A possible relationship between brain-derived adrenomedullin and oxytocin in the regulation of sodium balance

Meghan M White and Willis K Samson

Department of Pharmacological and Physiological Science, St Louis University School of Medicine, 1402 South Grand Boulevard, St Louis, Missouri 63104, USA

(Correspondence should be addressed to W K Samson; Email: samsonwk@slu.edu)

Abstract

Exaggerated thirst and salt appetite occurs when endogenous, brain-derived adrenomedullin (AM) production is compromised. In addition, the arginine vasopressin (AVP) response to hypovolemia is compromised. We hypothesized that AM acts in the hypothalamus to control oxytocin (OT) release and that the inhibitory action of AM on salt appetite is mediated via its effects on OT release in the rat. When plasma tonicity was elevated with sodium, ribozyme-induced compromise of central AM production significantly blunted the release of OT into plasma. OT responses to elevation of plasma osmolality without concomitant change in plasma sodium levels were not altered by compromise of AM production. Thus, brain-derived AM controls OT release in response to altered plasma sodium levels.

Furthermore, central AM-induced inhibition of NaCl intake can be reversed by pretreatment with an OT antagonist, and the increase in NaCl appetite seen following ribozyme compromise of central AM can be attenuated with central OT administration. These data support the hypothesis that endogenous, brain-derived AM is an essential participant in the hypothalamic response to hypernatremia via its actions on OT-expressing neurons. Together with our previous reports of the effects of AM on AVP secretion and ingestive behaviors, our results suggest that endogenous AM is a physiologically relevant regulator of the endocrine and behavioral mechanisms that maintain fluid and electrolyte homeostasis.

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Introduction

Adrenomedullin (AM), a potent vasoactive peptide originally identified in pheochromocytoma extracts, is now known to be produced in many sites including brain where multiple actions have been described (Hinson *et al.* 2000). AM has been shown to alter thirst, salt appetite, and food intake when given into brain. I.c.v. administration of AM inhibited water intake in rats stimulated by overnight fluid restriction (Murphy & Samson 1995). Selective compromise of brain-derived AM production with a ribozyme, a catalytic RNA, led to exaggerated water consumption in both the euvoletic and driven states (Taylor & Samson 2002, Taylor *et al.* 2005). Similarly, i.c.v. administration of AM inhibited salt intake in a two-bottle preference test (Samson & Murphy 1997), while antisense compromise of central AM increased salt appetite above control levels (Samson *et al.* 1999). Central administration of AM also inhibited food intake, although larger doses were needed to see the food inhibition than were used to study water and salt intake (Taylor *et al.* 1996). Nevertheless, ribozyme compromise of endogenous AM led to increased food intake in *ad libitum* fed rats (Taylor *et al.* 2005). These results led us to hypothesize that endogenous, brain-derived AM is a physiologic regulator of fluid and electrolyte balance.

In addition to actions on ingestive behaviors, AM has also been shown to exert effects on the release of hormones that can alter fluid and electrolyte balance. There are several reports that i.c.v. administration of AM increased *c-fos* mRNA and FOS protein in many areas of the hypothalamus, including the paraventricular nucleus (PVN) and supraoptic nucleus (SON; Serino *et al.* 1999, Ueta *et al.* 2000). Both the PVN and SON contain neurons that produce arginine vasopressin (AVP) and/or oxytocin (OT) and project not only to the posterior pituitary gland, but also elsewhere within the central nervous system (CNS). While AVP is well known to control fluid balance by its antidiuretic action, OT, particularly when acting as a neurotransmitter, has been implicated as an important regulator of sodium balance (Balment *et al.* 1980, Van Tol *et al.* 1987, Blackburn *et al.* 1992a,b, 1993, 1995, Verbalis *et al.* 1995, Amico *et al.* 2001, Fitts *et al.* 2003, Rigatto *et al.* 2003). We have previously demonstrated that brain-derived AM is a physiologic regulator of AVP secretion (Taylor *et al.* 2005). i.c.v. administration of AM stimulated AVP release, while ribozyme compromise of brain-derived AM prevented an appropriate AVP response to dehydration challenges (Taylor *et al.* 2005). Here, we examine the effects of AM within brain to regulate OT release and discuss the possible implications this has on mechanisms controlling sodium ingestion.

Plasma OT levels that can be correlated with central OT release have been demonstrated to change in response to increased plasma sodium levels. Chronic salt loading by increasing sodium intake in the diet led to increased *Oxt* (listed as *Oxt* in the MGI Database) mRNA in the PVN and SON (Van Tol *et al.* 1987, Dellmann *et al.* 1988, Ozaki *et al.* 2004). In addition, it appeared that there was a decrease in immunoreactive OT in the terminals of SON and PVN neurons, suggesting that more OT was being released into plasma and CNS sites (Dellmann *et al.* 1988). Indeed, i.v. infusion of hypertonic saline or chronic salt loading in rats led to increased plasma OT levels (Balment *et al.* 1980, Stricker & Verbalis 1986, 1996, Van Tol *et al.* 1987, Verbalis & Dohanics 1991, Verbalis *et al.* 1995). Not only does sodium stimulate OT production and release, but also OT acting within CNS sites inhibits further NaCl consumption, providing a negative feedback loop to maintain sodium homeostasis. i.c.v. administration of OT decreased sodium intake in a two-bottle preference test (Blackburn *et al.* 1992a,b). Rats treated with an OT antagonist (Blackburn *et al.* 1992a, Fitts *et al.* 2003), with a ricin OT conjugate designed to destroy OT-responsive neurons (Blackburn *et al.* 1995), and OT knockout mice (Rigatto *et al.* 2003, Ozaki *et al.* 2004) all displayed an exaggerated sodium appetite suggesting that endogenous OT is necessary for the normal control of Na⁺ intake. Based on the similar actions of AM and OT to regulate Na⁺ intake and the AM-induced Fos staining in OT neurons, we hypothesized that endogenous, brain-derived AM stimulates OT release and that AM inhibits sodium intake by activating OT-producing neurons.

Materials and Methods

Animals

Male Sprague–Dawley rats (*Rattus norvegicus*, 225–250 g, Harlan, Indianapolis, IN, USA) were housed individually with a 12 h light:12 h darkness cycle (0600–1800 h). Animals had free access to food and water. All animal procedures were approved by the St Louis University Animal Care Committee. Each animal was implanted with a right lateral cerebroventricular cannula as previously described (Samson & Murphy 1997) under ketamine/xylazine anesthesia (60 mg/kg Ketaset, Fort Dodge Animal Health, Fort Dodge, IA, USA; 8 mg/kg TranquiVed, Vedco, Inc., St Joseph, MO, USA), and animals were administered buprenorphine analgesia (0.05 mg/kg, Buprenex, Reckitt & Colman Ltd, Richmond, VA, USA). Animals were allowed to recover for a minimum of 5 days or until they returned to presurgery bodyweight. Cannula patency was tested by a dipsogenic response to 50 pmol angiotensin II (2 min of continuous drinking within 5 min of angiotensin II administration), 2–3 days prior to experimentation.

Peptides and ribozymes

Rat AM, OT, (d(CH₂)₅, Tyr (Me)₂, Orn₈)-vasotocin (OVT, OT antagonist), and angiotensin II were purchased from

Phoenix Pharmaceuticals, Inc (Burlingame, CA, USA). AVP and OT for RIA standards and iodination were also purchased from Phoenix Pharmaceuticals. A 2-O-methylated control ribozyme containing scrambled base antisense arms or 2-O-methylated AM-specific ribozyme (Taylor & Samson 2002) was purchased from IDT, Inc (Coralville, IA, USA). Peptides and ribozymes were resuspended in sterile 0.9% saline at an appropriate concentration prior to injection.

Oxytocin

Central AM-induced changes in plasma OT levels were examined by i.c.v. administration of 2 µl saline vehicle or vehicle containing 128, 256, or 1280 pmol AM in saline. Groups of rats were killed by rapid decapitation at 5, 10, 20, or 30 min following the injection, and trunk blood was collected. Effects of decreased endogenous AM levels in brain on plasma OT content were monitored by administering a ribozyme specific to *Am* (listed as *Adm* in the MGI Database) mRNA i.c.v. (Taylor & Samson 2002). Two treatment groups were employed: one treated with the control scrambled ribozyme (2 µg in 2 µl saline) and another administered the AM-specific ribozyme (2 µg in 2 µl saline; Taylor & Samson 2002). Eighteen hours following ribozyme administration, when hypothalamic AM protein levels were decreased by ~30% (Taylor & Samson 2002), rats were killed by decapitation and trunk blood was collected as before (Taylor *et al.* 2005). Because decreased plasma levels of OT were predicted following central AM ribozyme treatment, OT levels were elevated in rats by i.p. administration of 1 m/l NaCl (Sigma) or 2 m/l mannitol (Sigma; Blackburn *et al.* 1993). One group of rats was treated with saline vehicle or 256 pmol AM i.c.v. 10 min prior to receiving an i.p. injection of NaCl or mannitol (2 ml/rat). A second group was treated with the control ribozyme or the AM-specific ribozyme (i.c.v.) 18 h prior to NaCl or mannitol administration. Ten or thirty minutes after NaCl or mannitol was given, rats were killed by decapitation and trunk blood collected into heparinized tubes. Plasma was obtained from all samples by centrifugation (3000 g, 10 min, 4 °C) of whole blood samples and was frozen until OT and AVP levels were measured by RIA as previously described (Samson 1985, Samson *et al.* 1985). Data are expressed as pg hormone/ml plasma (mean ± S.E.M). For the analysis of significance between AM dose–response and NaCl ribozyme experiments, significance was determined by ANOVA with *post hoc* Scheffé's testing. All other experiments were analyzed at each time by independent samples *t*-tests with a probability of *P* < 0.05 considered significant.

Hypothalamo-neurohypophysial system explants

Rats (300–350 g) were killed by rapid decapitation, and a tissue explant containing the supraoptic nuclei and their neuronal projections to the posterior lobe of the pituitary was dissected as previously described (Sladek & Joynt 1979, Samson *et al.* 1987). The rostral limit of the explant was the

suprachiasmatic–supraoptic continuum. The caudal limit was arcuate nucleus at the exit of the pituitary stalk. Included in the hypothalamo–neurohypophyseal system (HNS) explants are the SON, ventral portions of the anterior hypothalamus and the medial preoptic area, as well as the rostral pole of the arcuate nucleus. The explants were immersed in 2 ml control medium (Dulbecco's Minimum Essential Medium (BioWhittaker, Inc., Walkersville, MD, USA) containing 0.1% w/v BSA (Sigma) and 1% v/v penicillin/streptomycin (Invitrogen)) and incubated at 37 °C in 5% v/v CO₂. Medium was replaced hourly for 4 h prior to experimentation. Six consecutive 20 min test periods were then conducted on each explant: 1, control medium; 2, control medium; 3, 100 nm/l AM in control medium; 4, control medium; 5, control medium; 6, 60 nm/l KCl in control medium. Medium from each test period was collected and frozen until subsequent measurement of OT content by RIA (Samson *et al.* 1985). Peptide release during test periods 2–6 was expressed as percent of OT released in test period 1, and differences among the groups were determined by ANOVA with *post hoc* Bonferroni testing.

Salt appetite

Salt appetite was examined with a two-bottle preference test (Stricker 1968). Rats were acclimated to two drinking bottles, one with tap water and the other with 0.3 m/l NaCl, for several days prior to experimentation. Hypovolemia was induced in rats by administering polyethylene glycol (PEG, Carbowax PEG 20 000; Fisher Scientific, Pittsburgh, PA, USA; 15% w/v in 0.9% w/v NaCl) subcutaneously in conjunction with 18 h of water and food restriction. Animals were anesthetized by isoflurane gas inhalation (3% v/v in O₂ for induction, 2% v/v in O₂ for maintenance of anesthesia, IsoSol, Vedco, Inc.), a small area on their back was shaved and cleaned with surgical scrub, and rats were injected subcutaneously with 5 ml of warmed (37 °C) PEG solution. At this time, one group of rats was treated i.c.v. with the control ribozyme, while a second group was administered the AM-specific ribozyme. Following 18 h of food and water restriction, PEG-treated rats were given free access to two drinking bottles containing tap water or 0.3 m/l NaCl, and cumulative intakes were measured every half an hour for 5 h, after which time food was returned to the cages, and again at 24 h after the return of fluid.

To determine whether AM's actions on sodium appetite were dependent upon OT release, a second series of salt appetite studies was conducted. Hypovolemia was induced with the PEG protocol as before. Eighteen hours following PEG treatment in conjunction with food and water restriction, one group of rats was treated with either saline or OVT, the OT antagonist (i.c.v., 10 µg/5 µl). Five minutes later, a second i.c.v. injection was made delivering either saline vehicle or 256 pmol AM. After 10 more minutes, salt and water bottles were returned to the cage and intakes were measured as before. In a second study, rats were treated i.c.v.

with either the control ribozyme or the AM-specific ribozyme at the same time as PEG administration. Eighteen hours later, a second i.c.v. injection of saline or OT (10 µg/5 µl) was given. Ten minutes later, salt and water bottles were returned and cumulative intakes were measured as before. Cumulative NaCl and water intakes are presented as means ± s.e.m. Significance between groups at each time was determined by ANOVA with Scheffé's *post hoc* testing or independent *t*-test when only two treatment groups were employed. A probability of <5% was considered significant.

Sucrose intake

Ad libitum fed and watered rats were given access to a 10% w/v sucrose solution for 8 h daily (0800–1600 h). Sucrose intakes were monitored and recorded at the end of each session. On the day of experimentation, rats were given an i.c.v. injection of saline vehicle or saline containing 256 pmol AM. Ten minutes later, they were given free access to the sucrose solution for 8 h as before. Total sucrose intake for each rat was recorded at the end of the test period and compared with the animal's sucrose intake on the previous day. Significance was determined by paired *t*-test, with *P* < 0.05 considered significant.

Results

In contrast to previously published data on AM's effects on AVP (Taylor *et al.* 2005), i.c.v. administration of 256 pmol AM into euvoletic, iso-osmotic rats did not significantly alter plasma OT levels compared with those in saline vehicle-injected rats at any time point examined (Fig. 1a). Doses of AM ranging from 128 to 1280 pmol had no significant effect on plasma OT levels (Fig. 1b). Similarly, treatment with the AM ribozyme to decrease brain-derived AM levels did not significantly alter plasma OT levels in normally hydrated rats: control ribozyme 52.3 ± 2.8 pg OT/ml plasma, *n* = 17; AM ribozyme 60.2 ± 3.9 pg OT/ml plasma, *n* = 11; *P* = 0.102.

Rats were challenged with hypertonic saline to determine whether they could mount an appropriate OT response when central AM levels were altered. Ten (Fig. 2a) and 30 (Figs 2a and 3a) minutes following i.p. administration of 2 ml of 1.0 M NaCl plasma OT levels were significantly elevated. I.c.v. administration of AM prior to the NaCl injection did not significantly alter the plasma OT levels compared with control-treated rats at 10 or 30 min after salt loading (Fig. 2a). Similarly, AM administration did not alter the AVP response to salt loading (Fig. 2b). However, when animals were pretreated with the AM ribozyme i.c.v. to decrease endogenous AM levels 18 h prior to NaCl administration, the OT response to salt loading was inhibited (Fig. 3a). While a decrease in endogenous AM levels did not completely reverse the OT response to hypertonic saline, the OT rise was significantly attenuated (*P* < 0.001 versus control ribozyme, 1.0 M NaCl). Again, the AVP rise to the salt load

Figure 1 i.c.v. administration of adrenomedullin (AM) did not significantly alter plasma oxytocin levels under basal conditions. At various time points following AM administration, trunk blood was collected and plasma oxytocin levels measured by RIA. (a) Time course of changes in plasma oxytocin levels following i.c.v. administration of 256 pmol AM or saline vehicle. $n=13$ each. (b) Plasma oxytocin levels 10 min following i.c.v. administration of saline vehicle or various doses of AM. $n=13$ each.

was not significantly altered by AM ribozyme pretreatment, even though there was a trend toward lower AVP levels in the AM-compromised rats (Fig. 3b). Unlike the hyperosmotic and hypernatremic stimulus provided by i.p. NaCl administration, mannitol when injected i.v. or i.p. will raise plasma osmolality but decrease plasma Na^+ levels (Blackburn *et al.* 1993). When mannitol was employed as the hyperosmotic stimulus, pretreatment with the AM ribozyme did not inhibit the OT response: control ribozyme 68.0 ± 4.4 pg OT/ml plasma, $n=13$; AM ribozyme 63.0 ± 4.1 pg OT/ml plasma, $n=13$; $P=0.406$. The AVP response to mannitol similarly was unaltered by pretreatment with the AM ribozyme: control ribozyme 7.2 ± 1.0 pg AVP/ml plasma, $n=13$; AM ribozyme 10.5 ± 3.5 pg AVP/ml plasma, $n=13$; $P=0.391$.

HNS explants were used to examine whether AM had direct effects on OT-secreting neurons. AM, in concentrations as high as 100 nm/l, failed to significantly alter OT

release from the explants. KCl treatment did, however, result in a large release of OT, suggesting that the HNS explants were intact and viable (Table 1).

Next, we sought to determine whether AM's inhibitory effects on salt appetite in a two-bottle preference test were due, at least in part, to release of OT. In the first experiment, PEG-treated rats were administered either saline or the OT antagonist, OVT, i.c.v., followed by a second injection of saline or AM. As previously reported (Samson & Murphy 1997), animals treated with saline followed by AM consumed significantly less NaCl than the saline/saline-treated controls did (Fig. 4a). Treatment with the OT antagonist alone did not significantly alter the amount of NaCl intake, although there was a trend towards elevated sodium consumption. However, pretreatment with OVT followed by AM reversed the ability of AM to inhibit salt appetite (Fig. 4a). In addition, i.c.v. administration of AM inhibited water intake (Fig. 4b). However, OVT pretreatment failed to alter the inhibitory effect of AM on water consumption. The effects of ribozyme compromise of central AM on sodium appetite mirrored

Figure 2 Failure of i.c.v. administration of adrenomedullin (AM) to alter the oxytocin response to hypertonic saline. Rats were administered saline vehicle or 256 pmol AM i.c.v. 10 min prior to receiving an i.p. injection of 2 ml of 1 M NaCl. At 10 and 30 min after saline administration, trunk blood was collected, and plasma oxytocin (a) and vasopressin (b) levels were measured by RIA. $n=12$ each.

Figure 3 Effects of ribozyme compromise of brain-derived adrenomedullin (AM) levels on the oxytocin response to hypertonic saline and mannitol. Central AM levels were compromised by AM ribozyme pretreatment (i.c.v.) 18 h prior to i.p. administration of 2 ml of 1 m/l NaCl, 2 m/l mannitol or isotonic saline. Thirty minutes after the i.p. injection, trunk blood was collected, and plasma oxytocin and vasopressin levels were measured by RIA. (a) Ribozyme compromise of brain-derived AM attenuated the oxytocin rise to hypertonic saline. $n=15$ each. (b) Central AM ribozyme pretreatment did not significantly alter the vasopressin rise to 1 m/l NaCl. $n=15$ each. ** $P<0.01$, *** $P<0.001$ versus control 0.15 M NaCl-injected rats; # $P<0.001$ versus control 1 m/l NaCl.

those seen with AM antisense (23); rats treated with the AM ribozyme had significantly exaggerated intake of 0.3 M NaCl (Fig. 5a). Water intake also tended to increase following AM ribozyme treatment, although significance was not attained until 24 h after bottles were returned to the animal's cages (Fig. 5b). In another experiment, rats were pretreated with the control ribozyme or the AM-specific ribozyme followed by saline or OT administration prior to monitoring NaCl intakes. Again, compromise of endogenous AM in brain resulted in exaggerated NaCl intake (Fig. 6a). Animals treated with the AM ribozyme followed by treatment with OT had a significant reversal of the stimulatory effects of AM loss on salt

intake (Fig. 6a). Again, there was no significant effect of central AM compromise on water intake during the two-bottle preference test (Fig. 6b). OT similarly did not alter water intake in either the scrambled ribozyme-treated rats or AM ribozyme-treated rats.

In addition to inhibiting saline intake, i.c.v. administration of AM also inhibited the intake of 10% w/v sucrose (Table 2). Rats acclimated to the presence of sucrose readily consumed the solution during the 8 h it was present each day. When rats were treated with saline i.c.v., there was no significant difference in that 8 h sucrose intake when compared with the previous day's intake. However, rats treated centrally with 256 pmol AM consumed significantly less sucrose than during the previous day.

Discussion

AM mRNA and protein are highly expressed in the hypothalamus (Ueta *et al.* 1995, Shan & Krukoff 2001), and AM colocalizes with OT and AVP in the PVN and SON (Ueta *et al.* 1995, Serino *et al.* 1999). In addition, i.c.v. administration of AM has been shown to induce *c-fos* expression in OT and AVP neurons (Serino *et al.* 1999), and AM alters the electrical activity of OT and AVP neurons in slice preparations (Follwell & Ferguson 2002). We have previously shown that endogenous, brain-derived AM is a potent regulator of the AVP system and is necessary for the proper AVP response to hypovolemia (Taylor *et al.* 2005). Here, we suggest that AM is also an important regulator of the OT system, specifically in the hormonal response to hypernatremia. Our current findings support the hypothesis that brain-derived AM is a physiologic regulator of sodium balance and that AM exerts its effects at least in part by altering OT secretion.

Under basal conditions, AM failed to alter OT release in a time- or concentration-dependent manner (Fig. 1a and b). We have previously shown that similar doses of AM caused a time-dependent elevation of plasma AVP levels

Table 1 Adrenomedullin (AM) failed to stimulate oxytocin release from hypothalamo-neurohypophyseal system explants. $n=8$

Treatment	Percentage of release during first incubation period (control media)
Second incubation period (control media)	107 ± 20
Third incubation period (100 nm/l AM)	119 ± 14
Fourth incubation period (control media)	158 ± 42
Fifth incubation period (control media)	127 ± 21
Sixth incubation period (60 mm/l KCl)	3521 ± 547, $P<0.001$

Figure 4 Central adrenomedullin (AM)-induced inhibition of salt appetite requires oxytocin. Eighteen hours prior to experimentation, rats were administered 5 ml of a 15% polyethylene glycol solution subcutaneously in conjunction with overnight food and water restriction. Animals were treated with either saline or OVT, the oxytocin antagonist (i.c.v., 10 μ g/5 μ l). Five minutes later, a second i.c.v. injection was made delivering either saline vehicle or 256 pmol AM. After 10 more minutes, salt and water bottles were returned to the cage, and salt (a) and water (b) intakes were measured. $n=10$. * $P<0.05$, ** $P<0.01$ versus saline/saline-treated rats.

(Taylor *et al.* 2005), suggesting that the amount of AM given was sufficient to determine whether AM would alter plasma OT levels. Our OT findings are in agreement with another group's data, which demonstrated that i.c.v. administration of AM did not alter plasma OT levels in an unstressed or stressed rat (Mimoto *et al.* 2001). However, Serino *et al.* (1999) have reported that doses of AM nearly 10 times the amount used in most of our studies (10 μ g) will significantly stimulate OT release at 5 and 10 min following i.c.v. administration, without altering levels of mRNA. Neither the total amount of AM in the hypothalamus or brain nor the amounts in the synaptic clefts surrounding magnocellular neurons of the PVN have been reported, so we cannot speculate on which doses of AM

are physiologically relevant. However, it is clear that doses of AM that do stimulate AVP release when given i.c.v. (Taylor *et al.* 2005) do not stimulate OT release in our hands.

Using a ribozyme, we can decrease brain-derived AM levels by $\sim 30\%$ without altering AM expression in other tissues (Taylor & Samson 2002). This seemingly small decrease in central AM levels has already been shown to result in potent physiologic changes in rats (Taylor & Samson 2002, Taylor *et al.* 2005). Here, we report that i.c.v. administration of AM failed to stimulate OT release in our hands (Fig. 1a and b), and compromise of endogenous AM within brain using a ribozyme also did not alter basal plasma OT levels. In addition, ribozyme compromise of AM production did not alter plasma AVP levels in the basal state despite the pharmacologic ability of AM to stimulate AVP

Figure 5 Ribozyme compromise of brain-derived adrenomedullin (AM) led to exaggerated salt intake. Hypovolemia was induced in rats by administering 5 ml of 15% polyethylene glycol (PEG) subcutaneously in conjunction with 18 h of water and food restriction. At this time, one group of rats was treated i.c.v. with the control ribozyme, while a second was administered the AM-specific ribozyme. Eighteen hours later, PEG-treated rats were given free access to two drinking bottles containing either 0.3 m/l NaCl (a) or tap water (b), and cumulative intakes were measured. $n=10$ each. * $P<0.05$, ** $P<0.01$ versus scrambled ribozyme-treated rats.

Figure 6 i.c.v. administration of oxytocin reversed the exaggerated salt intake seen in AM ribozyme-treated rats. Hypovolemia was induced in rats by administering 5 ml of 15% w/v polyethylene glycol subcutaneously in conjunction with 18 h of water and food restriction. At this time, one group of rats was treated i.c.v. with the control ribozyme, while a second was administered the AM-specific ribozyme. Following 18 h of food and water restriction, rats were administered saline vehicle or oxytocin (10 µg/5 µl) i.c.v. Ten minutes later, they were given free access to two drinking bottles containing either 0.3 M NaCl (a) or tap water (b), and cumulative intakes were measured. $n=10$. * $P<0.05$, *** $P<0.001$ versus AM ribozyme+saline; # $P<0.05$ versus scrambled ribozyme+saline.

release (Taylor *et al.* 2005). In fact, the effects of decreased endogenous AM levels on plasma AVP concentrations were only observed when AVP release was experimentally stimulated (Taylor *et al.* 2005).

Salt loading an animal will lead to significant increases in tissue *Or* mRNA levels and plasma OT concentrations (Balment *et al.* 1980, Stricker & Verbalis 1986, Van Tol *et al.* 1987, Dellmann *et al.* 1988, Verbalis & Dohinics 1991, Ozaki *et al.* 2004). The effects of altered central AM levels on hypertonic saline-stimulated plasma OT levels were evaluated. To induce a rapid change in plasma OT levels, rats were injected i.p. with 1 m/l NaCl. Administration of hypertonic saline, as expected, elevated plasma OT levels at 10 and 30 min after injection (Figs 2a and 3a). i.c.v. administration of

AM did not alter the OT response to hypertonic saline (Fig. 2a), a finding that is not surprising considering that AM did not alter plasma OT levels under basal conditions. Similarly, i.c.v. administration of AM did not alter the AVP response to hypertonic saline (Fig. 2b). This may be because AVP levels were elevated to such high levels by the hypertonic saline that the additional stimulus provided by AM administration was not able to be seen. In addition, we have previously reported (Taylor *et al.* 2005) that significant increases in AVP levels were only seen at 5 and 10 min after i.c.v. AM administration, with returns to control levels at 20 and 30 min following injection. Since we waited 10 min after AM injection to administer hypertonic saline, then 30 min after the saline to collect plasma, it is not surprising that we did not see any effects of AM on plasma AVP levels. Despite the failure of i.c.v. administration of AM to alter OT release, ribozyme compromise of central AM production significantly attenuated the OT rise following hypertonic saline (Fig. 3a), suggesting that brain-derived AM is necessary for a normal OT response to hypernatremia. This effect appears to be specific to OT since the AVP rise to hypertonic saline was not significantly affected by AM ribozyme pretreatment, although there was a trend towards lower AVP levels in AM ribozyme-treated animals (Fig. 3b).

It is possible that the effect of compromised central AM content on the OT response to hypernatremia was simply due to differences in the handling of sodium. However, we have previously reported (Taylor *et al.* 2005) that plasma osmolality and sodium concentration do not change from baseline levels 18 h following AM ribozyme administration and that these values are not different than those observed in scrambled ribozyme-treated rats. In fact, even during severe hypovolemia (water restriction and PEG treatment), plasma Na^+ levels were similar in AM ribozyme-treated rats and scrambled ribozyme-treated rats (Taylor *et al.* 2005). These data suggest that the handling of Na^+ is not different in ribozyme-treated rats and that brain-derived AM is important for the OT response to hypernatremia.

Plasma OT levels rise not only in response to hypernatremia *per se* but also to any hyperosmolar stimulus

Table 2 i.c.v. administration of adrenomedullin (AM) inhibits the intake of 10% sucrose in male rats. Male rats were given access to a 10% sucrose solution for 8 h daily. Cumulative intakes were measured at the end of each session. On the day of experimentation, rats were given an i.c.v. injection of 256 pmol AM or saline vehicle, and then given access to the sucrose solution for 8 h as before. Experimental intakes were compared with sucrose intake on the day prior to experimentation

	Preinjection intake (ml)	Postinjection intake (ml)
Treatment		
Saline	16.8 ± 4.6	14.6 ± 4.7
256 pmol AM	21.2 ± 5.5	11.6 ± 3.8*

$n=9$ each, * $P<0.05$ versus preinjection intake

(Stricker & Verbalis 1986, Verbalis & Dohanics 1991, Blackburn *et al.* 1993). A similar experiment to the hypertonic saline studies was conducted substituting hypertonic mannitol for saline. The compound mannitol can be used to raise plasma osmolality while decreasing plasma Na^+ concentrations (Blackburn *et al.* 1993). Interestingly, there was no difference in the plasma OT levels of AM ribozyme versus control-treated rats when given hypertonic mannitol. This finding should not be surprising, however, since we previously reported that AM ribozyme pretreatment did not affect the rise in plasma OT levels caused by several hyperosmotic, isonatremic stimuli such as overnight water restriction or PEG treatment (Taylor *et al.* 2005). These data in combination with our previous AVP findings (Taylor *et al.* 2005) suggest that there may be two populations of AM-producing neurons that independently regulate AVP and OT secretions. We hypothesize that one population of AM-producing neurons is regulated by changes in plasma volume and sends input to AVP-containing neurons, while a second population of AM-containing neurons responds specifically to changes in plasma Na^+ levels, and these neurons regulate OT secretion primarily.

Indeed, there are some correlations between AM, OT, and plasma Na^+ levels. It has been demonstrated that, like OT, AM levels will also increase with salt loading. One group demonstrated that after 28 days on a high-salt diet, rats had elevated plasma AM levels and increased expression of the AM receptor (Cao *et al.* 2003). It would be logical to conclude that AM levels in brain also must change or that plasma AM exerts actions on circumventricular organs of the brain in order for it to have effects on OT secretion. Indeed, it has been shown that substituting 2% w/v saline for the rat's normal drinking water led to a significant elevation of AM concentrations in the cerebrospinal fluid of rats (Chen *et al.* 2004). Furthermore, AM has been shown to exert effects within at least one circumventricular organ, the area postrema (Allen *et al.* 1997, Shan & Krukoff 2000), and it has been shown that area postrema lesions decrease the Fos-like immunoreactivity within the PVN of the hypothalamus observed following i.v. AM infusion (Shan & Krukoff 2000). Therefore, it is possible that AM from either peripheral or neuronal sources could affect OT secretion. Because i.c.v. ribozyme administration only alters brain-derived AM production (Taylor & Samson 2002), we would argue that AM from neuronal sources would have the major regulatory role in hypernatremic-induced OT secretion.

AM appears to act indirectly on OT-containing neurons since AM had no effect in HNS explants (Table 1), although once again this is not surprising given that i.c.v. administration of AM did not alter plasma OT concentrations (Figs 1a, b and 2a). A comprehensive study by Serino *et al.* (1999) demonstrated that i.c.v. administration of AM increased c-fos expression in OT-containing neurons, but saw no change in *Ot* mRNA levels at any time point examined following administration of AM. Changes in early gene expression can indicate cellular activation; however, they do not prove

a direct effect. Demonstration of AM receptors on or receptor subunit expression in OT-producing cells would be a more accurate indicator of potential direct cellular activation by AM. It has been demonstrated that magnocellular neurons in a PVN slice preparation depolarize to application of AM and that this effect is lost in the presence of tetrodotoxin, suggesting the effects of AM on OT neurons are indirect through an interneuron (Follwell & Ferguson 2002).

Both OT and AM within the CNS have been shown to be important inhibitors of sodium intake (Blackburn *et al.* 1992a,b, 1993, 1995, Samson & Murphy 1997, Samson *et al.* 1999). In addition, compromise of either central AM or OT levels led to exaggerated sodium appetites (Blackburn *et al.* 1992a, 1995, Samson *et al.* 1999, Amico *et al.* 2001, Fitts *et al.* 2003, Figs 5a and 6a). Based on our findings, we hypothesized that endogenous AM may stimulate OT release, which then mediates the inhibitory effects on salt intake. As hypothesized, in PEG-treated rats, i.c.v. administration of AM inhibited salt appetite, and this was reversed by pretreatment with OVT to block the actions of OT (Fig. 4a). OVT itself did not alter water intake in these animals (Fig. 4b), nor did it reverse the inhibitory effect of AM on water consumption. This agrees with a previous report that OT administration i.c.v. failed to significantly alter water consumption in the PEG treatment paradigm (Blackburn *et al.* 1993, Amico *et al.* 2001). As we had previously shown with antisense oligonucleotide treatment (Samson *et al.* 1999), compromise of endogenous AM by ribozyme treatment led to exaggerated salt intake (Figs 5a and 6a). The elevated saline intakes seen in rats with decreased central AM levels were reversed upon administration of OT (Fig. 6a). During the two-bottle preference test, PEG treatment is used to induce hypovolemia with no change in plasma sodium levels (see Taylor *et al.* 2005). As 0.3 m/l NaCl is aversive, animals must be driven to drink it. In the PEG paradigm, rats begin to consume water to compensate for their hypovolemia, but eventually they dilute their plasma (hyponatremia) to a point where they are driven to drink the hyperosmotic NaCl in an attempt to return plasma osmolality to normal. Under native circumstances, we believe that the increased intake of sodium triggers central AM release (from the Na^+ sensitive neuronal populations), which stimulates OT release into the CNS (and plasma), which then inhibits the rat from ingesting too much salt. When endogenous central AM levels have been lowered by ribozyme treatment, this break on sodium intake would be impaired and, consequently, animals would consume more Na^+ than necessary.

Previous studies demonstrated that AM inhibited not only salt intake (Samson & Murphy 1997, Samson *et al.* 1999) but also food (Taylor *et al.* 1996, 2005) and water intake (Murphy & Samson 1995, Taylor & Samson 2002, Taylor *et al.* 2005). Here, we also report a decrease in 10% w/v sucrose intake in *ad libitum* fed and watered rats treated centrally with AM. OT has been described as a general satiety factor (Verbalis *et al.* 1986, Lokrantz *et al.* 1997, Blevins *et al.* 2004) and has also been shown to inhibit food (Verbalis *et al.* 1986, Scalfani *et al.* 2007), water (Verty *et al.* 2004), salt (Dellmann *et al.* 1988,

Blackburn *et al.* 1992a,b, 1993, 1995, Murphy & Samson 1995, Allen *et al.* 1997), and sucrose intakes among others (Lokrantz *et al.* 1997, Scalfani *et al.* 2007). If central AM is, indeed, activating OT neurons as we hypothesize, then the effects of AM should not be specific but rather AM would be a satiation cue for all ingestive behaviors. This does not detract from the role that AM appears to play in fluid and electrolyte balance, but rather indicates that AM is of vital importance in the pathways that regulate homeostasis.

The studies presented here imply that brain-derived AM is necessary for the full OT response to altered plasma sodium levels. In addition, AM appears to control salt appetite at least in part through its effects on OT. Our findings in combination with other reports (Stricker 1968, Balment *et al.* 1980, Stricker & Verbalis 1986, 1996, Blackburn *et al.* 1992a,b, 1993, 1995, Verbalis *et al.* 1995, Serino *et al.* 1999, Amico *et al.* 2001, Fitts *et al.* 2003, Blevins *et al.* 2004, Chen *et al.* 2004) suggest a possible straightforward mechanism by which Na^+ stimulates AM release in brain and elsewhere, central AM stimulates OT release, and OT, acting at CNS sites, inhibits further Na^+ consumption. Future studies should determine whether brain-derived AM is necessary for long-term sodium balance. Furthermore, it would be important to understand how brain-derived AM selectively controls AVP secretion under hypovolemic conditions and OT secretion under hypernatremic conditions. Equally important would be a determination of which of the multiple AM receptors (Hay *et al.* 2006, Gibbons *et al.* 2007) transmits the AM signals under these conditions.

Our demonstrations of the importance of brain-derived AM on ingestive behaviors (Murphy & Samson 1995, Samson *et al.* 1999, Taylor *et al.* 2005) and AVP (Taylor *et al.* 2005) and OT plasma levels reinforce the hypothesis that AM of CNS origin is a physiologic regulator of fluid and electrolyte homeostasis. These effects of AM on mechanisms important to the maintenance of fluid and electrolyte homeostasis may contribute to the peptide's established protective actions on cardiovascular function and should be important considerations, as the list of potential clinical uses of AM in the treatment of cardiovascular diseases grows (Hinson *et al.* 2000).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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