventricular injection of both peptides promote migration to a seawater pool from land (Sakamoto et al., 2015)

In this study, we examined the possible involvement of neurohypophysial hormones, vasotocin and isotocin, in regulation of drinking in eels. An earlier report showed that vasopressin and oxytocin inhibited drinking with simultaneous increase in arterial pressure in eels (Hirano and Hasegawa, 1984). More recent data showed that isotocin relaxed the upper oesophageal sphincter (UES) muscle to increase drinking but vasotocin constricted the UES 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 injections using conscious eels with cannulas in the dorsal and ventral aortas 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*, were purchased from a local dealer. They were acclimated in SW (natural SW; about 33 psu) tanks without feeding for more than two weeks before use. Water in the tanks was filtered, aerated and maintained at 18ºC. 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

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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 M acetic acid (for isotocin) at a concentration of more than 10^{-4} M, and subsequently diluted with isotonic 0.9% NaCl (saline) as necessary before injection.

Effects of intra-arterial injection

SW-acclimated eels (170.4 ± 4.8 g, n = 8) were anesthetized in 0.1% (w/v) tricaine methanesulfonate (Sigma, St. Louis, MO, USA) for 15 min, and polyethylene tubes (OD: 0.8 mm) were surgically-inserted into the ventral and dorsal aortae for blood pressure measurement and injections. Vinyl tubes (OD: 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 (Nobata and Takei, 2011). After surgery, eels were placed in a plastic trough of their size, through which aerated water circulated at 18ºC. The outflow of 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 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 was recorded by a water balance monitoring system (MTS00658, Medical Try System, Tokyo, Japan).

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Peptide injections were started 3 days following surgery at 0.03, 0.1, 0.3 and 1.0 nmol/kg for isotocin, vasotocin and AngII and 1.0 nmol/kg for oxytocin in saline into the dorsal aorta. Each injection was immediately followed by a flush of 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 controls.

Effects of intra-cerebroventricular injection

SW-acclimated eels (184.9 ± 8.6 g, n = 8) were anesthetized, and cannulas were inserted as above with an additional one into the cerebral ventricle according to the published protocols (Nobata et al., 2011). Briefly, a stainless-steel guide cannula (ID: 0.35 mm, OD: 0.6 mm) was implanted into the third ventricle to a depth of 0.7 mm from the surface of the brain. An injector (OD: 0.3 mm) was connected to the polyethylene tube (ID: 0.28 mm, OD: 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 /eel. The same volume of saline injections served as controls.

Effect of area postrema (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 (approx. 0.5 mm diameter) was made in the skull by a drill (Nakanishi, Tochigi, Japan) and then in the exposed part of the arachnoid membrane by a 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 the lesioning, the hole was filled with Spongel (Yamanouchi,

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Tokyo, Japan) and sealed with Bone Wax (Lukens, Albuqueque, 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 for i.a. injections and 0.3 nmol/eel for i.c.v. injections.

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 injections 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 Steel-Dwass test. Changes in P_{VA} and P_{DA} after peptide injections were compared with controls by Dunnet test and among peptides by Tukey's test. Changes after APx were compared with those of sham controls by Student's *t*-test. Significance was determined at $P < 0.05$. All results were expressed as mean ± SEM.

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RESULTS

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

After i.a. injection of isotocin, drinking rate increased within 10 min and continued for 60 min (Fig. 1A). The increase was dose-dependent and reached maximum of 130% at 0.3 nmol/kg (Fig. 1C). Oxytocin had a similar effect, but vasotocin slightly decreased drinking rate for some time at all doses (Figs. 1A and 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, and reduced it for more than 1 h at 1.0 nmol/kg (Figs. 1A and C).

All peptides examined in this study increased ventral aortic pressure (P_{VA}) similarly at 1.0 nmol/kg, 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 it 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. All hormones increased heart rate in a dose-dependent manner, and isotocin (and oxytocin) exhibited the greatest effect (Supplemental Fig. S1A).

Effect of intracerebroventricular (i.c.v.) injections

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/eel (Fig. 2C). Vasotocin was inhibitory for drinking at a dose-dependent manner (Figs. 2A and C).

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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 (Figs. 2B, D and E). Vasotocin and oxytocin increased P_{VA} at 0.3 nmol/eel, but have no effects on P_{DA}. In contrast to i.a. injections, the i.c.v. injection of hormones had little effect on heart rate except for the increase after highest dose of isotocin (Supplemental Fig. S1B).

Effect of area postrema (AP) ablation

The dipsogenic effect of i.a. isotocin was completely abolished by AP lesioning (APx) 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 initial transient increase after AP ablation (Fig. 3B, Table 2). Heart rate, P_{VA} and P_{DA} were not influenced by the AP lesioning (Table 2). Vasotocin induced delayed increase in drinking rate in sham controls as in intact fish, but it disappeared in APx eels (Fig. 3C, Table 2).

In contrast to the i.a. injection of isotocin, the dipsogenic effect of i.c.v. AngII was not influenced by APx (Fig. 3D, Table 1) as are the 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

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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 for water, motivated by thirst before drinking. Therefore, the mechanisms inducing thirst are primary for terrestrial animals, while inhibitory mechanisms appear to have developed in fishes to avoid overdrinking (Takei, 2002; Takei, 2015). Consistently, dipsogenic potency of AngII is much greater in terrestrial animals than in fishes. In fact, only a brief drinking was induced at doses higher than 1.0 nmol/kg (the highest dose used in this study), and the transient drinking was followed by a long inhibition as observed in this study (Takei, 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, 2002; Danzler 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).

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Effect on drinking

In the present study, we found that isotocin induces copious drinking for more than one hour after injection at a dose as low as 0.1 nmol/kg, 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 hormone that is more potent and efficacious than AngII in vertebrates.

The dipsogenic effect of isotocin was abolished by APx, indicating that circulating isotocin acts on the AP and probably causes reflex swallowing to initiate drinking. In the reflex swallowing system of fishes, the glossopharyngeal-vagal motor complex (GVC) nuclei innervate drinking-related muscles in the pharynx and oesophagus, including the UES 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 UES 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 UES muscle for water ingestion. Although the presence of isotocin receptors has not yet been demonstrated in the AP neurons, this study confirms again that in teleosts, the AP is an important component in the neural network for regulation of drinking (Nobata and Takei, 2011). Consistently, the action of peripheral oxytocin on the AP is suggested in rats an mice (Morton GJ et al., 2012; Maejima et al., 2014; 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., 2003), we anticipated that isotocin injected into the CSF would induce swallowing through action at the AP located near the fourth ventricle. Absence of the

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anticipated action may be due to the inability of isotocin to cross the CSF-brain barrier via ependymal cells of the AP. Supportively, 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 APx. 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 neurophypophysis (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 because of simultaneous baroreflexogenic inhibition of drinking. As vasopressor action of AngII is mediated in part through an adrenergic activation in American eels (Oudit and Butler, 1995b), it is possible that α-adrenergic blockade attenuates the baroreflexogenic inhibition of drinking. In preliminary study, however, phentolamine failed to diminish the vasopressor effect and antidipsogenic effect of AngII (S. Nobata, 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 of AngII is required to detect the transient stimulation of drinking and the stimulation is mediated by the AP in eels (Nobata and Takei, 2011). As the inhibition by 1.0 nmol/kg of AngII was exaggerated after APx (Fig. 3C), it is apparent that transient stimulation occurred at this dose but it was too small to override the inhibition.

In contrast to the results seen with peripheral injection, APx 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 (Mukuda et al., 2013). AngII acts on the OVLT and/or the median preoptic nucleus (MnPO) in mammals and

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bird (Johnson et al., 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., 2003). However, it is unlikely that i.c.v. AngII enhanced drinking through isotocin secretion because APx eels still display 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. At least, 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 the expression in the OVLT of the immediate early gene c-fos (Katayama et al., 2018). Although the nuclei activated by i.c.v. AngII remains unknown, the local thirst induced by i.c.v. AngII in the terrestrial goby may be induced through a different pathway 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 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; Yamaguch et al., 2012). However, as vasotocin concentration in plasma does not increase even in extreme dehydration in eels (S. Nobata, unpublished data), vasotocin-induced drinking may not be significant in the natural state to enhanced drinking in dehydrated SW eels (Nobata and Takei, 2011).

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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 the 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 UES by directly acting there, resulting in stimulation of drinking, and this action is inhibited by the antagonist H-9405 (oxytocin receptor antagonist) (Watanabe et al., 2007). As the dipsogenic effect of isotocin is completely diminished by AP-lesioning in this study but is not inhibited by the antagonist (S. Nobata, unpublished data), the direct action on the UES is likely minor in the dipsogenic effect of circulating isotocin. These findings suggest that isotocin is stronger as a candidate hormone for regulation of drinking than is AngII, which is evidently different from pattern seen in terrestrial animals. Probably, it is due to the difference in accessibility to water and the necessary of thirst arousal between two groups.

Cardiovascular effects

AngII increased P_{VA} and P_{DA} equally, but isotocin elevated only P_{VA} in this study. Since 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, 1983). Indeed, urotensin II, a potent vasopressor hormone at both P_{VA} and P_{DA}, strongly inhibits drinking in eels and the inhibition was not abolished by APx (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

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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 on 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 have restricted the arterio-arteriolar pathway and increased blood flow to the arterio-venous pathway as does vasotocin (Oudit and Butler, 1995a), with possibly resultant increase in Na^{+} and Cl^{-} excretion by SW-type ionocytes (Evans et al., 2005). Isotocin may be able to bind the ionocytes as does vasotocin in eels (Guibbolini et al., 1988), and to stimulate Cl^{-} excretion by gill respiratory cells in 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 dipsogenic hormone that is essential for body fluid homeostasis in SW teleosts. The unique dipsogenic potencies of isotocin in teleosts may be due to their aquatic life, as isotocin acts specifically on induction of reflex swallowing. Plasma levels, storage in the pituitary, and mRNA levels in the hypothalamus are almost the same in sea breams under hyper- and hypoosmotic 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 the APx eels can increase drinking volume after SW transfer to almost the same levels as controls (Nobata and Takei, 2011). Transient

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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 the 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.

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Data availability

The data that support the findings of this study are available from the corresponding author, S.N., upon reasonable request.

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Figures

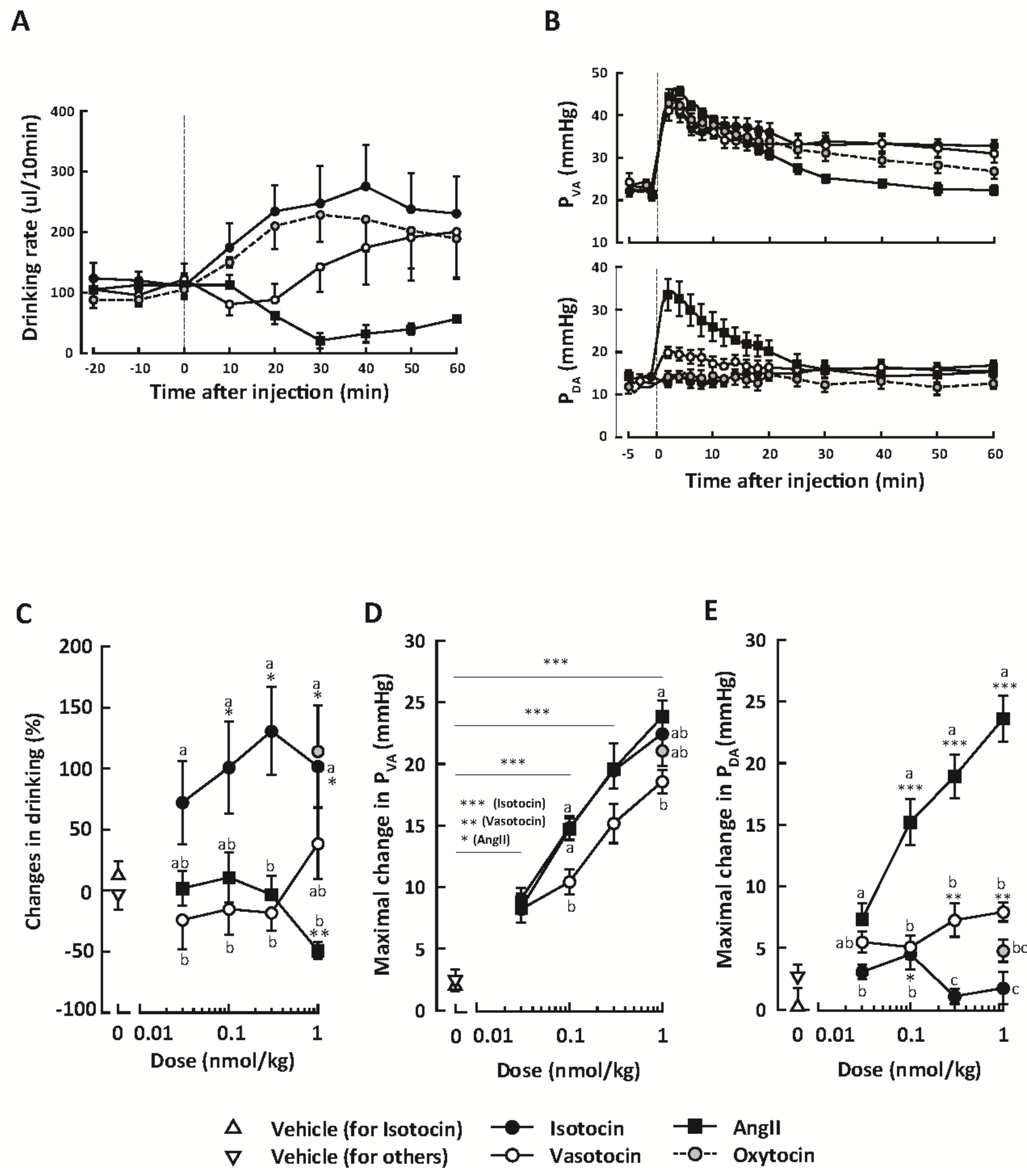

Figure 1. Effects of isotocin, vasotocin, angiotensin II (AngII) and oxytocin injected intra-arterially on drinking and cardiovascular function in conscious eels. Time course changes in drinking rate (A), ventral aortic pressure (P_{VA}) and dorsal aortic pressure (P_{DA}) (B) after injection of each peptide at 1.0 nmol/kg (n=8). Dose-response relationship of drinking rate (C), P_{VA} (D) and P_{DA} (E) after injection of each peptide in which changes in drinking was

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expressed as a percent change after injection and maximal changes were used for P_{VA} and P_{DA}. 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.

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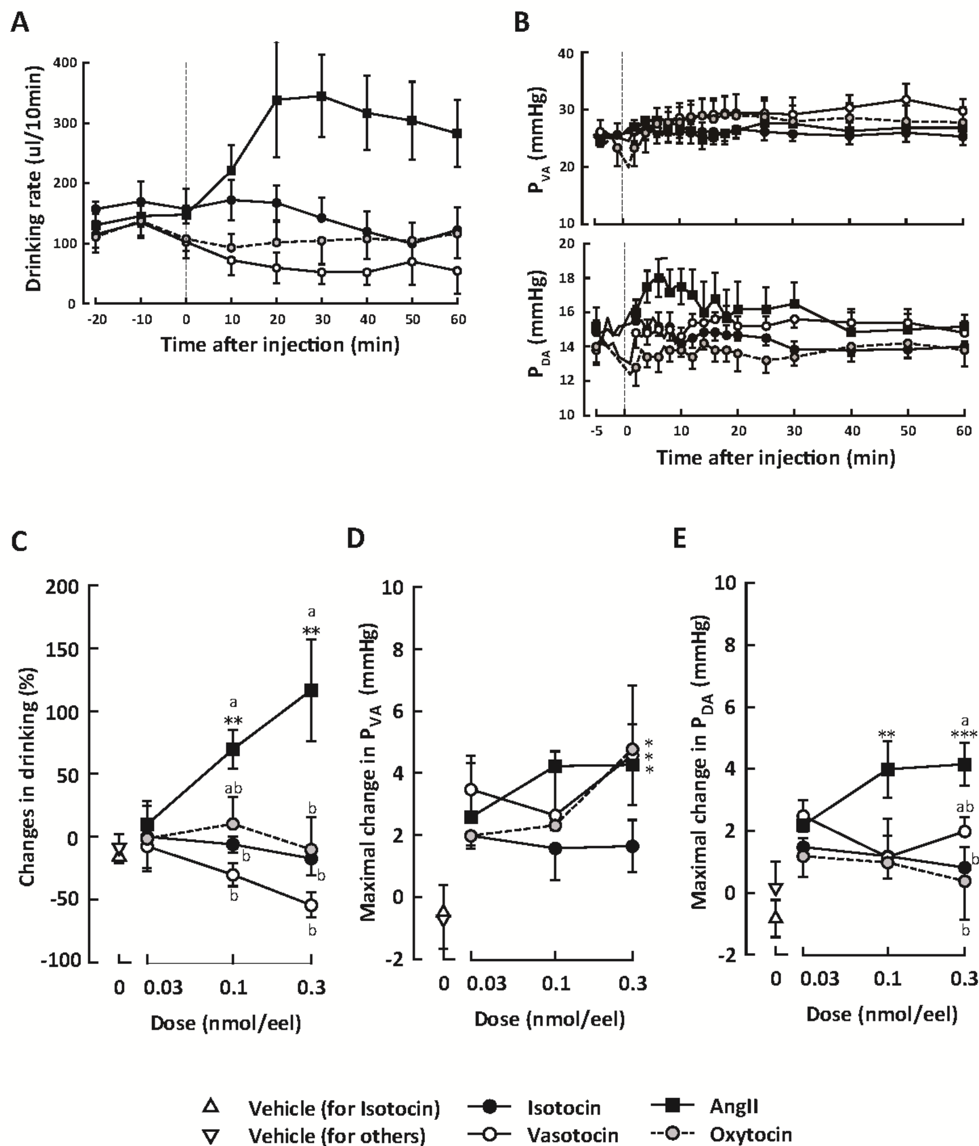

Figure 2. Effects of isotocin, vasotocin, angiotensin II (AngII) and oxytocin injected intra-cerebroventricularly on drinking and cardiovascular function in conscious eels. Time course changes in drinking rate (A), ventral aortic pressure (P_{VA}) and dorsal aortic pressure (P_{DA}) (B) after injection of each peptide at 0.3 nmol/eel (n=8). Dose-response relationship of drinking rate (C), P_{VA} (D) and P_{DA} (E) after injection of each peptide as in Fig. 1. 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.

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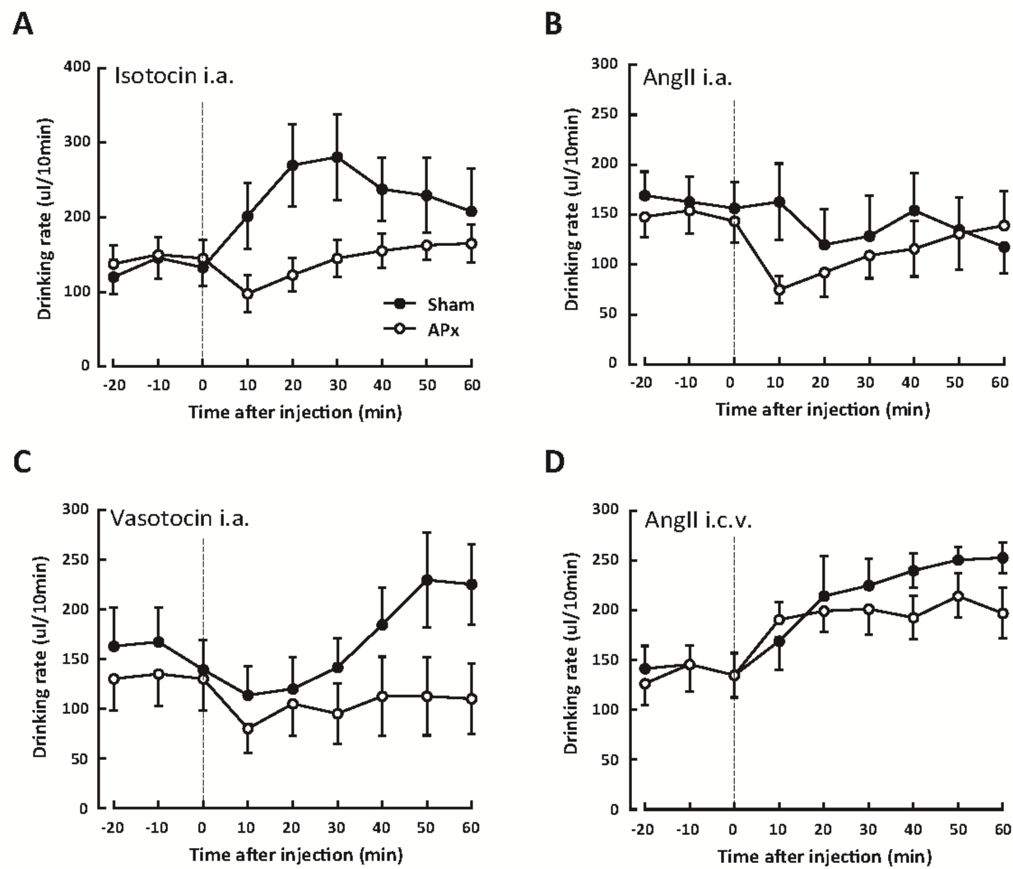

Figure 3. Effects of area postrema (AP) ablation on hormone actions in conscious eels. Time course changes in drinking rate after intra-arterial (i.a.) injections of isotocin (A), angiotensin II (AngII) (B), vasotocin (C) at 1.0 nmol/kg and intra-cerebroventricular (i.c.v.) injection of AngII (D) at 0.3 nmol/eel into sham controls and APx eels (n=7 in each group).

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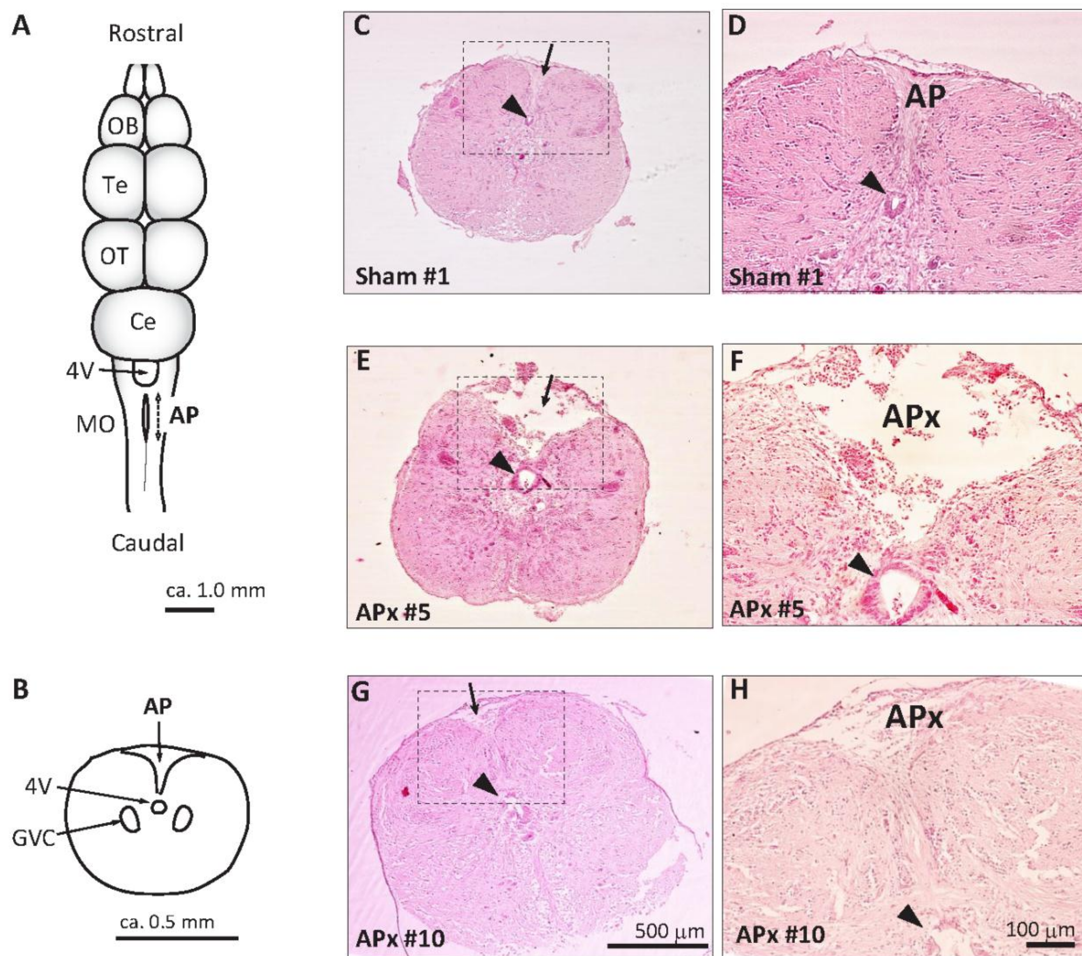

Figure 4. Typical cross sections at the medulla oblongata (MO) showing AP ablation in an eel of sham controls and APx eels used in this study. Schematic representation of eel whole brain from the dorsal side (A) and cross section at the MO (B). Cross section of the MO of sham control (C) and the enlarged view of dot square drawn in C (D). Cross sections of the MO of APx eels (E, G) and the enlarged views of dot squares (F, H). OB, Olfactory bulb; Te, Telencephalon; OT, Optic tectum; Ce, Cerebellum, AP, area postrema; 4V, forth ventricle; GVC, glossopharyngeal-vagal motor complex.

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Table 1. Changes in drinking rate, heart rate, and ventral aortic pressure (P_{VA}) and dorsal aortic pressure (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 the area postrema lesioned (APx) eels (n=7).

		Drinking rate (%)	Heart rate (beats/min)	P_{VA} (mmHg)	P_{DA} (mmHg)
Isotocin (1.0 nmol/kg 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/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

Changes in drinking rate are represented as a percentage of averaged drinking rate (μl/30 min) for 60 min after injection to drinking rate for 30min 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 changes, and they were compared between sham controls and APx eels by Student's *t*-test.

Table 2. Changes in drinking rate, heart rate, ventral aortic pressure (P_{VA}) and dorsal aortic pressure (P_{DA}) after intra-arterial (i.a.) injections of angiotensin II and vasotocin in sham controls (n=7) and the area postrema lesioned (APx) eels (n=7).

		Drinking rate (%) (0-30 min)	Drinking rate (%) (30-60 min)	Heart rate (beats/min)	P_{VA} (mmHg)	P_{DA} (mmHg)
Vasotocin (1.0 nmol/kg 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 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 for the first 30 min (0-30 min) and the later (30-60 min) after injection were represented as a percentage to that for 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} were represented as the maximum changes and were compared between sham controls and APx eels by Student's *t*-test (*P < 0.05).

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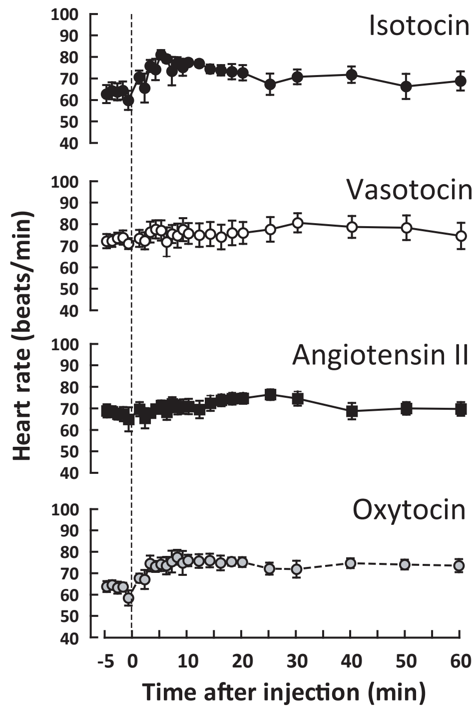
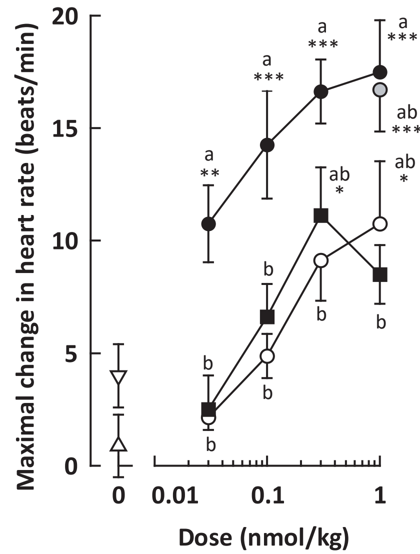
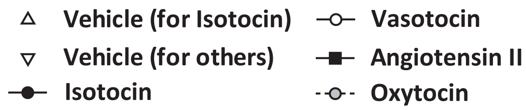
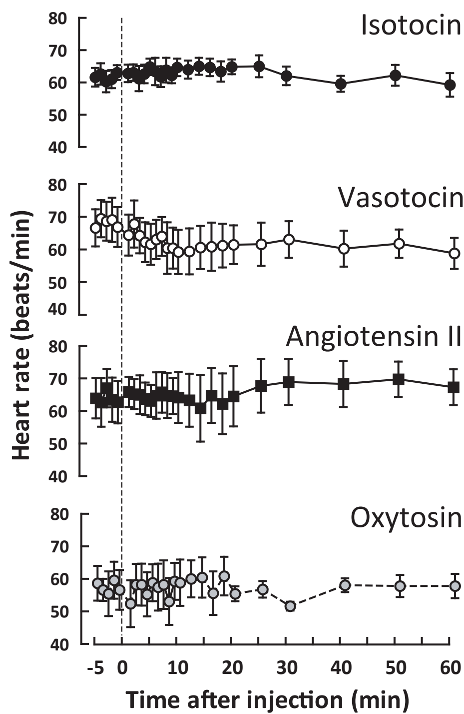
A
intra-arterial injection

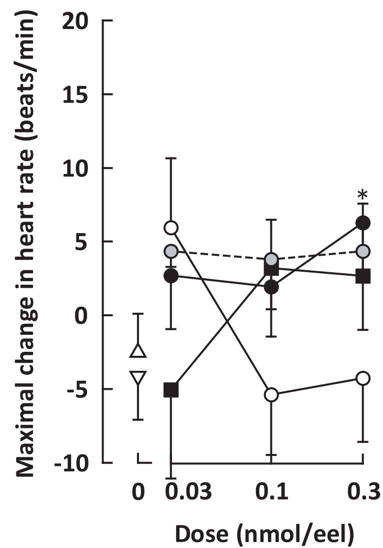






B
intra-cerebroventricular injection





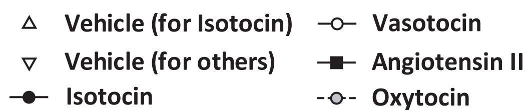


Fig. S1. Effects of isotocin, vasotocin, angiotensin II (AngII) and oxytocin injected intra-arterially (i.a.) (A) and intra-cerebroventricularly (i.c.v.) (B) on heart rate in conscious eels. (A) Time course changes in heart rate after i.a. injection of each peptide at 1.0 nmol/kg (n=8) (left) and the 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.