学位論文（要約）

Regulation of drinking behavior in the amphibious mudskipper
（両生魚トビハゼにおける飲水制御機構の
行動生理学的研究）

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Abstract

Tetrapods and ray-finned fish maintain their body-fluid osmolality at levels approximately one third to that of seawater. In terrestrial and marine environments, they are exposed to dehydration. To cope with this problem, vertebrates have developed behavioral/physiological mechanisms such as drinking during their evolution. Terrestrial tetrapods have thirst as a conscious sensation of a need for water and a motivation for drinking behavior in response to water depletion. Thirst aroused in the tetrapod forebrain is regulated by humoral inputs (e.g., plasma osmolality and osmoregulatory hormones) and by the afferent input of local sensation. The former is defined as “general thirst”, while the latter is defined as “local thirst”. Thirst sensation motivates animals to seek and consume water and thereby restores the parameters to their physiological set points.

On the contrary, aquatic fish can drink surrounding water just with swallowing reflex generated in the medulla oblongata without seeking water. Since any forebrain region involved in drinking has not been identified in fish, it is generally accepted that fish do not have thirst. Meanwhile, amphibious fish, such as the mudskipper, *Periophthalmus modestus*, prefers to stay on land and possesses water-searching behavior to refill water in the buccal/opercular cavity. I hypothesized that the mudskipper has thirst as a motivation for drinking behavior.

In my PhD thesis, I first examined whether the mudskipper has general-thirst mechanisms such as the direct action of dipsogenic/antidipsogenic hormones on the forebrain. In Chapter 1A, I showed that angiotensin II (AngII) facilitated migration to water and enhanced swallowing. I then analyzed the action of AngII on the brain by detecting the expression of AngII receptor mRNA and by semi-quantitatively measuring the number of c-Fos expressing neurons. However, the direct action of AngII was found in the medulla oblongata, but not in the diencephalon. In Chapter 1B, I showed that atrial

natriuretic peptide (ANP) inhibited AngII-induced drinking. The action of ANP was also found in the medulla oblongata. Thus, AngII and ANP regulated swallowing of water via the medulla oblongata similar to other aquatic teleost fish. The results in chapter 1 suggest the absence of general thirst in the mudskipper although AngII changed the migratory behavior. I then hypothesized that the loss of buccal/opercular water by AngII-induced swallowing may motivate the mudskipper to migrate into water for refilling. To test this hypothesis, in Chapter 2, I confirmed that swallowing of buccal/opercular water is facilitated by AngII on the land area, and then showed that artificial removal of buccal/opercular water motivated the mudskipper to move to water. Thus, buccal/opercular water is a key factor for the migratory behavior, indicating the existence of sensory mechanism of buccal/opercular water. In Chapter 3, I hypothesized presence of undetermined receptors for buccal/opercular water and its salinity, and examined effects of AngII and ANP injection on salt-intake behavior. I showed that salt appetite of the mudskipper was enhanced by AngII, suggesting that the undetermined receptors in the buccal/opercular cavity is sensitive to salt concentration, as well as water, to regulate the salt-intake behavior.

In this thesis, I pointed out that sensory system(s) in the buccal cavity controls migratory behavior for drinking, suggesting thirst in fish for the first time. This behavioral control seems to be similar to the local thirst in mammals, and thus the thirst mechanism might be important for terrestrial adaptation in vertebrates. In the General Discussion part, I compared the thirst mechanism of the mudskipper with known mechanisms in terrestrial tetrapods and aquatic fish, and propose a hypothetical evolutionary process of thirst during land invasions by vertebrates.

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General Introduction

Constancy of the internal environment known as homeostasis is necessary for living organisms. The term homeostasis was first introduced by Cannon as “the physiological rather than the physical arrangements for attaining constancy” (Cannon, 1929). Body-fluid concentration is an important internal parameter that is tightly controlled. For terrestrial vertebrates, dehydration is one of the most serious problems and water retention in the body is crucial to survive on land. Terrestrial tetrapods drink water, and have developed body integuments, such as the layers of keratinized skin cells, to reduce water loss from the body surface. In addition, the mammalian kidney with juxtamedullary nephrons that can produce hyperosmotic urine is an adaptation to reduce water loss from excretion.

Teleosts are osmotic and ionic regulator similar to tetrapods, and the composition of their body fluid is similar to those of tetrapods, at which plasma osmolality is approximately one third to that of seawater regardless of the inhabited salinity (Evans, 2008). The osmoregulatory ability might have allowed them to flourish in a wide range of aquatic environments including freshwater and seawater, and in particular cases even on the land. Furthermore, euryhaline teleost fish have the ability to maintain their plasma composition in both freshwater and seawater environments. In seawater environment, teleost fish are exposed to a severe dehydration since seawater is three times more concentrated than their body fluid (Takei et al., 2014). Marine teleosts drink seawater to cope with this problem (Hirano et al., 1972; Kobayashi et al., 1983; Perrott et al., 1992; Takei, 2015). After drinking, seawater is desalinated in the esophagus by some teleost species, and then water is absorbed together with NaCl in the intestine (Nagashima and Ando, 1994; Parmelee and Renfro, 1983). The excess ions are excreted from the branchial

or cutaneous ionocytes (Sakamoto et al., 2000; Seo et al., 2015;Uchida et al., 1996) and from the kidney, respectively (Takei and Hirose, 2002).

As already mentioned, both terrestrial tetrapods and marine teleost fish are exposed to dehydration. However, an obvious difference between aquatic and terrestrial habitats is the ease of access to water. Aquatic fish are always surrounded by water, so that they do not need search for water for drinking. Meanwhile, terrestrial/amphibious vertebrates such as the amphibians, reptiles, birds, and mammals appear to have developed "thirst" to mobilize them to seek water (Cannon, 1918; Fitzsimons, 1979; Fitzsimons, 1998; Takei, 2015) (Fig. 1).

General and local thirsts as motivations to drink.

One of the most remarkable features of animals is their ability to sense the changes in body-fluid concentration and to trigger specific behavioral responses that restore the homeostasis. Thirst is defined as a conscious sensation of a need for water and a motivation to drink (Fitzsimons, 1979; Robertson, 1991). Early theories diverged into two school of thoughts: (1) thirst is a local sensation of dryness in the mouth and throat, which was defined as "local thirst"; (2) thirst is an internal sensation generated from deficits in body-fluid volume, which was defined as "general thirst" (Cannon, 1918; Montgomery, 1931).

With regard to the local thirst, it is difficult to test in aquatic animals since the surrounding water continuously moist their mouth and throat, and thus it was considered that the local thirst might have evolved during land invasion of vertebrates (Cannon, 1918). In terrestrial tetrapods, the importance of local thirst has been overlooked for several decades (e.g., Fitzsimons (1979) called "secondary thirst"). However, recent

studies reaffirmed the importance of local thirst as an anticipatory thirst that prevents future water deficits in mammals (Krashes, 2016; Zimmerman et al., 2016). The neural basis of the local thirst is not well understood, but the SFO involvement was recently suggested (Fig. 2a).

On the other hand, many physiological studies in the 20th century have recognized the general thirst as the homeostatic responses to changes in body-fluid composition such as increased plasma osmolality (cellular dehydration), decreased plasma volume (extracellular dehydration), and an increase in dipsogenic hormones (Fitzsimons, 1998). Thirst sensation motivates animals to find and consume water and thereby restores these parameters to their physiological set points (Fitzsimons, 1979) (Fig. 2a).

Involvement of hormones in regulation of general thirst and salt appetite in tetrapods.

1) Dipsogenic hormone: angiotensin II

The renin-angiotensin aldosterone system (RAAS) is known to regulate body-fluid homeostasis and cardiovascular functions in response to decreases in blood pressure and renal perfusion (Carey and Siragy, 2003; Kobayashi and Takei, 1996; Olson, 1992). The RAAS cascade involves the secretion of renin that cleaves angiotensin I (AngI) from the N-terminus of the angiotensinogen. AngI is a decapeptide and biologically inactive, and angiotensin converting enzyme cleaves 2 amino acids from the C-terminus of AngI to form biologically active angiotensin II (AngII). Changes in amino acids were found in AngII during evolution, with [Asp^1] residue in tetrapods while [Asn^1] residue in fish (Takei et al., 2004). AngII is one of the major vasoconstricting peptides and is also involved in thirst, salt appetite, angiogenesis, glucose metabolism, and cellular growth

and migration (Fyhrquist and Saijonmaa, 2008). In addition, AngII stimulates the adrenal glands to release aldosterone. Aldosterone stimulates reabsorption of Na^+ and water from the distal tubules and the collecting duct to increase blood volume and pressure. Aldosterone also acts synergistically with AngII to stimulate salt appetite (Alhadeff and Betley, 2017; Fluharty and Epstein, 1983).

Since Fitzsimons and his colleagues discovered that infusion of AngII into the forebrain of rats produced thirst (Epstein et al., 1970; Fitzsimons, 1971), AngII has been considered to be the most potent dipsogenic hormone in most vertebrates, including fish (Fitzsimons, 1998; Kobayashi and Takei, 1996; McKinley and Johnson, 2004; Takei, 2000). In mammals and birds, systemic AngII has been shown to act on the sensory circumventricular organs (sCVOs) in the forebrain that lack the blood-brain barrier (BBB) to induce a sequence of fluid-intake and salt-intake behaviors (Alhadeff and Betley, 2017; Geerling and Loewy, 2008; Johnson and Buggy, 1978; McKinley, 2003; Simpson and Routtenberg, 1973; Takei, 1977) (Fig. 2a). The organum vasculosum of the lamina terminalis (OVLT) and the subfornical organ (SFO) are known as forebrain sCVOs where angiotensin type 1 receptor (AT1) is localized (Johnson and Buggy, 1978; McKinley, 2003). Activation of these regions by AngII is also linked to other neuroendocrine systems that modulate water and salt retention by the kidneys (Ferguson and Bains, 1996; Ferguson and Kasting, 1987). These renal responses are mediated primarily by arginine vasopressin (AVP) in mammals (Huang et al., 1995; Rasmussen et al., 2003; Rasmussen et al., 2004; Verbalis et al., 1991). AVP is produced by magnocellular neurosecretory cells in the paraventricular and supraoptic nuclei of the hypothalamus, and are released into circulation from the neurohypophysis, under a direct control of descending input from the

sCVOs (Antunes-Rodrigues et al., 2004). Thus, the sCVOs in the forebrain have an important role in AngII-stimulated general thirst and salt appetite in tetrapods.

2) Anti-dipsogenic hormones: natriuretic peptides.

Natriuretic peptides (NPs) are hormones with 22-36 amino acids and characterized by an intramolecular ring structure of 17 amino acid residues formed by a disulphide bridge. Since the first discovery of a natriuretic factor in the rat heart in 1981 (De Bold et al., 1981), it has become clear that NPs play important roles in body-fluid and cardiovascular homeostasis in vertebrates (Galli and Phillips, 1996; Suzuki et al., 2001; Takei, 1999). The NP system consists of three types of hormones [atrial NP (ANP), brain or B-type NP (BNP), and C-type NPs (CNPs)] and three types of receptors [NP receptor (R)-A, NPR-B, and NPR-C] in tetrapods (Potter et al., 2005). ANP and BNP are circulating hormones secreted from the heart, whereas CNPs are mostly neural origin. Cardiac NPs act on various tissues and cells that are involved in body-fluid and cardiovascular regulation. Biological actions of BNP are overlapped with those of ANP because they share the same receptor, NPR-A. ANP and BNP reduce blood volume by increasing the excretion and by decreasing the uptake of both water and Na^+.

Regarding excretion, ANP induces strong diuresis and natriuresis by stimulating dilatation of afferent renal arterioles and constriction of efferent arterioles, which increase the pressure in the glomerular capillaries and thus glomerular filtration rate (Marin-Grez et al., 1986). In addition, ANP inhibits AngII-stimulated water and Na^+ reabsorption in the proximal tubules (Harris et al., 1987). ANP also inhibits the secretion of AVP and aldosterone, a potent antidiuretic and antinatriuretic hormone, respectively, thereby indirectly further promoting diuresis and natriuresis (Dillingham and Anderson, 1986; Metzler and Ramsay, 1989). Regarding the decrease in water and Na^+ uptake, ANP

administration in the cerebral ventricle inhibits AngII-induced thirst, osmotically stimulated thirst, and salt appetite (Antunes-Rodrigues et al., 1985; Antunes-Rodrigues et al., 1986; Burrell et al., 1992). Suppression of thirst and salt appetite by ANP was suggested to be mediated by NPR-A in the SFO (Blackburn et al., 1995; Ehrlich and Fitts, 1990).

Given that both dipsogenic AngII and anti-dipsogenic NPs act on the sCVOs such as OVLT and SFO, the sCVOs in the forebrain are considered to have central roles in the endocrine control of general thirst and salt appetite in tetrapods.

Endocrine control of drinking in aquatic fish: Do fish have thirst mechanisms?

As already mentioned, aquatic teleosts, which have never experienced a terrestrial lifestyle in the evolutionary history, drink water in their marine environment. Drinking of aquatic fish is regulated by hormones such as AngII and ANP as in tetrapods, but aquatic fish can complete drinking solely by swallowing water inside their mouth. This fact implies unnecessity of a motivation to move to water for drinking, and swallowing reflex generated in the medulla oblongata is sufficient for drinking in aquatic fish. Indeed, removal of the whole forebrain did not affect the drinking induced by AngII in eels (Takei et al., 1979). Recently, the site of action of AngII was suggested to be the area postrema (AP), one of the hindbrain sCVOs (Mukuda et al., 2005) (Fig. 2b). Electric lesioning of the AP impaired AngII-induced drinking (Nobata and Takei, 2011). These data strongly suggest that AngII acts on the AP to induce swallowing reflex without an involvement of the forebrain. Since the forebrain region implicated in fish drinking was not identified (Takei, 2015), it has been considered that thirst is a terrestrial adaptation acquired during the evolution of terrestrial tetrapods (Takei, 2015).

Differences between terrestrial tetrapods and aquatic teleosts have also been found in the potency of dipsogenic/antidipsogenic hormones. Dipsogenic regulation is dominant in mammals, whereas antidipsogenic control is more potent in teleosts (Takei, 2015). For instance, 100-fold higher doses are required for AngII to induce drinking in teleosts compared to mammals (Takei, 2002). In contrast, ANP injected into the circulation of seawater eels inhibited drinking at doses that do not lower arterial pressure, while ANP has only antidipsogenic effect in mammals at high hypotensive doses (Takei and Hirose, 2002). Furthermore, some hormones (e.g., bradykinin), which have been identified as dipsogenic hormones in mammals, exert antidipsogenic action in eels (Takei et al., 2001). Collectively, although hormones regulating drinking have been identified in teleosts, thirst mechanisms may not have evolved during the evolution of aquatic fish (Fig. 2b).

The mudskipper goby as a model of drinking behavior in fish.

In the evolutionary history of vertebrates, the land invasion by vertebrates in evolution occurred not only in lobe-finned fish that lead to tetrapods, but also in ray-finned fish such as amphibious mudskippers, which are independent of the tetrapod lineage (Graham and Lee, 2004; Ord and Cooke, 2016). Mudskippers spend a greater part of their lives out of water to feed and avoid predation (Clayton, 1993; Graham, 1997). Naturally, their habitat is brackish water but they can survive in a wide range of salinity environments from full-strength to diluted seawater (5 ppt seawater). Therefore, the mudskipper has been used as a model animal for analyses of behaviors related to osmoregulatory control, such as salinity preference and movement between aquatic and land environments (Gordon and Gabaldon, 1985; Kagawa et al., 2013; Sakamoto et al., 2005; Sakamoto et al., 2011; Sakamoto et al., 2015). For adaptation to a semi-terrestrial

lifestyle, they have developed unique behavioral and physiological features (Clayton, 1993; Graham, 1997; Ishimatsu and Gonzales, 2011; Sakamoto et al., 2002). For example, they can close opercula and stop ventilating the gills, because cutaneous respiration greatly contributes to their gas exchange (Tamura et al., 1976). This enables them to store water in the buccal and/or opercular cavities when they are on land. Buccal/opercular water prevents the gills from desiccation and maintains the gill function for ion regulation and respiration (Sayer, 2005; Tamura et al., 1976). It is frequently observed that mudskippers move on mudflat to search for water to drink, suggesting the existence of general and/or local thirst similar to terrestrial tetrapods. Recently, an OVLT-like structure was identified in the diencephalon of the mudskipper (Hamasaki et al., 2016). Therefore, the mudskipper is a valuable experimental model to provide new insights into thirst mechanisms in vertebrates (Fig. 1 and 2b). Has thirst evolved independently in the amphibious fish as a regulatory mechanism of drinking behavior?

Aim of this study.

Previous evidence suggests that general and local thirst are essential for terrestrial adaptation in tetrapods. However, in fish, existence of "thirst" is still under debate since previous studies did not support the existence of general and local thirsts in aquatic fish. The final aim of my study is to clarify whether fish have thirst and to deduce how thirst mechanisms emerged during the vertebrate evolution. To achieve this, I used the amphibious mudskipper, *Periophthalmus modestus*, as my experimental model. In my thesis, I investigated the regulation of drinking behavior in the mudskipper. Drinking in mudskipper is composed of a sequence of behaviors (Fig. 1 and 3). In this thesis, I divided the sequence into each behavior and defined them as follows. "Drinking behavior" means

whole sequence of behaviors. The drinking behavior is composed of 1) migration to water areas, 2) buccal refilling, and 3) swallowing. “Buccal refilling”, refers to the action of taking water into the mouth (the buccal/opercular cavity), while “swallowing” means movement of water from the buccal cavity to the esophagus. “Dipsogenic” and “antidipsogenic” actions are determined by increased and decreased amount of water swallowed, respectively, regardless of migratory behaviors. “Thirst” is defined as a motivation for “drinking behavior” aroused in the forebrain.

In Chapter 1A and 1B, I examined effects of AngII and NPs on the drinking behavior, respectively. I found that AngII prolonged the aquatic stay and increased swallowing in the mudskipper. I also examined whether AngII directly acts on the forebrain sCVOs, as a possible mechanism of general thirst, by detecting the expression of AngII receptor and by semi-quantitatively measuring the number of c-Fos expressing neurons in the sCVOs. From these investigations, I showed that AngII exerts dipsogenic action via the AP (a sCVO in the hindbrain) similar to other aquatic teleost, suggesting general thirst mechanism could be absent in the mudskipper.

The results of Chapter 1 not only showed that AngII promoted migration of the mudskipper to water, but also implied direct action of AngII on swallowing of buccal/opercular water. Therefore, I hypothesized that the loss of buccal/opercular water by AngII-induced swallowing may motivate the mudskipper to migrate into water for refilling. To test this hypothesis, in Chapter 2, I first confirmed that swallowing of buccal/opercular water is facilitated by AngII on land, and then examined whether artificial removal of buccal/opercular water would motivate the mudskipper to move to water. The results showed that buccal/opercular water is a key factor for the migratory behavior, indicating the existence of a sensory mechanism of buccal/opercular water in

the buccal cavity, which is similar to the local thirst reported in mammals (Cannon, 1918; Zimmerman et al., 2016).

The results of Chapter 2 implied that afferent signals from undetermined receptors for water in the buccal/opercular cavity are involved in the refilling behavior in the mudskipper. Therefore, in Chapter 3, I attempted to show a possible mechanism of sensory detection of buccal water in the mudskipper. I hypothesized that the receptors might detect salt in the buccal/opercular water, and examined effects of AngII and ANP injection on salt-intake behavior.

The overall data in the thesis showed that 1) AngII facilitates swallowing of buccal water by acting through the AT1 receptor located in the AP (a sCVO in the hindbrain) as has been shown in aquatic teleost fish, 2) the depletion of buccal water in turn triggers the buccal refilling behavior. My results imply that the amphibious mudskipper does not have the general thirst mechanism, but I firstly pointed out that sensory system(s) in the buccal cavity controls migratory behavior for refilling, and this behavioral control is similar to the local thirst. In the General Discussion, I compared the thirst mechanism of the mudskipper with known mechanisms in terrestrial tetrapods and aquatic fish, and propose a hypothetical evolutionary process of thirst mechanisms during invasions of land environment by vertebrates.

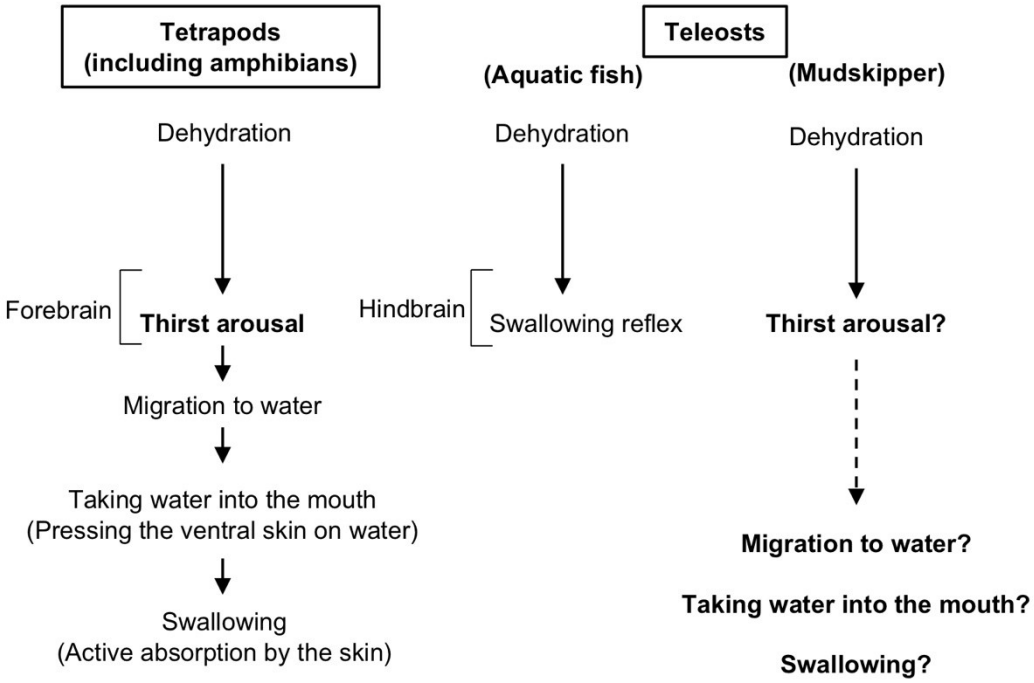

Figure 1. Differences in the drinking process between tetrapods and teleosts.
In tetrapods, dehydration evokes thirst to motivate a sequence of drinking behaviors ("general thirst" in Fig. 2). Fully aquatic fish can complete drinking by the swallowing reflex of buccal water without thirst induction, because water is always present in the mouth. Mudskippers are amphibious gobies that spend a great part of their lives out of water and it is frequently observed that mudskippers move to water to drink. Therefore, it is of interest to investigate whether the mudskipper has thirst mechanisms.

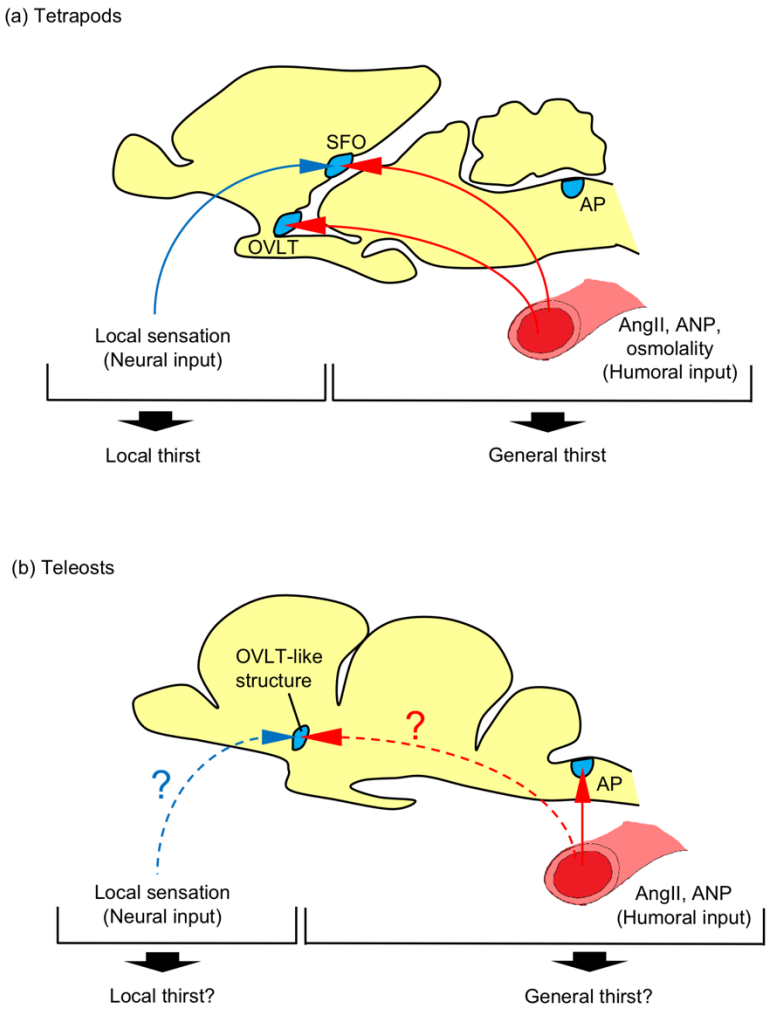

Figure 2. Differences in the regulatory mechanism of drinking between tetrapods and teleosts. (a) In tetrapods, humoral inputs act on the sensory circumventricular organs (sCVOs) in the forebrain that lack the blood-brain barrier to regulate general thirst. The organum vasculosum of the lamina terminalis (OVLT) and the subfornical organ (SFO) are the well-recognized forebrain sCVOs. Angiotensin II (AngII) is a dipsogenic hormone, and atrial natriuretic peptide (ANP) is an anti-dipsogenic hormone. The SFO neurons also receive neural inputs (e.g., sensory detection of oropharyngeal water) to regulate local thirst. (b) In teleosts, regulation of drinking has been investigated in aquatic fish. Humoral inputs act on the area postrema (AP), one of the hindbrain sCVOs, to induce swallowing. Although the OVLT-like structure was recently found in eels and mudskippers, its involvement in drinking has not been shown. Local thirst regulation in teleosts has never been examined.

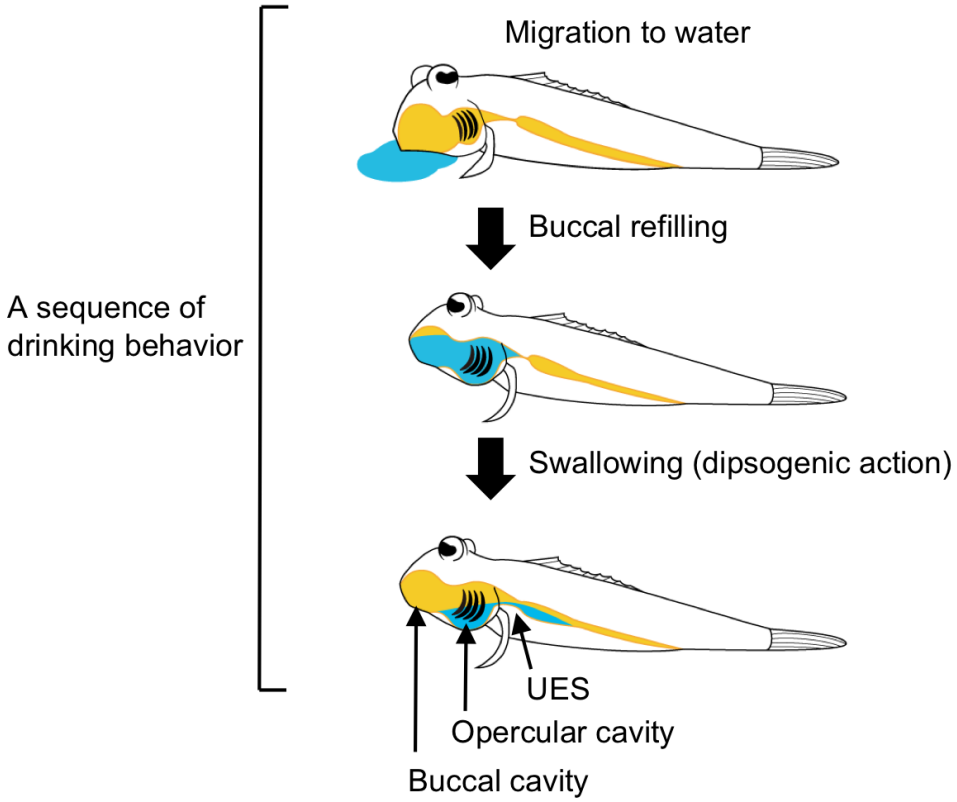

Figure 3. Processes of drinking behavior in the mudskipper. A sequence of drinking behaviors in the mudskipper includes migration to water areas, taking water into the buccal and opercular cavities ("buccal refilling"), and swallowing of buccal/opercular water to the esophagus ("swallowing"). UES, upper esophagus sphincter.

Chapter 1A

Dipsogenic effect of angiotensin II in the mudskipper

Abstract

Dipsogenic action of angiotensin II (AngII) was investigated in the mudskipper. Intracerebroventricular (ICV) injection of 3×10^{-8} M AngII significantly prolonged period spent in water during the initial 60 min after the injection. ICV injection of 3×10^{-6} M and 3×10^{-10} M AngII also prolonged time spent in water at 4 hrs and 8 hrs after the injection, which are considered to be secondary effects via other hormones. The ICV injection of AngII also increased drinking rate dose-dependently. Expression of AngII receptor (AT1 receptor) mRNA was detected in the area postrema (AP), but not in the OVLT-like structure in the mudskipper diencephalon. Elevation of neuronal activity indicated by c-Fos immunoreactivity was also found in the AP but not in the OVLT-like structure after the ICV injection of AngII. The results in this chapter showed that AngII is a dipsogenic hormone in the mudskipper, and I firstly found that AngII motivates them to stay in water. Similar to eels, AngII could directly act on the AP neurons that control the swallowing reflex. Although AngII-induced migration suggests an involvement of possible thirst mechanisms in the mudskipper forebrain, this behavior is likely not mediated by the general thirst.

Introduction

The existence of thirst is under debate in aquatic fish. Aquatic fish can complete drinking solely by swallowing water inside the mouth. Therefore, they are not necessary to search and move to water for drinking, and thus it has been considered that thirst do not exist in aquatic fish. Indeed, removal of the whole forebrain did not affect the drinking induced by angiotensin II (AngII) in eels (Takei et al., 1979). The site of action of AngII was suggested to be the area postrema (AP), one of the sensory circumventricular organs (sCVOs) in the hindbrain (Mukuda et al., 2005); electric lesioning of the AP impaired AngII-induced drinking (Nobata and Takei, 2011). These data suggest that the swallowing reflex generated in the medulla oblongata is sufficient for drinking in aquatic fish. In this regard, the mudskipper provides a key research model for evolution of thirst, because they frequently search and move to water to drink when they are on mudflat. Since injection of AngII potently increased water ingestion in many vertebrates such as terrestrial tetrapods, seawater fish, and euryhaline fish (Kobayashi et al., 1983; Takei, 1977, 2000), AngII is a likely candidate for a dipsogenic hormone motivating drinking behavior in the mudskipper.

The neural mechanisms regulating AngII-induced thirst have been extensively investigated in tetrapods (Fitzsimons, 1998). Since the circulating hormones cannot pass the blood-brain barrier (BBB), they act on the regions outside the BBB such as the sCVOs (Ferguson and Bains, 1996; McKinley and Johnson, 2004). In mammals, the sCVOs including the subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT), and AP, are structures that lack the functional BBB and serves as a blood-brain interface (Ferguson and Bains, 1996). Particularly, the forebrain sCVOs including SFO

and OVLT are important for the central regulation of body fluid balance and for receiving humoral AngII signals in mammals (Fitzsimons, 1998; Matsuda et al., 2017; McKinley and Johnson, 2004; Oka et al., 2015; Simpson and Routtenberg, 1973) and in birds (Takei, 1977; Tsuneki et al., 1978). Angiotensin type 1 receptor (AT1) in the forebrain sCVOs mediates AngII-induced thirst (McKinley et al., 2003; McKinley and Johnson, 2004).

In this chapter, I first examined effects of ICV injection of AngII on migratory behavior between land and water, and on drinking rate in the mudskipper. I found that the ICV injection of AngII prolonged time spent in water and increased drinking rate. Since the parvocellular preoptic area anterior part (PPa) including the OVLT-like structure was recently identified as a region outside the BBB in the mudskipper diencephalon (Hamasaki et al., 2016), I then investigated possible sites of action of ICV-injected AngII in the mudskipper brain, by examining AT1 receptor expression and c-Fos immunoreactivity following the ICV injection of AngII.

Methods

Animals.

One year-old mudskippers of both sexes (*Periophthalmus modestus*) weighing 3 to 5 g were collected from the estuary of the Fujii River, which flows into the Inland Sea of Seto (34° N: 134° E). The environmental salinity changes from 10 to 30 ppt. No sex differences have been found in their amphibious behavior in the previous reports (Sakamoto et al., 2011; Sakamoto et al., 2015). Fish were acclimated for 2–5 weeks in laboratory tanks (3 L) with brackish water (10 ppt, 149 mM Na^+, 176 mM Cl^-, 3.8 mM Ca^{2+}, 346 mOsml/kg), which is almost isotonic to the mudskipper plasma. All specimens

were maintained at room temperature of 22-25°C under a constant photoperiod cycle of 12-hrs light/12-hrs dark (lights on at 7:00 a.m.) and were fed daily with Tetrafin flakes (TetraWerke, Melle, Germany). Small plates were placed in each tank to allow mudskippers an opportunity to climb onto them. Fish were anesthetized in 0.01% tricaine methanesulfonate (Sigma, Tokyo, Japan) neutralized with sodium bicarbonate before injection and sampling. All experiments were approved by the Animal Experiment Committee of the University of Tokyo, and performed in accordance with the manuals prepared by the committee.

ICV injection of hormones.

The mudskipper angiotensinogen cDNA was previously cloned from the liver (Watanabe, unpublished data) to determine the amino acid sequence of AngII. The putative amino acid sequence of mudskipper AngII is NRVYVHPF. The synthetic peptide (Peptide Institute, Osaka, Japan) was dissolved in an artificial cerebrospinal fluid (140 mM NaCl/3 mM KCl/1.0 mM $MgCl_2$/1.3 mM $CaCl_2$/2 mM Na_2HPO_4/2 mM NaH_2PO_4, pH 7.4). ICV injection was performed according to the previous reports (Kagawa et al., 2013; Sakamoto et al., 2011; Sakamoto et al., 2015). AngII (3×10^{-12} M to 3×10^{-6} M) or vehicle (artificial cerebrospinal fluid) was injected post-orbitally along the midline into the third ventricle with 0.1 μl/g volume. Evans blue (0.1%) was used to confirm the success of ICV injection. To minimize the leakage from the injection site, the needle was withdrawn 30 seconds after the injection.

Behavioral analysis.

Immediately after injection of AngII, each fish was placed in the 10-ppt seawater area (10 ppt, 149 mM Na^+, 176 mM Cl^-, 3.8 mM Ca^{2+}, 346 mOsml/kg) of experimental tank (Fig. 4). The land area was made of plastic mesh, and care was taken to ensure that

there was minimum water on the land area. Water in the tank was constantly aerated. The period in water and the frequency of migration between water and land area (defined as the "frequency of migration") were recorded for 8 hrs following the injection. The parameters were measured at 0.25 hr (0.13-0.38 hr), 0.5 hr (0.25-0.75 hr), 1 hr (0.75-1.25 hr), 2 hr (1.75-2.25 hr), 4 hr (3.75-4.25 hr), and 8 hr (7.75-8.25 hr) after injection.

Drinking rate.

Fish were put in a columnar tank (diameter, 65 mm; height, 92 mm) filled with 0.004% phenol red in 10-ppt seawater (10 ppt, 149 mM Na^+, 176 mM Cl^-, 3.8 mM Ca^{2+}, 346 mOsml/kg) after ICV injection with vehicle or 0.1 μl/g of 3×10^{-8} to 3×10^{-6} M of AngII. The amount of water in the whole gastrointestinal tract was measured according to the method of Kobayashi and others (Kobayashi et al., 1983). Briefly, the whole tracts were removed on a petri dish and washed by 1 ml saline. Then, 0.5-ml aliquots of the samples were mixed with 0.5 ml 5% trichloroacetic acid (Sigma-Aldrich), and centrifuged at 10,000 rpm for 5 min. The supernatant was mixed with 0.5 ml of 1 M NaOH, and absorbance was determined at 550 nm by a spectrophotometer (DU640, Beckman Coulter, CA, USA).

Cloning of AT1 cDNAs (*agtr1*).

After anesthesia, the brains were dissected out from intact mudskippers and immediately frozen in liquid nitrogen. Total RNA was extracted using ISOGEN (Nippongene, Toyama, Japan). Single-stranded cDNAs were prepared from RNA of the brain, using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, MA, USA). For *agtr1* cloning, a partial sequence was first cloned by a nested PCR with four degenerate primers (TBRTSGGDAAYASCATGGTGGT, GACCGBTACCTKGCYATYGTSCA, GCAGCTRTTRAMGTARGCDATGCA,

TGSACRATRGCMAGGTAVCGGTC). Then gene specific primers (GAAGAGCTCCCAGTCCCGGGACG and CAGTCATCGTGGCCCACATGGGC) were used for 3' and 5' RACE (SMARTer^{TM} RACE cDNA Amplification Kit, Clontech, Takara Bio USA, CA, USA), respectively. Finally, cDNAs encompassing the whole coding regions were amplified using gene-specific primers (ATGAAGAACATAACCTCAGGAGCAG, TTAATGTCAGAGGAAGACTTG) designed based on the partial sequences determined by the RACE method. cDNAs were amplified with high-fidelity Ex Taq DNA polymerase (TaKaRa, Tokyo, Japan), ligated into pGEM T-easy (Promega, Madison, WI, USA). Amplified products were sequenced by an ABI3130*xl* DNA sequencer (Applied Biosystem, Foster City, CA, USA) and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Deduced amino acid sequences of AT1 were aligned with those of other fish and phylogenetic trees were generated by the neighbor-joining method using the ClustalW program (version 2.1) with default settings in a worldwide web server of the DNA Data Bank of Japan (http://clustalw.ddbj.nig.ac.jp/). For comparison of amino acid sequences of mudskipper AT1 with those of other members of the same family, AT1, angiotensin type 2 receptors (AT2), bradykinin receptors, and apelin receptors were included in the phylogenetic tree.

***In situ* hybridization of *agtr1*.**

The brains of intact fish were fixed in 4% paraformaldehyde in phosphate buffer (PB) and embedded in Paraplast (McCormick Scientific, Richmond, IL, USA). Serial sections were prepared at 7 μm and mounted onto MAS-coated slides (Matsunami Glass, Osaka, Japan). The cDNA fragment (1092bp) encoding AT1 receptor was transcribed to synthesize digoxigenin-labeled antisense and sense cRNA probes (DIG RNA Labeling

Kit; Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocols. The deparaffinized and rehydrated sections were treated with 5 mg/ml proteinase K (Sigma-Aldorich, MO, USA) in Tris-EDTA buffer [100 mM tris(hydroxymethyl)aminomethane and 50 mM ethylenediaminetetraacetic acid, pH 7.5] and then hybridized with 1 μg/ml DIG-labeled cRNA probes in hybridization buffer [50% formamide, 5 × Saline Sodium Citrate Buffer (5 × SSC, 750 mM NaCl and 75 mM sodium citrate, pH 7.0), 40 μg/ml bovine calf thymus DNA] at 58°C for 40 h. After hybridization, sections were washed in 2 × SSC for 30 min at room temperature, followed by 1 × SSC for 1 hr at 65°C, and finally in 0.1 × SSC for 1 hr at 65°C. Following immunohistochemical reaction with alkaline phosphatase-conjugated anti-DIG antibody (Roche Applied Science, Upper Bavaria, Germany), hybridization signals were visualized with 4-nitro blue tetrazolium chloride (450 mg/ml) and X-phosphate/5-bromo-4-chloro-3-indolyl-phosphate (175 mg/ml). Stained sections were dehydrated with ethanol, cleared with xylene, and coverslipped. Micrographs were taken using a digital camera (DMX1200; Nikon, Tokyo, Japan).

c-Fos immunohistochemistry.

The brains were dissected out 1 hr after ICV injection of vehicle (artificial cerebrospinal fluid, ACSF) or AngII (3×10^{-8} M in ACSF), and fixed in 4% paraformaldehyde in PB and embedded in Paraplast (McCormick Scientific, Richmond, IL, USA). Serial sections were prepared at 7 μm and mounted onto MAS-coated slides (Matsunami Glass, Osaka, Japan). The sections were deparaffinized, rehydrated and then rinsed in phosphate buffered saline (PBS). The sections were immersed in methanol containing 0.3% H_2O_2 for 30 min at room temperature. After rinsing in PBS, the sections were incubated with proteinase K (10 mg/ml in PBS) for 10 min at room temperature.

The sections were rinsed thoroughly in PBS and then pretreated with a blocking solution (2% normal goat serum, 0.01% NaN_3 in PBS) for 1 hr at room temperature and incubated with a polyclonal antibody raised against human c-Fos (1:200, sc-253, SantaCruz, Dallas, TX, USA) for 12 hrs at 4°C. Specificity of the antibody against c-Fos protein of the mudskipper was previously confirmed by Western blotting analysis (Hamasaki et al., 2016). After rinsing in PBS, the sections were treated with ABC Elite kit (PK-6101, Vector, Burlingame, CA, USA) according to the manufacturer's instruction. The sections were rinsed in 0.1 M PB and immersed in 0.01% 3, 3' -diaminobenzidine tetrahydrochloride (DAB) in PB for 3 min to intensify colorization and then incubated in 0.01% DAB solution containing 0.01% H_2O_2 for 3 min in dark. Finally, the sections were rinsed in PB and distilled water, and immunoreactive c-Fos signals were examined and photographed as above. The photomicrographs were binarized by graphic software Image J (https://imagej.nih.gov/ij/) and the number of c-Fos positive neurons was counted manually and compared between the cohorts. The PPa area was defined from the rostral tip of the anteroventral region of the third ventricle to the level where the anterior commissure emerged caudally. Every three sections was analyzed in order not to count positive cells repeatedly.

Statistical analyses.

The data are expressed as means ± s.e.m. Kyplot 5.0 (KyensLab, Tokyo, Japan) was used for statistics analyses. Time course data were analyzed by two-way ANOVA followed by Dunnett's post-hoc test. Effects of AngII on drinking rate were analyzed with one-way ANOVA followed by Dunnett's post-hoc test wherever appropriate. Unpaired *t*-test was used for analyses of the number of c-Fos immunoreactive cells.

Results

Effects of AngII on the migratory behavior.

In the intact condition, mudskippers stayed on the land area of the experimental tank about 80% of 8-hrs observation period (Fig. 5a). After the ICV injection of 3×10^{-8} M AngII, the mudskippers spent more time in water than vehicle-injected controls within the first 30-min period (Fig. 5a). The prolonged stay in water by 3×10^{-8} M AngII continued for at least an hour, and then decreased nearly to the control level. The tendency to extend time stayed in water was also observed for the initial 30 min by injection of 3×10^{-12} M AngII, although no statistically significant difference was detected. Significant increase in time stayed in water was also observed after 4 hrs by 3×10^{-6} M injection and after 8 hrs by 3×10^{-10} M injection. No significant difference was observed in the frequency of migration between water and land areas at any concentration and time point (Fig. 5b).

Effects of AngII on drinking rate.

After the injection of AngII or vehicle, fish were kept for 1 hr in a columnar tank without the land area, and the amount of water ingested was measured. The amount of ingested water was increased by AngII injection dose-dependently (Fig. 6), and the increase was statistically significant at 3×10^{-6} M AngII.

cDNA cloning of angiotensin type-1 receptor (*agtr1*).

The cDNA encoding mudskipper AT1 receptor was cloned from the mudskipper brain (Fig. 7; Accession number, LC171725) using teleost *agtr1*-like cDNA sequences as queries. The phylogenetic analysis of the AT1/AT2/bradykinin receptor/apelin receptor family showed that the newly identified mudskipper AT1 receptor was grouped in a cluster of AT1 with other teleost AT1/AT1a receptors (Fig. 8). The deduced amino acid

sequence of mudskipper AT1 receptor showed high sequence identity to AT1 receptors of other teleosts and lamprey; in particular, the putative transmembrane domains were well conserved among teleost AT1/AT1a receptors (Fig. 9).

Expression of mRNA encoding AT1 receptor in the mudskipper brain.

When the antisense cRNA probe for the mudskipper *agtr1* mRNA was used for *in situ* hybridization, neurons expressing *agtr1* mRNA were detected at least in several neuronal areas in diencephalon and medulla oblongata. In medulla oblongata, many neurons having intense positive signals were detected in the area postrema (AP) and the glossopharyngeal-vagal motor complex (GVC), which control swallowing reflex in teleosts (Fig. 10a and 10c). Meanwhile, no signal for *agtr1* mRNA was observed in the forebrain sCVOs, such as PPa including the OVLT-like structure (Fig. 10b). No signal was observed in the negative control sections incubated with the sense probe (data not shown).

Detection of c-Fos immunoreactive neurons in the mudskipper sCVOs.

As an indirect marker of neuronal activity, I investigated c-Fos immunoreactivity in the sCVOs of the mudskipper brain. In this experiment, I focused on the AP and the PPa including the OVLT-like structure. In the AP of vehicle injected fish, c-Fos positive neurons were scattered. ICV injection of AngII increased the number of c-Fos positive neuronal cells in the AP (Fig. 11b and d). c-Fos positive neurons were located in the PPa (Fig. 11a and c), but no change in the number of c-Fos positive neurons was observed in the PPa by the ICV injection of AngII. When only the positive neurons in the OVLT-like structure (indicated by red circles in Fig. 11a and c) were counted, the number of c-Fos positive neurons did not differ between the vehicle controls and the AngII-injected group.

Discussion

In this chapter, I demonstrated that ICV injection of AngII increased drinking rate in the mudskipper as previously shown in aquatic teleosts (Ogoshi et al., 2008), implying that AngII is a potent dipsogenic hormone commonly in vertebrates. In addition, AngII injection also prolonged time spent in water. This is the first finding of "motivation for migration to water areas" in fish, suggesting that the amphibious mudskipper possesses the thirst-like mechanism similar to tetrapods. On the other hand, I further demonstrated that plausible site of action of AngII in the mudskipper brain is the AP in the medulla oblongata.

In my study, the ICV injection procedure was chosen because this route is more consistent and potent than the peripheral injection for studying the migratory behavior in my preliminary experiments. This tendency is consistent with the results in eels; the dipsogenic effect of AngII injected into the circulation is only transient, and is followed by profound and prolonged inhibition of drinking in eels (Nobata et al., 2013). It is likely that the dipsogenic effect is masked by a secondary inhibition that is induced by baroreflex caused by concurrent hypertension. In my study, the effect of ICV AngII on the migration to water was not dose-dependent; 3×10^{-8} M of AngII was the most effective concentration for the initial 15-60 min after the injection, a duration which is similar to that shown in eels (Kozaka et al., 2003). After the injection of higher concentration of AngII (3×10^{-6} M), mudskippers frequently showed jumping behaviors possibly due to the increased blood pressure as shown in other teleosts (Fuentes and Eddy, 1998). Significant increases in period spent in water were also observed at 4 hrs and 8 hrs after the injection of 3×10^{-6} M and 3×10^{-10} M of AngII, respectively. These responses may represent a

secondary effect of injected AngII via elevated secretion of other hormones (e.g., vasotocin and cortisol) (Arnold-Reed and Balment, 1994; Saavedra, 1992) since these hormones also prolonged the period spent in water (Sakamoto et al., 2011; Sakamoto et al., 2015).

Since the behavioral analyses showed that AngII motivated the mudskipper to migrate to water as in tetrapods, it was suggested that the mudskipper possesses general thirst. Therefore, I examined reception areas of AngII in the mudskipper brain by localization of AT1 receptor and by c-Fos neuron abundance by immunohistochemistry. In these analyses, I focused on the OVLT-like structure in the diencephalon and the AP in the medulla oblongata, because these regions are known to lack the BBB in teleosts (Hamasaki et al., 2016; Takei, 2015). The sCVOs that lack the BBB are the sites that receive systemic hormones, and are important for the general thirst elicitation. The AT1 mRNA was located in the AP but not in the OVLT-like structure. Similarly, AngII injection increased the number of c-Fos immunoreactive neurons in the AP, but not in the OVLT-like structure. Therefore, in the mudskipper brain, it is highly probable that AngII acts directly on the hindbrain AP rather than the forebrain OVLT-like structure. These results coincided well with the studies in eels (Nobata et al., 2013; Nobata and Takei, 2011). The dipsogenic effects of AngII is diminished after AP lesion in eels (Nobata and Takei, 2011), but is not diminished after the lesioning of forebrain (Takei et al., 1979). The AP neurons send tyrosine hydroxylase (TH)-positive fibers to the GVC (Ito et al., 2006), which in turn control the upper esophageal sphincter (UES) muscle (Mukuda and Ando, 2003; Nobata et al., 2013). The UES muscle is the last gate of the alimentary tract and relaxation of the UES muscle initiates swallowing. Taken together with results of the previous (Ito et al., 2006) and present studies, I suggest that, in teleosts, AngII acts

directly on the AP neurons, which control the swallowing reflex. Although AngII-induced migration suggests an involvement of possible thirst mechanisms in the mudskipper forebrain, this behavior is likely not mediated by the general thirst.

Table 1. The accession numbers of genes used in the analysis

Gene name	Accession number
Mudskipper AT1	LC171725
Medaka AT1	XM_011488970
Medaka apelin receptor a	XM_004072521.3
Medaka apelin receptor b	XM_011481847.2
Japanese eel AT1	GU726141.1
Japanese eel AT2	GU726142.1
Japanese eel apelin receptor a	GU726143.1
Zebrafish AT1a	XM_017351685.2
Zebrafish AT1b	XM_005162528.4
Zebrafish AT2	NM_001013350.2
Zebrafish apelin receptor a	NM_001075105.1
Zebrafish apelin receptor b	NM_001030197.2
Zebrafish bradykinin receptor B1	NM_183073.2
Zebrafish bradykinin receptor B2	XM_009292772.3
Mouse AT1a	NM_177322.3
Mouse AT1b	NM_175086.3
Mouse AT2	NM_007429.5
Mouse apelin receptor	BC039224.1
Mouse bradykinin receptor B1	NM_007539
Mouse bradykinin receptor B2	NM_009747.2

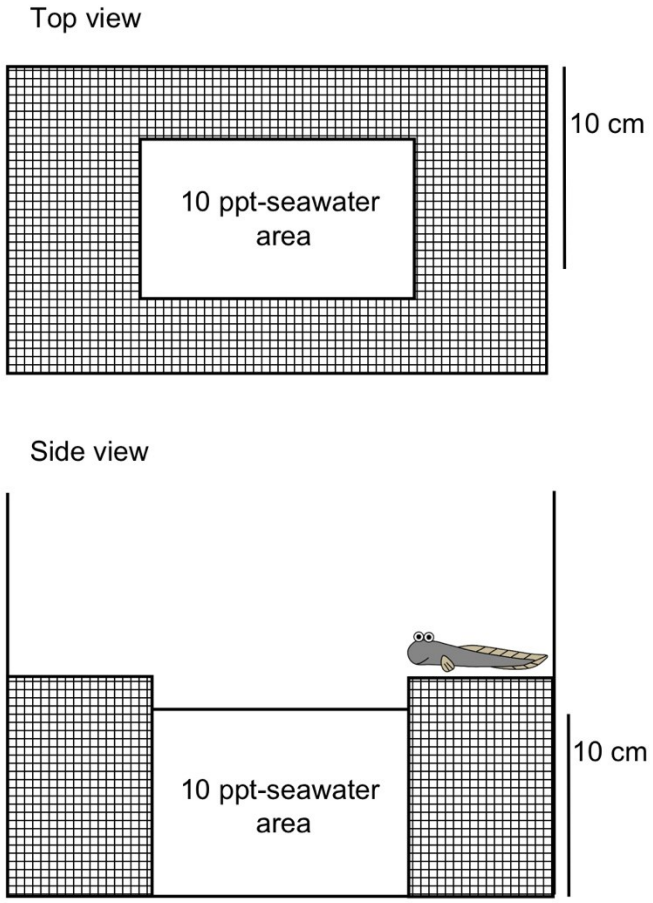

Figure 4. Schematic diagram of the experimental tank used for behavioral analysis. Brackish water (10-ppt seawater), which resembles natural environment of the mudskipper was filled in the water area. The mudskippers were allowed to migrate freely between the land area and water area.

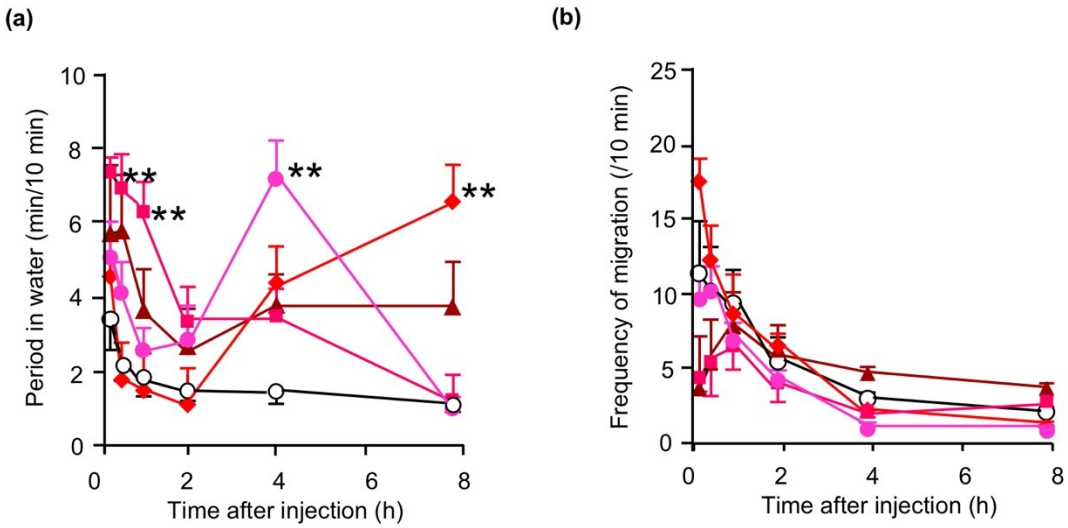

Figure 5. Changes in migration between the land area and water area after injection of AngII. The period of time in water (a) and the frequency of migration (b) were measured after intracerebroventricular (ICV) injection with 0.1 μl/g of 3×10^{-6} M (●, n = 6), 3×10^{-8} M (■, n = 5), 3×10^{-10} M (◆, n = 7), 3×10^{-12} M (▲, n = 7) of AngII, and vehicle (○, n = 8). The measurement was conducted at 0.25 hr (0.13-0.38 hr), 0.5 hr (0.25-0.75 hr), 1 hr (0.75-1.25 hr), 2 hr (1.75-2.25 hr), 4 hr (3.75-4.25 hr), and 8 hr (7.75-8.25 hr) after injection. Data are shown as mean ± s.e.m.; the absence of error bars indicates a small s.e.m. ** indicates significant difference among AngII groups versus vehicle group ($P < 0.01$).

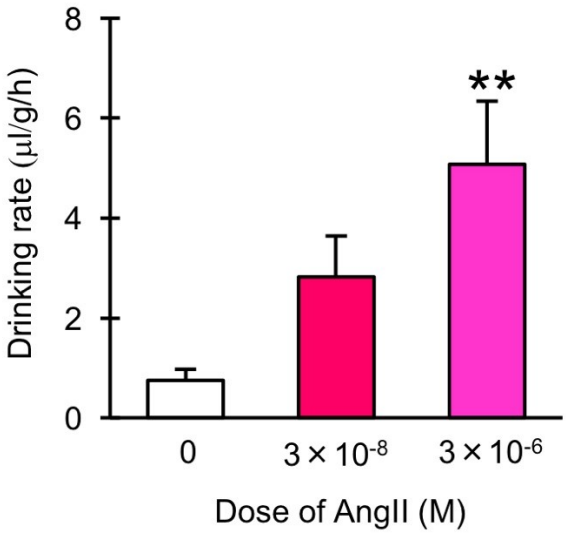

Figure 6. Effects of ICV injection of AngII on drinking rate. Fish were kept in a columnar tank (diameter-65 mm, height-92 mm) containing phenol red in 30% seawater for 1 hr after ICV injection. Data are shown as mean ± s.e.m. ** indicates significant difference among AngII groups versus vehicle group ($P < 0.01$).

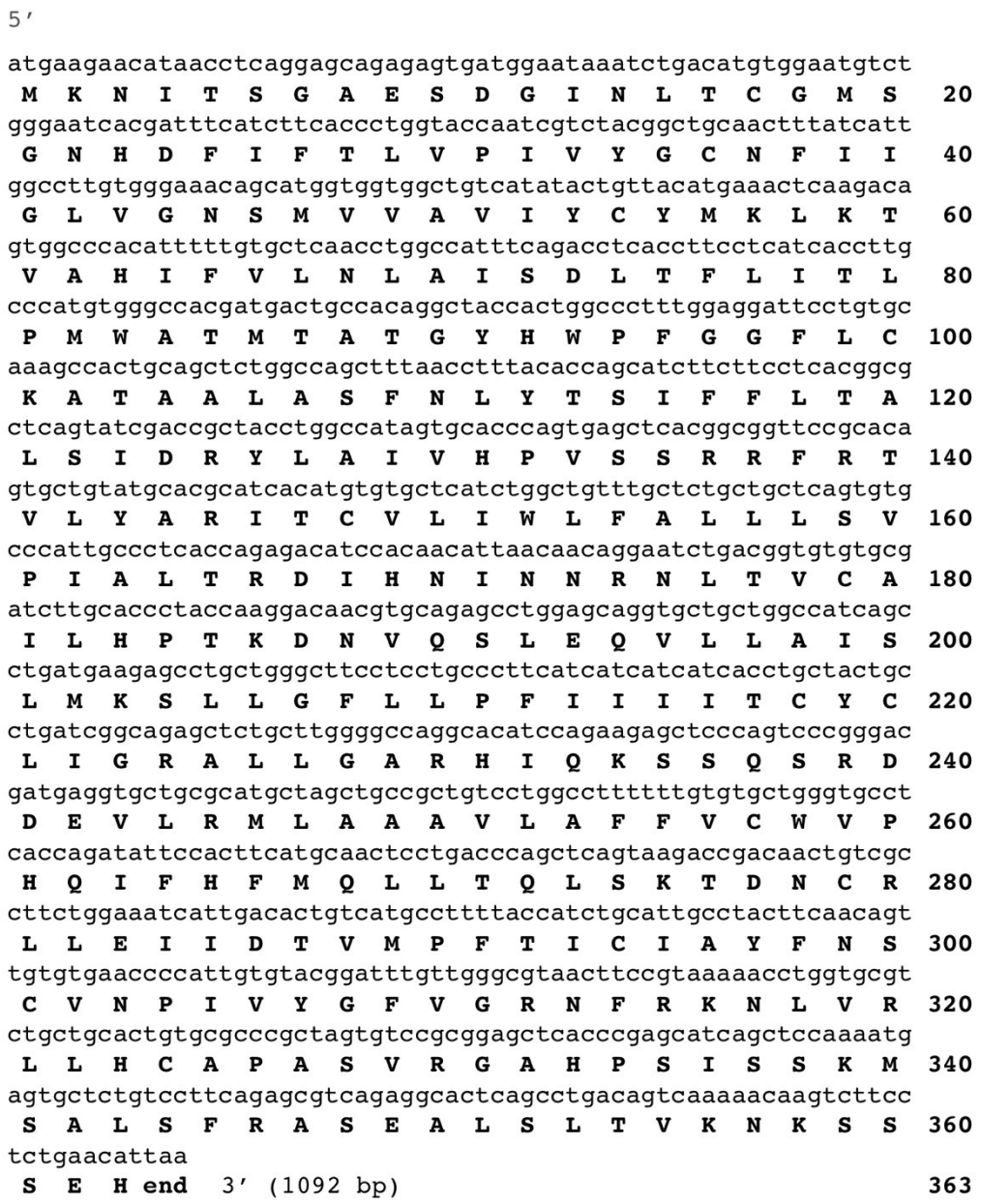

Figure 7. Nucleotide sequence of cloned cDNA encoding the mudskipper AT1 receptor. The coding region of the nucleotide sequence and the putative amino-acid sequence of mudskipper AT1 are shown. This nucleotide sequence was used for the design of probe for *in situ* hybridization (see text and Fig. 10).

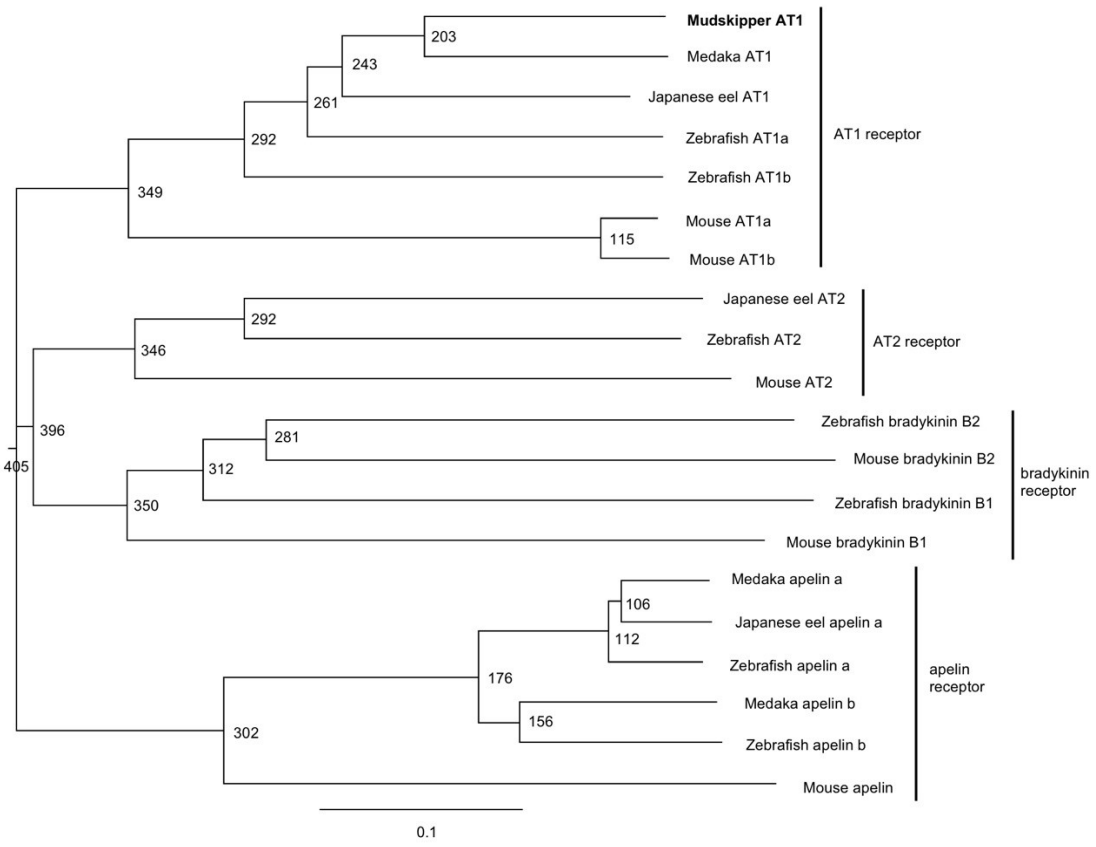

Figure 8. **The molecular phylogenetic tree of angiotensin receptors (type 1: AT1 and type 2: AT2), apelin receptors, and bradykinin receptors in vertebrates (mouse and teleosts).** Molecular phylogenetic tree was estimated by a neighbor-joining method. The number at each node represents bootstrap value in per-mil. Accession numbers of genes and mRNAs used in the analysis are listed in **Table 1**.

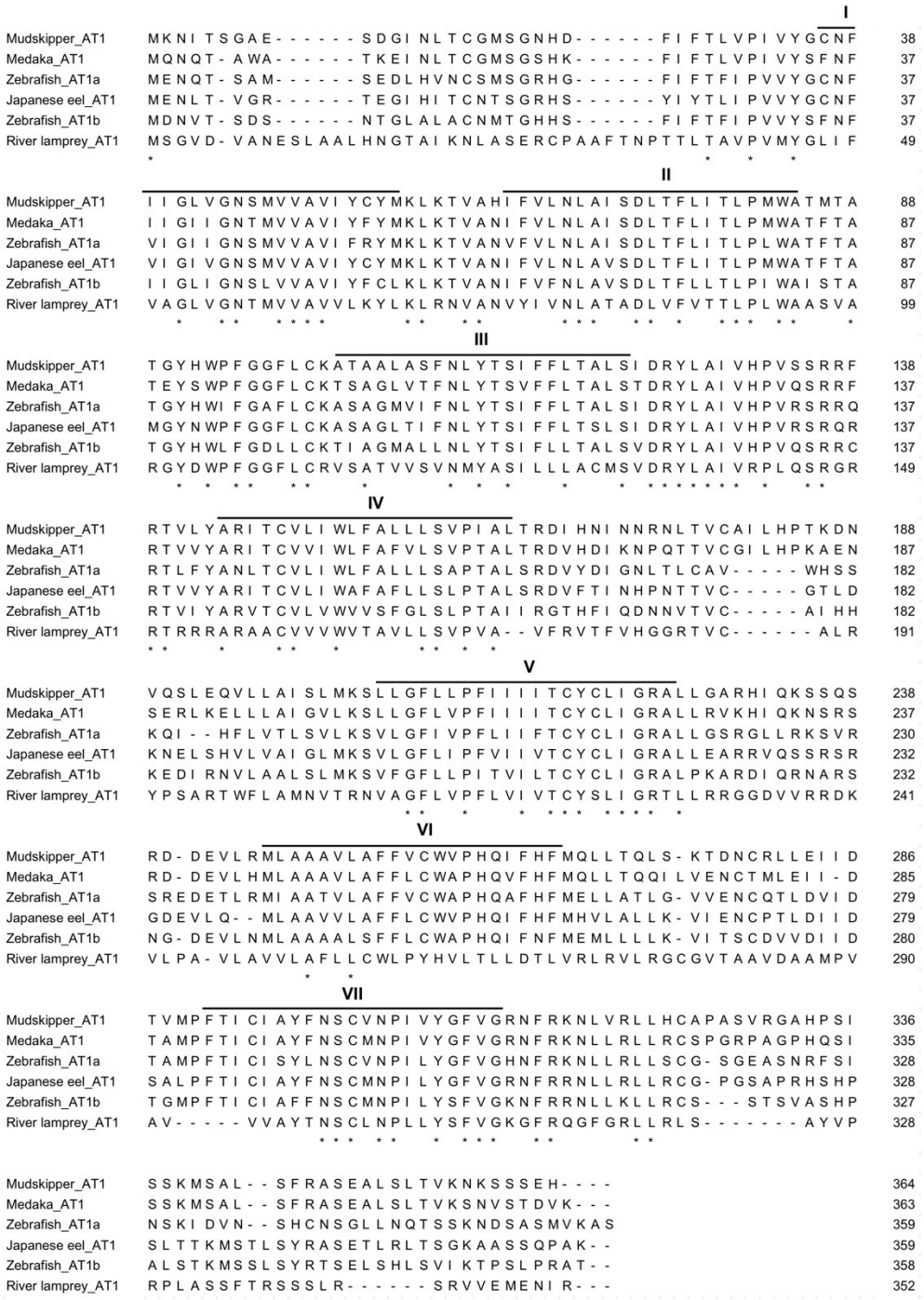

Figure 9. Comparison of the deduced amino acid sequences of fish AT1 receptors. Sequences of AT1 isoforms (zebrafish AT1a/AT1b) are incorporated. The 7 putative transmembrane domains are indicated with upperlines (I-VII).

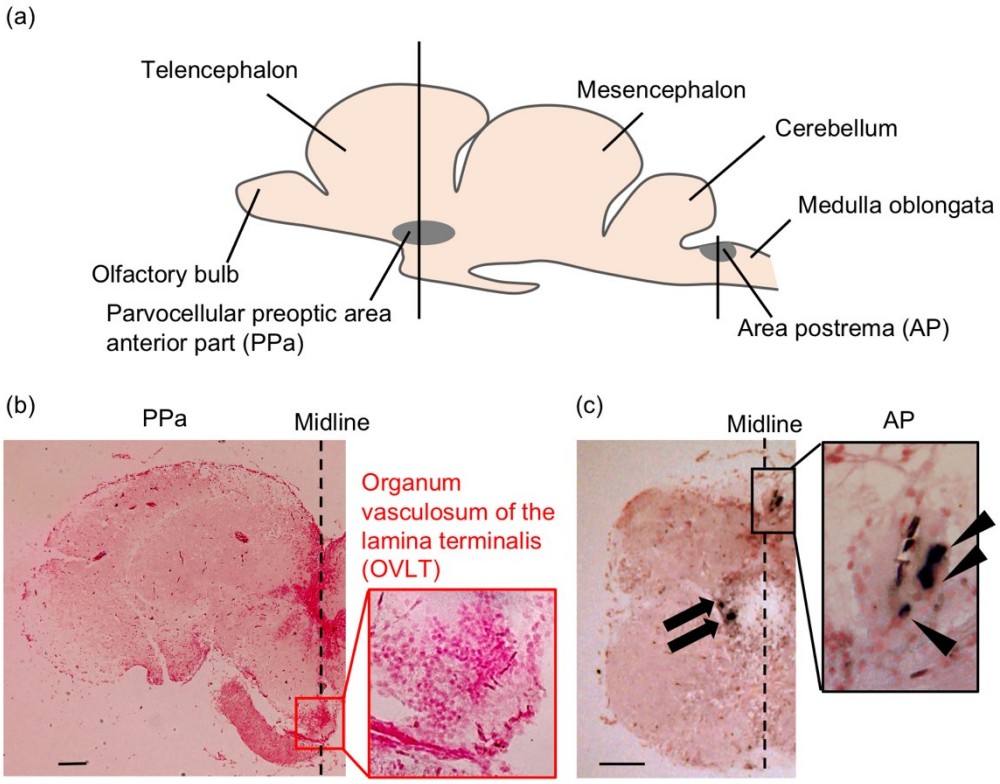

Figure 10. Localization of AT1 receptor-expressing neurons in the mudskipper brain. (a) Gross anatomy of the mudskipper brain at the mid-sagittal plane showing localization of the sensory circumventricular organs (sCVOs, gray-shaded regions). Vertical lines show the sites where cross sections were made. (b, c) *In situ* hybridization of AT1 mRNA. Dash lines indicate the midlines and arrows and arrowheads show the positive cells. No mRNA signal was detected in the parvocellular preoptic area anterior part (PPa) including the possible organum vasculosum of the lamina terminalis (OVLT, inset). Positive neurons (arrowheads) were detected in the area postrema (AP). Several positive neurons (arrows) were also found in the glossopharyngeal-vagal motor complex (GVC). The sections were counterstained with Nuclear Fast Red. Scale bars, 100 μm.

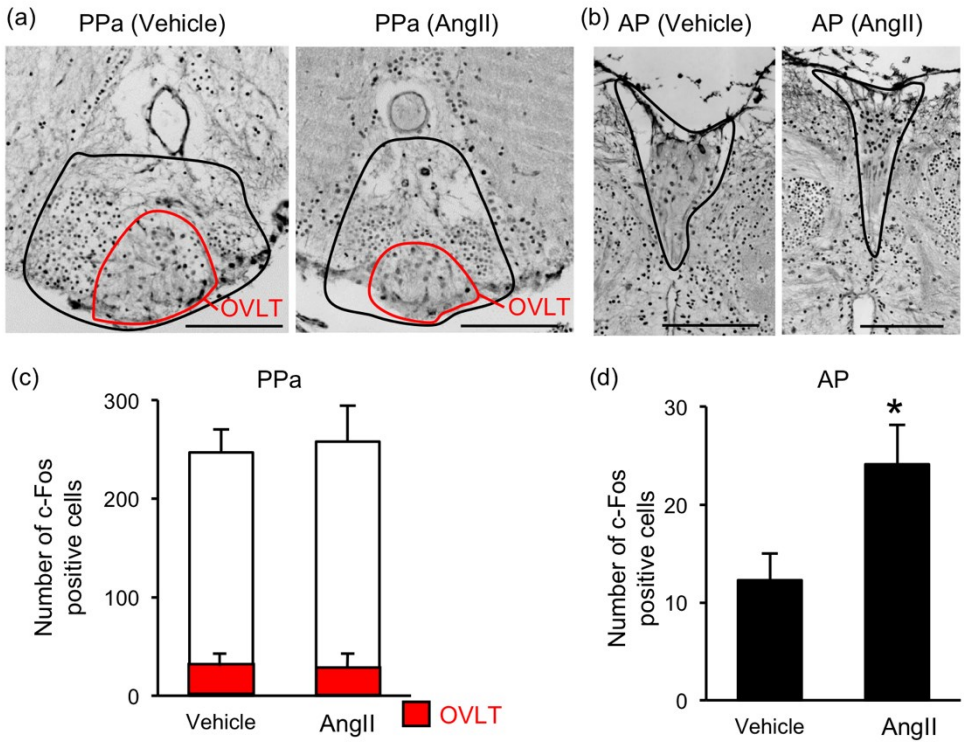

Figure 11. Changes in the number of c-Fos immunoreactive neurons after ICV injection of AngII in the PPa and the AP. (a, b) The c-Fos positive cells in the sCVOs after ICV injection with 0.1 μl/g of 3×10^{-8} M AngII or vehicle. Black lines indicate the regions of PPa (a) and the AP (b), while red lines indicate the OVLT within PPa (a). (c, d) Number of c-Fos positive cells in the PPa including the OVLT (red columns) and in the AP after the injection of AngII or vehicle. Data are shown as mean ± s.e.m. (n = 5). *P < 0.05 versus vehicle group. Scale bars, 100 μm.

Chapter 1B

Antidipsogenic effect of natriuretic peptides in the mudskipper

*このセクションは、今後刊行される予定の内容を含むため、現時点では公表できない。５年以内に出版予定である。

Chapter 2

Role of buccal water in a motivation for migration to water in the mudskipper

Abstract

In Chapter 1, I showed that angiotensin II (AngII) stimulated a sequence of drinking behavior in the mudskipper, but the site of action of AngII was considered to be the area postrema (AP), a sensory circumventricular organ in the hindbrain. Therefore, I hypothesized that AngII would enhance swallowing of water and the depletion of buccal/opercular water may motivate migration to water for buccal refilling. To test this hypothesis, I examined effects of artificial removal of buccal/opercular water on the migratory behavior. X-ray images of buccal/opercular water revealed that the mudskipper can swallow the stored water on land. By using a “drain tank”, it was confirmed that the swallowing of buccal/opercular water is enhanced by intracerebroventricular (ICV) injection of AngII on land. Artificial removal of buccal/opercular water by piercing holes in the opercula prolonged the aquatic stay. Similarly, absorption of the buccal/opercular water by dry resin significantly shortened the latency of the first migration into water. These results support my hypothesis that depletion of buccal/opercular water on land facilitates the migration to water in the amphibious mudskipper, possibly by the dry sensation in the buccal/opercular cavity. This regulatory mechanism is most likely homologous to the local thirst (or anticipatory thirst) found in mammals.

Introduction

Restoration of total body water after dehydration largely depends on drinking water controlled by thirst, and on water retention by antidiuresis. These responses were primarily regulated by humoral factors such as plasma osmolality and osmoregulatory hormones including angiotensin II (AngII) and natriuretic peptides (NPs). In mammalian brain, these hormones act on the sensory circumventricular organs (sCVOs) in the forebrain and control the thirst (Fitzsimons, 1998). This central circuit caused by general dehydration has been thus defined as general thirst (Cannon, 1918).

In Chapter 1A and 1B, I revealed that AngII and atrial NP (ANP) are dipsogenic and antidipsogenic hormones, respectively in mudskippers, as shown in other aquatic teleost fish (Kobayashi et al., 1983; Tsukada et al., 2005). Furthermore, the intracerebroventricular (ICV) injection of AngII prolonged the aquatic stay (Chapter 1A), and this was the first finding in fish that AngII motivated migration to water. My results thus suggest that the amphibious mudskipper has “thirst”. Since AngII increased the motivation for migration to water, I examined whether AngII acted on the sCVO in the diencephalon of mudskipper. However, the results of Chapter 1A showed that the site of action of AngII is not the sCVOs in the forebrain, but the area postrema (AP), a sCVO in the hindbrain. Then, how did AngII motivate the migration to water?

The mudskipper stores water in the buccal and opercular cavities when they are on land. Buccal/opercular water prevents the gills from desiccation and maintains the gill function such as ion regulation and respiration (Sayer, 2005; Tamura et al., 1976). The ICV-injected AngII, which acts directly on the AP neurons, enhanced swallowing of water. If this occurs on land, buccal/opercular water will be depleted. I hypothesized that

depletion of buccal/opercular water by AngII-induced swallowing on land may motivate mudskippers to move into water. To test this hypothesis, I examined effects of artificial removal of buccal/opercular water on migration to water.

Methods

Animals.

One year-old mudskippers of both sexes (*P. modestus*) weighing 3 to 5 g were collected and maintained as described in the Methods of Chapter 1A. For injection and sampling, fish were anesthetized in 0.01% tricaine methanesulfonate (Sigma, Tokyo, Japan) neutralized with sodium bicarbonate. All experiments were approved by the Animal Experiment Committee of the University of Tokyo, and performed in accordance with the manuals prepared by the committee.

X-ray imaging of swallowing and buccal refilling.

The real-time drinking behavior was observed with microfocus X-ray angiography system (HITEX MFX-80, Hitex, Osaka, Japan) by modifying the method used in the previous research (Sonobe et al., 2015). Mudskippers were put into 100-mm polystyrene culture dishes with covers. In the dish, the mudskipper could move horizontally, but could not jump. From the side of dish, diluted seawater containing iodinated contrast medium (Iomeron 350; Bracco-Eisai, Tokyo, Japan) to make the water radiopaque was gradually supplied through a tube using a syringe pump (PHP4400 Programmable, Harvard Apparatus, MA) in the X-ray angiography system. During each cine-scan, monochromatic X-rays at 80 kV passed through the mudskipper, and the images were recorded at 30 frames/s for 3-5 minutes. Still images taken by the X-ray camera were

converted to movies by Image J (http://imagej.nih.gov/ij/).

ICV injection of AngII.

ICV injection of AngII was conducted as described in the Methods of Chapter 1A.

Measurement of swallowing volume induced by AngII on land.

After ICV injection of 3×10^{-8} M AngII or vehicle, fish were put in a 1,000-ml Büchner funnel (TPP rapid 1000, TPP Techno Plastic Products, Switzerland) containing aerated diluted seawater (10 ppt, 149 mM Na^+, 176 mM Cl^-, 3.8 mM Ca^{2+}, 346 mOsml/kg, 500 ml) with 0.004% phenol red (Sigma-Aldrich). The water was completely drained by aspiration without disturbing the fish in 15 min after the injection, and then mudskippers were subsequently allowed to stay without water for additional 45 min. The first group ("in water", n = 6) was sampled at 15 min, just after the water was drained. The second group of fish ("on land", n = 6) was sampled after 60 min to measure volume of ingested water. Fish were sacrificed with deep anesthesia, and the amount of water in the whole gastrointestinal tract was measured (Kobayashi et al., 1983). Briefly, the whole tracts were removed on a petri dish and washed by 1 ml saline. The 0.5-ml samples were mixed with 0.5 ml 5% trichloroacetic acid (Sigma-Aldrich), and centrifuged at 10,000 rpm for 5 min. The supernatant was mixed with 0.5 ml of 1 M NaOH, and absorbance was determined at 550 nm by a spectrophotometer (DU640, Beckman Coulter, CA, USA).

Artificial removal of buccal/opercular water.

After anesthesia, a hole was drilled in each side of opercula, and a polyethylene tube (0.8 mm ID, 1.6 mm OD, Natsume, Tokyo, Japan) was inserted into the hole (Fig. 21a). The site of the hole was determined according to the previous report that showed the water storage capacity of the opercular cavity (Michel et al., 2015) and the X-ray image showing buccal/opercular water mainly stored in the opercular cavity (Fig. 21b). Sham operation

controls were prepared by sealing the tube with instant glue. After surgery, fish were allowed to recover overnight. Each fish was placed in the water area of experimental tank (Fig. 4). The land area was made of plastic mesh, and care was taken to ensure that there was minimum water on the area. Water in the tank was constantly aerated. The period in water and the frequency of migration between water and land area (defined as the "frequency of migration") were recorded for 30 min (n = 6). Alternatively, buccal/opercular water was removed by filling the buccal and opercular cavities with 0.05 g of dry Sephadex G-25 resin (GE health care, Tokyo, Japan) after light anesthesia. As controls, fish were treated with the same amount of the resin pre-incubated with 0.9%-NaCl. After treatments, fish were placed on the land area to test migration behavior (Fig. 4). After the recovery from anesthesia, the latency for migration into water was recorded. In this experiment, same individuals were used in both control and treatment experiments (n = 8).

Statistics analyses.

The data are expressed as means ± s.e.m. Kyplot 5.0 (KyensLab, Tokyo, Japan) was used for statistics analyses. The amount of drinking was analyzed with two-way ANOVA. Behavioral data were analyzed by non-parametric Wilcoxon single-rank test.

Results

Effects of AngII on swallowing of buccal/opercular water on land.

X-ray observation using a contrast medium successfully visualized buccal/opercular water. When the upper esophagus sphincter was relaxed, water held in the buccal/opercular cavity and in the most anterior part of the oesophagus (Fig. 3 and 21b)

flowed into the intestine; the mudskipper does not have a stomach and the oesophagus is connected directly to the proximal intestine. In the drain tank experiment (Fig. 22), fish drank approximately 2 µl (/g fish/15 min) during the "in water" phase. There was no difference in the ingested water volume between the vehicle-injected group and the AngII-injected group. During the following "on land" phase, no increase in the volume of ingested water was observed in the vehicle-injected group. On the other hand, the AngII-injected group ingested more water during the "on land" phase (Fig. 22). Since all water was drained from the tank in this phase, fish must have swallowed water stored in the buccal/opercular cavity.

Effects of artificial removal of buccal/opercular water on the migratory behavior.

In the piercing experiment, the period in water and the frequency of migration between water and land areas were recorded for 30 min, because long-term recording of behavior without buccal/opercular water could be stressful to fish. The artificial removal of buccal/opercular water by piercing holes in both sides of the opercular skin (Fig. 21a) significantly prolonged the period in water compared with controls with sealed holes (Fig. 23). The frequency of migration between water and land areas was not changed by artificial removal of buccal/opercular water (Fig. 23). In addition, effects of artificial removal of buccal/opercular water were further tested by placing water-absorptive dry resin in the buccal/opercular cavity. In this experiment, the latency of the first migration into water was measured. The depletion of buccal/opercular water with dry resin significantly shortened the latency of the first migration into water compared with controls with wet resin (no absorption of water occurs) (Fig. 24).

Discussion

In this chapter, I first confirmed that mudskippers store water in the buccal/opercular cavity on land by using X-ray imaging, as suggested by a previous study (Michel et al., 2015). I also showed that AngII enhanced swallowing of buccal/opercular water on land in the mudskipper. The treatments that reduced buccal/opercular water stimulated mudskippers to move to water area, supporting my hypothesis that AngII-induced swallowing of buccal/opercular water on land may motivate migration to water for buccal refilling. In the mudskipper, it is highly possible that migration to water is directly stimulated by the sensory detection of buccal/opercular drying. This behavioral regulation can be categorized as local thirst that motivates buccal refilling in the sequence of drinking behaviors.

Swallowing of buccal/opercular water on land implies that buccal/opercular water is important as storage of drinking water, in addition to roles to prevent the gills from desiccation and to maintain the gill function for ion regulation and respiration. Furthermore, in other mudskipper species (*Periophthalmus barbarus*), it has recently been shown that buccal/opercular water is used as a protruding and retracting "hydrodynamic tongue" during the initial capture and subsequent intra-oral transport of food (Michel et al., 2015). Buccal/opercular water is crucial for mudskippers to survive on land, and mudskippers refill buccal/opercular water soon after the water is depleted.

The results of Chapter 1 and 2 revealed that the mudskipper does not have the general thirst, but has local thirst. Local thirst is probably stimulated by sensory mechanism(s) in the buccal/opercular cavity as suggested in mammals (Krashes, 2016; Zimmerman et al., 2016). Recent studies reaffirmed the importance of local thirst as an anticipatory thirst

that prevents future water deficits in mammals (Krashes, 2016; Zimmerman et al., 2016). However, little is known about the underlying molecular mechanism for water sensation. In fruit fly, sodium channel family, PPK28, is considered to be an osmosensing ion channel that mediates the cellular and behavioral response to water (Cameron et al., 2010). In mice, it was recently reported that PKD2L1-expressing acid-sensing receptor cells, which were previously suggested as the sour taste sensors, mediate taste responses to water (Zocchi et al., 2017). In zebrafish, genes of PKD family are expressed in regions that may correspond to taste receptors (England et al., 2017). Taste receptor cells expressing PKD family in the buccal/opercular cavity might be involved in sensory detection of water in the mudskipper.

It is reported in mammals that oropharyngeal water and its drying are detected by afferent fibers of the Vth, IXth and Xth cranial nerves (Norgren, 1991; Norgren and Smith, 1988). The signals from these afferent fibers are relayed to the forebrain, such as the SFO and the limbic system, and regulate thirst (Denton et al., 1999; Norgren, 1991; Zimmerman et al., 2016). In eels, the Xth cranial nerve (vagus nerve) that innervates the buccal/opercular cavity is critical for anticipatory drinking in response to Cl^- in the buccal/opercular water (Mayer-Gostan and Hirano, 1976). Similarly, the cranial nerves may sense the presence of buccal/opercular water also in the mudskipper. In mammals, oropharyngeal-forebrain neural signaling is involved to regulate the secretion of vasopressin (VP), as well as the thirst, without changes in systemic water balance (McKenna and Thompson, 1998; Norgren, 1991; Stricker and Hoffmann, 2005; Stricker and Stricker, 2011; Thrasher et al., 1981). This anticipatory mechanism has recently been investigated, and it was showed that ingestion of water and learned sensory cues predicting water lowered the activities of the VP secreting neurons (Mandelblat-Cerf et

al., 2017). In addition to the anticipatory mechanism, VP neurons play an important role in anticipatory thirst (Gizowski et al., 2016). In the mudskipper, ICV injection of vasotocin induced migration to water (Sakamoto et al., 2015), suggesting that vasotocin neurons are involved in the regulation of local thirst also in the mudskipper. Taken together, the signal from the cranial nerves may be relayed to the forebrain to induce local thirst in the mudskipper as shown in mammals.

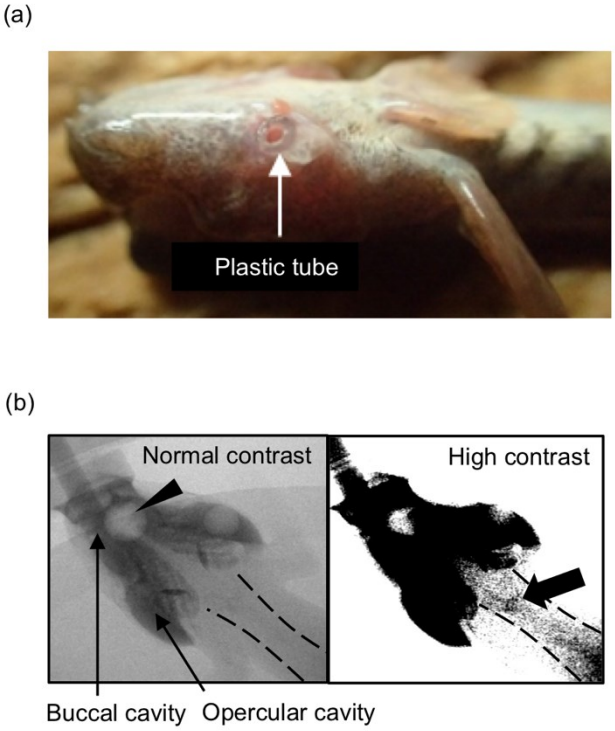

Figure 21. Representative pictures showing piercing surgery and buccal/opercular water. (a) A picture showing an implanted plastic tube in the opercular cavity of the mudskipper to drain buccal/opercular water. (b) X-ray images showing the presence of water (deep gray) in the buccal and opercular cavities. The arrowhead indicates air in the cavities. The arrow in the high-contrast image shows water in the most anterior part of the oesophagus just before swallowing. Broken lines outline the gastrointestinal tracts.

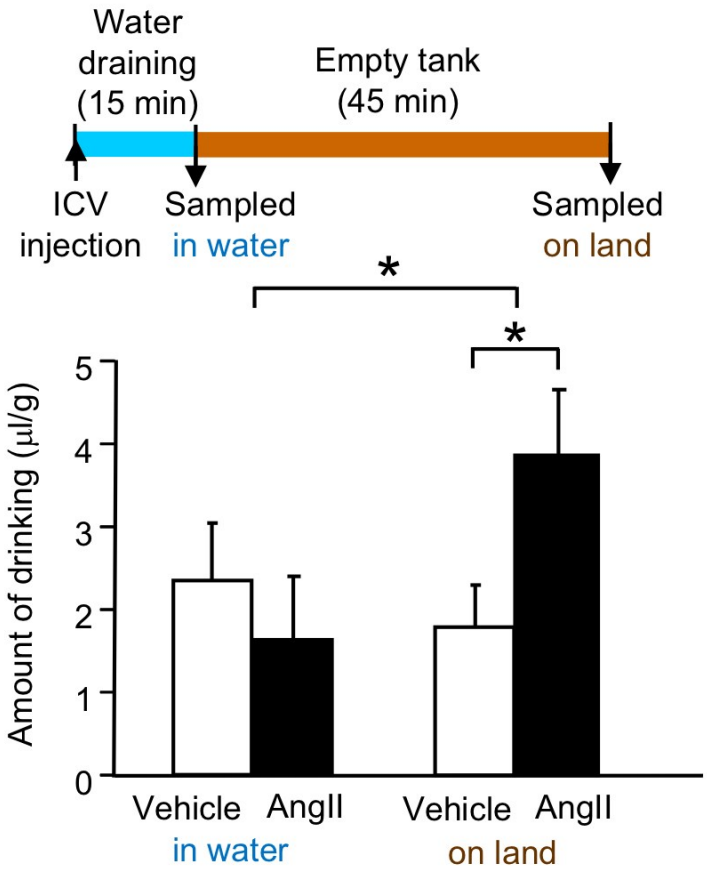

Figure 22. Effects of ICV injection of AngII on the amount of drinking on land.
The diagram shows the experimental design. After the ICV injection of AngII or vehicle, water in the tank was completely drained out within 15 min. Fish were sampled at 15 min and 60 min, and the volume of ingested water was measured. Mudskippers can retain water in the buccal/opercular cavity and drink it even in the empty tank. Data are shown as mean ± s.e.m. * indicates a significant interaction and a significant difference among AngII group ("on land") versus vehicle group ("on land"). $P < 0.05$, n = 6.

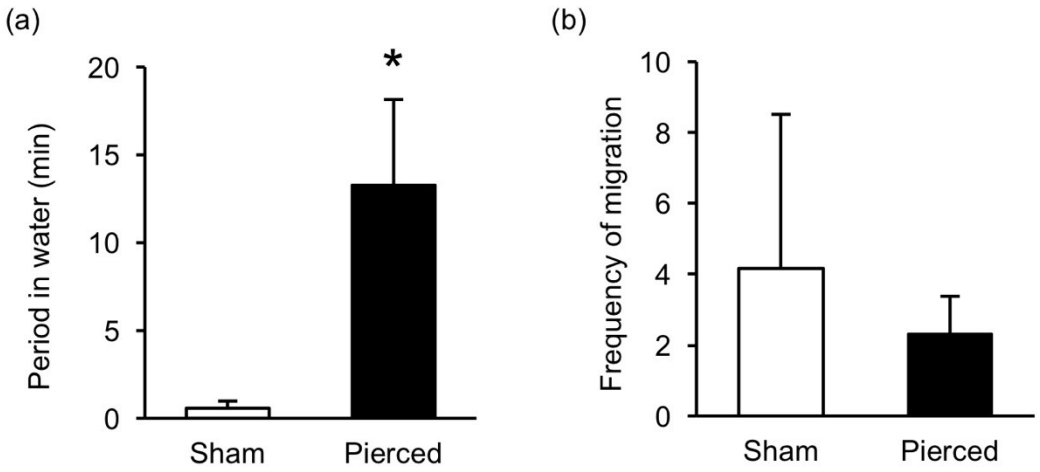

Figure 23. Effects of artificial removal of buccal/opercular water by piercing on migratory behavior. The opercular skin was pierced to drain buccal/opercular water (see Fig. 21), and the migratory behavior was observed. Sham controls were prepared by sealing the plastic tubes. Period in water (a) and frequency of migration (b) were measured for 30 min. Data are shown as mean ± s.e.m. *$P < 0.05$ versus control, n = 6.

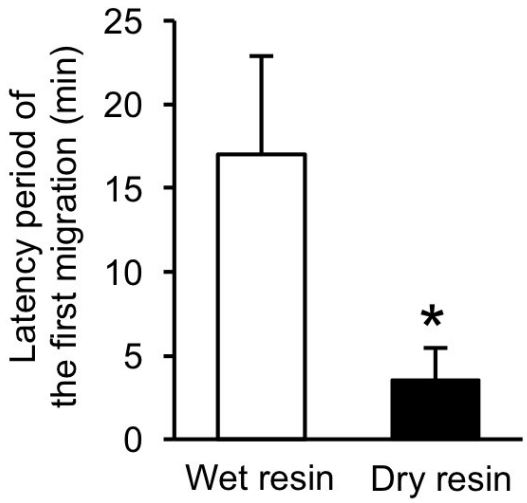

Figure 24. Effects of removal of buccal/opercular water by inserting water-absorptive dry resin on migratory behavior. Control fish were treated with the water pre-incubated resin ("wet resin"). In this experiment, latency period for the first migration to water was measured. Data are shown as mean ± s.e.m. **P* < 0.05 versus control, n = 8.

Chapter 3

Hormonal control of buccal refilling behavior and salt appetite in the mudskipper

*このセクションは、今後刊行される予定の内容を含むため、現時点では公表できない。５年以内に出版予定である。

General Discussion

To understand the evolution of thirst mechanism in vertebrates, I have focused on an amphibious ray-finned fish, the mudskipper, as a research model in my thesis. My results showed that angiotensin II (AngII) and atrial natriuretic peptide (ANP) are dipsogenic and antidipsogenic hormones, respectively, which regulated the migratory behavior to water in this fish. It is of great interest whether the mudskipper possesses thirst as a motivation for the hormonally induced drinking behavior. In tetrapods, systemic AngII stimulates the subfornical organ (SFO) regions in the forebrain to induce thirst (Simpson and Routtenberg, 1973; Takei, 1977; Uchiyama, 2015), and such mechanism is known as general thirst (Cannon, 1918). Another type of thirst evoked by oropharyngeal cues (e.g., dry mouth) is local thirst (Cannon, 1918). In my investigations, I discovered the unique drinking behavior of the mudskipper, in which swallowing of buccal water was accelerated by AngII on land as well as in water. The migration to water appears to be directly stimulated by local sensation of buccal/opercular drying, and thus categorized as local thirst. In the General Discussion, I compared the thirst mechanism of the mudskipper with the known mechanisms of terrestrial tetrapods and attempted to offer a hypothesis about the process of thirst emergence during land invasion of vertebrates.

Finding of local thirst in the mudskipper and its involvement in anticipatory motivation.

In tetrapods, general thirst is induced by increases in systemic AngII and Na^+ levels ($[Na^+]$), the most potent dipsogenic hormone thus far known in all vertebrates including fish (Fitzsimons, 1998; Kobayashi et al., 1979; McKinley and Johnson, 2004; Takei, 2000). In mammals, birds and amphibians, systemic AngII binds to the receptor in the sensory circumventricular organs (sCVOs) in the forebrain that lack the blood-brain

barrier to induce a sequence of drinking behaviors (Simpson and Routtenberg, 1973; Takei, 1977; Uchiyama, 2015). The organum vasculosum of the lamina terminalis (OVLT) and SFO are known to be forebrain sCVOs that are crucial for monitoring [Na^+] and dipsogenic action of AngII (Johnson and Buggy, 1978; McKinley, 2003; Matsuda, 2017). In aquatic teleost fish, however, the forebrain sCVOs were not implicated in elicitation of drinking, since removal of the whole forebrain did not affect the drinking induced by seawater exposure (Hirano et al., 1972) or by AngII stimulation (Takei et al., 1979) in eels. The area postrema (AP) in the hindbrain is proposed to be the sCVO for receiving systemic AngII (Nobata et al., 2013).

In my thesis, central administration of AngII motivated the amphibious mudskipper to move to water, suggesting the presence of thirst in fish. Recently, OVLT-like structure was histologically identified in the diencephalon of the mudskipper (Hamasaki et al., 2016), but my studies did not support the direct action of AngII in this region. Nax channel, coded by *Scn7a* gene, specifically expressed in the glial cells (ependymal cells and astrocytes) of the SFO and OVLT as a brain [Na^+] sensor, and was involved in regulation of AngII-induced thirst (Matsuda, 2017). However, a brain [Na^+] sensor has not been identified in fish (Zakon, 2012). I also revealed that AngII acts on the AP to increase swallowing of buccal/opercular water on land, and the loss of buccal/opercular water motivated mudskippers to move to water for buccal refilling. This behavior could be evoked by local sensation of buccal drying, which is equivalent to the local thirst in tetrapods. Such local sensation is referred to a motivation for drinking known as anticipatory thirst, which occurs before blood-osmolality fluctuates in mammals (Zimmerman et al., 2016). In the amphibious mudskipper, the local thirst may contribute to an anticipatory mechanism to prevent potential dehydration on land. Although

migratory behavior induced by local sensation has not been observed in aquatic fish, it is well recognized that eels detect an increase of Cl^- concentration in the buccal/opercular water, which enhances swallowing of water (Hirano, 1974). This "chloride response" could prevent future dehydration in hyperosmotic marine environments, and thus was referred to as an anticipatory drinking (Hirano, 1974). The sensation of buccal ions in aquatic fish may be similar to the sensation of buccal water underlying local thirst of tetrapods and mudskippers. In basal vertebrates such as river lamprey (*Lampetra fluviatilis*), the transfer from seawater to freshwater rapidly decreased drinking rate without a change in plasma osmolality (Rankin, 2002). Thus, the anticipatory-drinking mechanism by local sensation appears to be widely distributed among vertebrates (Fig. 29).

If the local sensation for anticipatory drinking would be connected with the behavioral output of migration to water, local thirst could have emerged in terrestrial and amphibious vertebrates. The mudskipper, which evolved from the aquatic teleosts to invade the terrestrial environment (Ord and Cooke, 2016; You et al., 2014), has developed local thirst, in addition to the AngII-induced swallowing at the medulla oblongata (Fig. 29). The phylogenetically distant vertebrates (ray-finned fish and tetrapods) appeared to acquire local thirst that expedited a sequence of drinking behaviors when they were exposed to a desiccative environment. In addition to the osmoregulatory purpose, local thirst also prevents the gills from desiccation, and maintains the gill function for ion regulation and respiration in the mudskipper. Thus, moistening the gill could be one of the selection pressure for the development of the local/anticipatory thirst.

Similar to other aquatic teleost fish, my results on AngII receptor and neuronal activity after the injection of AngII and ANP revealed that mudskippers appear not to

have general thirst. In contrast, terrestrial tetrapods with pulmonary respiration cannot physically store water readily available for drinking, and thus they must move to water source driven by the AngII-induced thirst in the forebrain (Hillyard et al., 1998; Kobayashi et al., 1979; Nobata et al., 2013). The general thirst is likely a characteristic of terrestrial tetrapods and may have been acquired when they lost their gills during the evolution (Fig. 29).

The mudskipper as a unique model to investigate the mechanism of sensory detection of buccal water.

The molecular and neural mechanisms regulating local thirst remain unclear in the mudskipper. In mammals, it was recently suggested that acid-sensing receptor cells expressing PKD2L1, which was previously identified as the sour-taste sensors, mediate taste responses to water (Zocchi et al., 2017). Oropharyngeal water and dryness are detected by afferent fibers of the Vth, IXth and Xth cranial nerves (Norgren, 1991; Norgren and Smith, 1988) and the signals are relayed to the forebrain, such as the SFO and the limbic system, to regulate thirst (Denton et al., 1999; Norgren, 1991; Zimmerman et al., 2016). Also in "chloride response" of eels, the Xth cranial nerve (vagus nerve) that innervates the buccal/opercular cavity is critical for detection of Cl^- in buccal/opercular water (Mayer-Gostan and Hirano, 1976). In mammals, several studies have demonstrated that such neural mechanisms are involved in the regulation of antidiuretic vasopressin secretion as well as the thirst regulation without changes in systemic water balance (Figaro and Mack, 1997; McKenna and Thompson, 1998; Norgren, 1991; Stricker and Stricker, 2011; Thrasher et al., 1981). The vasopressin neurons that innervate the OVLT have recently been shown to play an important role in anticipatory thirst (Gizowski et al.,

2016). In the mudskipper, injection of vasotocin, the teleost homologue of vasopressin, induced migration to water (Sakamoto et al., 2015), suggesting an involvement of vasotocin neurons in the regulation of their thirst. Together with mammalian studies, it is possible that the signal derived from the cranial nerves is relayed to the forebrain to induce local thirst in the mudskipper. The SFO neurons monitor blood factors for the regulation of general thirst, as well as mediate the sensation of oropharyngeal cues in mammals (Zimmerman et al., 2017; Zimmerman et al., 2016). In the mudskipper, however, the local thirst regulation in the forebrain appears to be independent from the major site of AngII action in the medulla oblongata (Fig. 29). Therefore, the local thirst regulation can be specifically analyzed in this fish by excluding the impact of general thirst. Together with the less complicated fish brain and recent publication of genome databases of mudskipper species (You et al., 2014), mudskippers might become a unique and excellent model to investigate the thirst mechanism.

Local thirst followed by buccal refilling behavior may contribute to land invasion in semi-terrestrial vertebrates.

In the present thesis, I suggest that local thirst may play a critical role in the early stage of land invasion of vertebrates. The mudskipper lives in brackish tidal flats, which may be similar to the habitats of early tetrapods that diversified to invade land habitats (Graham and Lee, 2004; Long and Gordon, 2004; Ord and Cooke, 2016; Schultze, 1999). Tidal pools often dry up in the seasonal drought of the Devonian period, and such environments imposed selection pressures for “walking” and the acquisition of limbs to move to adjacent pools to acquire water (Romer, 1967). Similarly, such selection pressures might have led to the emergence of local thirst as a motivation to move to water.

Acanthostega and *Tiktaalik*, the Devonian vertebrates, are possible ancestral tetrapods and still possessed gills (Clack, 2002; Daeschler et al., 2006). Buccal/opercular water held by the mudskipper and possibly by ancestral tetrapods may originally prevent the gills from desiccation, but also allow them to stay on land longer as a source of drinking water. It conferred protection against dehydration and thus might contribute to land invasion of vertebrates. Understanding the early emergence of local thirst should provide a fresh insight into the characters acquired during the land invasion of vertebrates. More evidence is required in various vertebrates including aquatic fish and amphibious lungfish to generalize the functions and mechanisms of local thirst in the vertebrate evolution (Fig. 29).

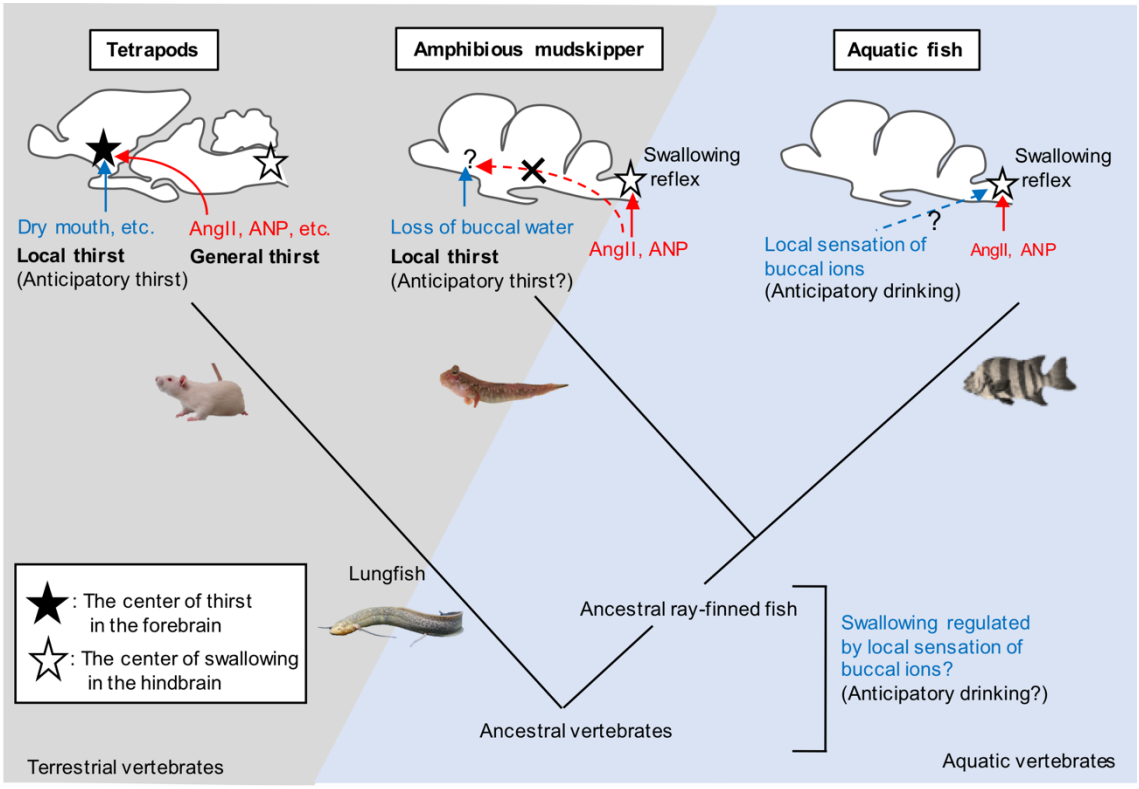

Figure 29. Schematic diagram showing evolution of the thirst mechanisms proposed in my thesis. Black and white stars indicate the brain regions controlling thirst and swallowing reflex, respectively. In terrestrial tetrapods, dipsogenic angiotensin II (AngII) and antidipsogenic atrial natriuretic peptide (ANP) directly act on the forebrain to regulate general thirst. The oropharyngeal signals (e.g., dry mouth) also reach to the forebrain to induce local thirst (anticipatory thirst). These signals are integrated in the forebrain and regulate motivation for migration to water. On the other hand, in aquatic and the amphibious ray-finned fish, AngII and ANP act primarily on the medulla oblongata to induce swallowing of buccal water. In addition, in the amphibious mudskipper, loss of buccal water on land induces local thirst to evoke migration to water, possibly through the forebrain. Because local thirst exists both in tetrapods and in the amphibious fish, this thirst appears to be important for invasion onto the land during the evolution of vertebrates. Drinking by local sensation occurs in teleosts (e.g., eel) and basal vertebrates (e.g., river lamprey) as an anticipatory drinking. Thus, the anticipatory mechanism by local sensation may be universally important for osmoregulation in vertebrates.

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