Osmoregulatory fluid intake but not hypovolemic thirst is intact in mice lacking angiotensin

1,2M.J. McKinley, 1L.L. Walker, 1T. Alexiou, 2A.M. Allen, 3D.J. Campbell, 2R. Di Nicolantonio, 4B.J. Oldfield, 2D.A. Denton

Howard Florey Institute1 and Department of Physiology2, University of Melbourne, Parkville, Victoria, 3010; St Vincent’s Institute of Medical Research and the Department of Medicine3, University of Melbourne, St Vincent’s Hospital, Fitzroy, Victoria; and Department of Physiology, Monash University, Clayton4, Victoria; Australia

Short title: Thirst in angiotensin-deficient mice

Mailing Address: Michael J. McKinley,
Howard Florey Institute,
University of Melbourne, Parkville,
Victoria, 3010
Australia
Tel: 61383447303
Fax: 61 393481707
e-mail: michael.mckinley@florey.edu.au
Abstract

Water intakes in response to hypertonic, hypovolemic and dehydrational stimuli were investigated in mice lacking angiotensin II as a result of deletion of the angiotensinogen gene (Agt-/- mice), and in C57BL6 wild-type mice (WT-mice). Baseline daily water intake in Agt-/- mice was approximately threefold that of WT-mice because of a renal developmental disorder of the urinary concentrating mechanisms in Agt-/- mice. Intraperitoneal (i.p.) injection of hypertonic saline (0.4 and 0.8 mol/l NaCl) caused a similar dose-dependent increase in water intake in both Agt-/- and WT-mice during the hour following injection. As well, Agt-/- mice drank appropriate volumes of water following water deprivation for 7 hours. However, Agt-/- mice did not increase water or 0.3 mol/l NaCl intake in the 8 hours following administration of a hypovolemic stimulus (subcutaneous 30% polyethylene glycol), whereas WT-mice increased intakes of both solutions during this time. Osmoregulatory regions of the brain (hypothalamic paraventricular and supraoptic nuclei, median preoptic nucleus, organum vasculosum of the lamina terminalis (OVLT) and subfornical organ) showed increased number of neurons exhibiting Fos-immunoreactivity in response to i.p. hypertonic NaCl in both Agt-/- mice and WT-mice. PEG treatment increased Fos-immunoreactivity in the subfornical organ, OVLT, and supraoptic nuclei in WT-mice, but only increased Fos-immunoreactivity in the supraoptic nucleus in Agt-/- mice. These data show that brain angiotensin is not essential for the adequate functioning of neural pathways mediating osmoregulatory thirst. However, angiotensin II of either peripheral or central origin is probably necessary for thirst and salt appetite that results from hypovolemia.

Key words: angiotensinogen gene, mice, thirst, hypertonicity, dehydration, hypovolemia, Fos, angiotensin-deficient.
INTRODUCTION

While it was proposed more than 30 years ago that a brain renin-angiotensin system generated angiotensin in the brain (13,17), the physiological roles and modus operandi of this system are still incomplete (10,39,40). All components of a renin-angiotensin system including renin, angiotensinogen, angiotensin converting enzymes, angiotensin I, II, and III, AT₁ and AT₂ receptors and angiotensinases have been identified within the central nervous system (12, 23-25,29,35,44). While these different components of the renin-angiotensin system may not all be co-localised within specific parts of the brain, it is likely that angiotensin peptides are generated within the brain. It is thought that the active peptides angiotensin II, III or IV may have roles within the brain in functions as diverse as the regulation of cardiovascular and fluid homeostasis (2,3,11,19,35,40,41,46), reproduction (15), thermoregulation (28), memory (49) cognition, emotional responses to stress and anxiety, cerebral blood flow regulation and brain developmental processes (39). However, some have questioned whether angiotensin is a neuropeptide because of its very low abundance in brain (23), and have proposed that an alternative endogenous ligand may act on angiotensin receptors in the brain (6).

Centrally administered angiotensin stimulates water drinking, vasopressin secretion and a brisk natriuresis that promote a positive fluid balance and reduce plasma osmolality (19,29,38). One of the postulated physiological functions of brain angiotensin II is that of a signalling molecule in osmoregulatory neural pathways, probably as a neurotransmitter or modulator (5). A wealth of evidence in support of such a function for brain angiotensin comes from many reports of the potent effect of centrally administered angiotensin antagonists such as losartan, in blocking water drinking, vasopressin secretion, increased arterial pressure and natriuresis in response to centrally administered hypertonic saline (5,18,27,36,50). The inhibitory influence on osmoregulatory function of central antisense oligonucleotides directed against the synthesis of angiotensinogen in the brain is also consistent with this idea (43). In the present investigation, we have tested the hypothesis that angiotensin has an essential role in osmoregulatory function.

Genetically modified strains of mice lacking the gene encoding angiotensinogen, the only known source of the biologically active angiotensin peptides, have been
developed in two laboratories (22,48). These mice exhibit lower arterial blood pressure, higher plasma renin levels, lower body weight, and an increased fluid turnover compared with wild-type mice (1,21,22,26,47,48). The lower body weight results from reduced white adipose tissue mass (26). The increased fluid turnover is due to a renal urinary concentrating defect resulting from a lack of angiotensin during kidney development (21,47). In the case of mice with a homozygous lack of the angiotensinogen gene (Agt-/- mice), most do not survive the first post-natal week unless they are rescued by fluid and electrolyte replacement during the post-natal period or angiotensin treatment (47). We have observed that adult Agt-/- mice rescued by perinatal saline treatment, as expected, lack any angiotensin peptides in the blood, peripheral organs or brain (1). Thus they offer an experimental model to test whether brain angiotensin is necessary for the operation of central osmoregulatory neural pathways.

The aims of this investigation were first to investigate the role of angiotensin in osmoregulatory fluid intake using Agt-/- mice by testing whether osmotically stimulated fluid intake is disrupted in this mouse strain. It is not clear whether angiotensin, either of peripheral or central origin is necessary for hypovolemic thirst in mice (20,37). Therefore, we also investigated whether water drinking in response to hypovolemia, induced by subcutaneous colloid injection, is disrupted in angiotensin deficient mice. A further aim was to compare the expression in the brain of the proto-oncogene c-fos, an indicator of neuronal activity (31), in Agt-/- and wild-type mice in response to hyperosmotic and hypovolemic challenges.

**MATERIALS AND METHODS**

*Animals*

Experiments were approved by the Animal Ethics Committee of the Howard Florey Institute which adheres to the Code of Practice of the National Health and Medical Research Council of Australia for the care and welfare of experimental animals. Experiments were performed on mice of the C57BL6 strain in which the coding region of the angiotensinogen gene had been deleted by homologous recombination. C57BL6 mice,
heterozygous for the angiotensinogen gene (Agt+/- mice) were obtained from the laboratory of Prof. P. Meneton, INSERM, Paris, France and were derived from the angiotensinogen gene knockout mice described by Kim et al. (22). Several generations of mice were bred to obtain sufficient numbers of homozygous Agt-/- mice, and wild-type Agt+/+ litter mates for experiments. Because there is a high mortality rate in Agt-/- mice in the perinatal period, we administered a daily subcutaneous injection of isotonic 0.15 mol/l NaCl to all pups during the first week of life to prevent fatal dehydration occurring in these animals (47). Mice were toe-clipped at 8-14 days of age to obtain DNA for genotyping, which involved a polymerase chain reaction procedure using primers to label both neomycin and angiotensinogen DNA sequences. Due to the defective renal concentrating mechanism, Agt-/- mice have a phenotype of high water intake (approximately 3 times that of wild-type C57BL6 mice). Thus, we checked that daily water intakes of mice matched the appropriate Agt phenotype in all mice.

After 1-2 months, mice of either sex were housed individually in cages with ad libitum access to pelleted food and water, except during experiments involving water or food deprivation. All female mice were nulliparous. Water and 0.3 mol/l NaCl solution was provided from glass drinking spouts attached to plastic syringes that allowed measurement of water intake and NaCl intake each day. Room temperature was kept at 22°C, and a 12h light:12h dark cycle was maintained.

**Experimental protocols**

**Daily intakes of water.** In 6 age and sex matched pairs of mice (2 males, 4 females, aged 8.8 ± 1.0 months), water intake was measured during the course of the day at 3h intervals from 0900h to 2100h, and the overnight intake measured again at 0900h of the next morning. Lights were switched off at 1800h and on again at 0600h.

**Water intake in response to intraperitoneal (i.p.) injection of hypertonic NaCl.** Wild-type and Agt-/- mice, matched for sex (3 males, 6 females) and aged 9.9 ± 1.5 and 12.1 ± 1.0 months respectively were allowed access to water and food. They were injected i.p. with hypertonic 0.4 or 0.8 mol/l NaCl solution (2% of body weight) or isotonic 0.15mol/l
NaCl (controls) at 1000-1030h. Water intake during the following hour was measured. This period of observation was chosen, because in preliminary experiments we observed that the drinking response to i.p. hypertonic saline in wild-type mice was completed within 1 hour of the injection of hypertonic saline. As well, the high baseline water intake of Agt-/- mice necessitated that this period of observation be as short as possible.

Water and NaCl intakes in response to water deprivation. Wild-type and Agt-/- mice (matched for sex, 4 males, 2 females) and aged 9.1 ± 2.6 and 11.2 ± 3.0 months respectively, were provided with water and 0.3 mol/l NaCl to drink for several days prior to the day of water deprivation. At 0900 h on this day, both solutions were removed, and the body weight of the mouse measured. In the first experiment, the period of water deprivation was 24 h, and all mice were allowed access to food during this time. At 0900h on the next day, mice were again weighed, food intake measured, and then water and 0.3 mol/l NaCl solution were placed back on their cages. Water and NaCl intakes were then measured after 1 hour. Because Agt-/- mice became more dehydrated than WT-mice when deprived of water, another experiment was undertaken in an attempt to produce a similar degree of dehydration. In the next experiment, different groups of WT- and Agt-/- mice were used (matched for age = 6.4 ± 0.9 months and sex; 5 males, 1 female) that had been provided with food and water (but not NaCl solution) during the days prior to water deprivation. A similar protocol was employed, except that the period of deprivation from water was 7 h in Agt-/-, and 24 h in wild-type mice. When water was placed back on the cage at 1600h, intake was measured after 1 h.

Effect of subcutaneous (s.c.) injection of polyethylene glycol on water intake. Age (6.5 ± 1.5 months) and sex matched (all males) Agt-/- and wild-type mice were provided with both water and 0.3 mol/l NaCl to drink several days prior to experiments. Daily water, NaCl and food intakes were measured. On the experimental day, mice were weighed at 0930h and injected s.c. with 30% polyethylene glycol (molecular weight 20,000) in 0.15 mol/l NaCl solution (0.2 ml/10g body weight). Intakes of water, 0.3 mol/l NaCl and food were measured at 2, 4, 6, 8, 24 and 48 h after the injection.
Statistical analysis of intakes of water, food and sodium solution. Results are expressed as mean ± standard error of mean. Analysis of the intakes during each period of observation (non-cumulative) by two factor analysis of variance for repeated measures followed by a post-hoc multiple comparison test (Newman-Keuls) was used to evaluate differences between WT- and Agt-/- mice. While cumulative intakes are shown for convenience in Figs 1 and 3, these were not tested statistically except for the final total values that were compared by either Student t-test or Mann Whitney U test. Where appropriate, square root transformation of data was utilised to obtain homogeneity of variance.

Fos immunohistochemistry in response to hypertonicity, hypovolemia or no treatment. Agt-/- and WT-mice were injected either intraperitoneally with hypertonic 0.8 mol/l NaCl (0.2 ml/10g, n = 5) or subcutaneously with 0.2 ml/10g of 30% PEG (n = 4), then killed by i.p. injection of sodium pentobarbital (1mg/10g) at either 2 h (hypertonicity) or 6 h (PEG treatment) respectively after the stimuli were administered. In addition, control untreated Agt-/- mice (n = 4), wild-type mice (n = 4) or wild-type mice injected i.p. with isotonic NaCl solution 2 hours earlier (n = 5) were also killed with sodium pentobarbital (1mg/10g). Drinking water was provided on the cage during the period following treatments to prevent confounding effects of dehydration that could be additional to the PEG-induced hypovolemia, particularly in the Agt-/- mice. The brains of all these mice were then perfused via the heart with isotonic saline (30 ml) followed by 4% paraformaldehyde/0.1 M phosphate buffered saline (PBS), removed, post-fixed in this solution for 1 hour then immersed in 20% sucrose/PBS overnight. Serial coronal sections (40µm) of the brain were then cut on a freezing microtome and subjected to standard immunohistochemical procedures for the detection of Fos protein using a polyclonal antisera (Ab-5, Oncogene Science) as previously described (33). Diaminobenzidine was used as the chromogen to visualise the final reaction product. Counts were made of cell nuclei exhibiting Fos-immunoreactivity (Fos-IR) in 5 osmoregulatory regions of the brain (8,30,33), the supraoptic nucleus (SON), hypothalamic paraventricular nucleus (PVN), organum vasculosum laminae terminalis (OVLT), the subfornical organ (SFO) and the median preoptic nucleus (MnPO) in each brain. Using an eyepiece fitted with a graticule
to avoid repeat-counting, we averaged the counts of cell nuclei exhibiting Fos-IR in 3 sections for each specific region, using corresponding rostro-caudal stereotaxic levels for the C57BL6 mouse brain (16) for the particular nuclei in each brain. For the median preoptic nucleus, we standardised the part from which counts were made by counting Fos-labelled nuclei within a window of 400 x 75 µm ventral from the mid-level of the anterior commissure in the midline immediately anterior to this structure (see Fig. 9).

Results are expressed as mean and standard error of the mean. Comparisons of effect of treatments on Fos-IR counts for each brain site between WT- and Agt-/- mice were made by two factor analysis of variance and subsequent Newman-Keuls multiple comparison test. Significance was assigned at p < 0.05.

RESULTS

Normal pattern of fluid and food intake in Agt-/- and wild type mice. Water intake during 24 hours was 3-fold greater in Agt-/- mice than in wild types matched for age and sex. Increased fluid intake was evident within 3 hour of the commencement of measurements and continued throughout the 24 h of observations (Fig. 1). Food intake in the Agt-/- mice was not significantly different from WT-mice (Fig. 1). Body weight was significantly lower in the Agt-/- mice than in the WT-mice (23.8 ± 2.7 vs 29.2 ±7.6 g, p < 0.05, Wilcoxon paired sample test).

Comparison of water intake in response to systemic injection of hypertonic saline in Agt-/ and wild-type mice. Intraperitoneal injections of hypertonic NaCl (0.4 and 0.8 mol/l) caused a dose-dependent drinking response during the hour following the injection in both groups of mice when compared to the intakes following control injections of isotonic NaCl (Fig. 2). There was a small but significantly greater intake of water in Agt-/- mice compared to the wild-type animals, consistent with the baseline intake of Agt-/- being greater than that of WT-mice.

Water intake in response to fluid deprivation in Agt-/- and wild-type mice.
In the first experiment in which both strains of mice were deprived of water for 24 hours, the amount of water drunk by the mice upon regaining access to water was much greater
in the Agt-/− than WT-mice (1.01 ± 0.11 versus 0.59 ± 0.07 ml/10g body weight, n = 6 pairs) during the first hour of access to water. Neither group consumed 0.3 mol/l NaCl solution. However, it was evident that Agt-/− mice had probably incurred a much greater fluid deficit than WT-mice during the period of water deprivation because their loss of body weight (25.2 ± 1.3 to 19.1 ± 1.1 g) was ~25% compared with that of ~10% in WT-mice (28.6 ± 2.0 to 25.8 ± 1.9 g). Therefore in another group of Agt-/− mice (n = 6), we attempted to produce a similar weight deficit in response to water deprivation as occurred in their age and sex matched wild-type litter mates deprived of water for 24 h. Thus, we observed that the body weight of Agt-/− mice fell by 2.0 ± 0.3 g in Agt-/− when deprived of water for 7 h. This weight loss was not significantly different from that lost by WT-mice (2.3 ±0.3 g) when deprived of water for 24 h, although it was a marginally but significantly greater percentage weight loss than wild-types (9.5 ± 1.3% in Agt-/− mice versus 7.5 ± 0.9% in WT-mice). When the 24 h water-deprived WT-mice were given water to drink, they consumed 0.9 ±0.3 ml during the following hour, which represented 43 ± 11 % of the weight lost, while Agt-/− mice that had been deprived of water for 7 h drank 1.7 ± 0.5 ml in the ensuing hour, equivalent to 94 ± 41% of their weight loss incurred during the period of water deprivation.

Comparison of water and sodium intake in response to hypovolemia resulting from subcutaneous injection of polyethylene glycol (PEG) in Agt-/− and wild-type mice.

Subcutaneous injection of 30% PEG in WT-mice caused a significant increase in water intake within 2 h when compared to baseline intake of mice injected with isotonic saline subcutaneously (Fig. 3). There was a small but significant intake of 0.3 mol/l NaCl solution following PEG administration (Fig. 3). However, in the Agt-/− mice, no such effects were observed and intakes of water and NaCl were similar to intakes observed during the 8 h following a control subcutaneous injection of isotonic saline solution (Fig. 3). PEG treatment did not have any effect on food intake in either group of mice during 8 hours following PEG treatment. The dipsogenic and natriorexic effects of PEG treatment in WT-mice were also reflected in increased water and NaCl intakes in the 24 h intakes following treatment. However, in Agt-/− mice, there was a pronounced reduction in the 24 h intakes of water, NaCl and food following PEG treatment (Fig. 4). As well, 3 of the 5
Agt-/- mice died 24-48 h following PEG administration, whereas no deaths occurred in the WT-mice during this period.

**Expression of c-fos in the brains of Agt-/- and wild-type mice normally, and in response to hypertonicity or PEG-induced hypovolemia.** Fos-immunoreactivity was investigated in regions of the brain known to have a role in body fluid homeostasis and cardiovascular regulation. These regions were the SON (Fig. 5) and PVN (Fig. 6) in the hypothalamus, and the OVLT (Fig. 7), SFO (Fig. 8) and MnPO (Fig. 9) in the lamina terminalis. Many neurons exhibited basal Fos-IR in commissural and medial sub-nuclei of the nucleus of the solitary tract (NTS) and area postrema in the hindbrain of untreated Agt-/- mice (not shown), but not in WT-mice. Because of the pronounced difference in baseline Fos-IR in NTS and area postrema between WT- and Agt-/- mice, we did not make counts of these regions with hypertonic or hypovolemic stimuli. Baseline Fos-IR in the SON and PVN tended to be greater in Agt-/- mice than in WT-type mice, however the differences were not statistically significant (Table 1). Although some Agt-/- mice exhibited considerable Fos-IR in the OVLT and MnPO, it was not significantly different from that in WT-mice. Baseline Fos-IR was very low in the SFO of both Agt-/- and WT-mice (Fig. 8).

Intraperitoneal injection of hypertonic 0.8 mol/l NaCl caused a large and significant increase in the number of neurons expressing Fos-IR in the SON, PVN (predominantly but not exclusively in its lateral and medial magnocellular subnuclei), OVLT, SFO and OVLT in both Agt-/- mice and WT-mice (Figs 5-9). There were no significant differences in Fos-IR counts between the two strains of mice for any of these brain regions (Table 1).

Hypovolemia resulting from PEG treatment caused a large increase in the number of neurons expressing Fos-IR in the SON, OVLT and SFO of WT-mice compared to baseline counts of Fos-IR (Table 1, Figs 5-9). Although the increased Fos-IR in the PVN did not reach significance, this may be due to low number of mice (n = 4). However, isotonic saline control injection also appeared to cause a comparable increase in Fos-IR in WT-mice, and handling may have contributed in part to the response of the PVN in WT-mice to PEG. In Agt-/- mice, PEG treatment increased the number of neurons
expressing Fos-IR in the SON (Figs. 5), but not in the OVLT, SFO, MnPO or PVN (Table 1, Figs 6-9). Water intakes over the 6 h from injection of PEG until mice were killed were similar in both groups being 1.5 ± 0.4 in WT-mice and 1.3 ± 0.2 in Agt-/- mice.

DISCUSSION

Mice in which the angiotensinogen gene had been deleted exhibited a baseline level of daily water intake that was almost three times that of age and sex matched WT-mice, consistent with earlier results of others in Agt-/- mice (22,48). An increased water turnover occurs in Agt-/- mice because they have a partial nephrogenic diabetes insipidus resulting from a developmental impairment of renal concentrating mechanisms caused by a perinatal lack of angiotensin peptides (21,47). The elevated daily water intake in Agt-/- mice is probably secondary to fluid loss from the kidney which would have a dehydrating effect. This loss of body fluid should provide osmotic and non-angiotensin mediated volemic stimuli for thirst. The dipsogenic responses to hyperosmolar and hypovolemic stimuli that we report, are set against this high baseline level of water drinking that occurs in angiotensin-deficient Agt-/- mice.

The primary aim of these experiments was to determine whether angiotensinergic mechanisms in the brain are necessary for the function of osmoregulatory thirst mechanisms. Previously, we and others had shown that centrally administered angiotensin antagonist drugs were capable of blocking water drinking, vasopressin release and natriuresis in response to intracerebroventricularly injected hypertonic saline, suggesting angiotensin signalling in osmoregulatory pathways (5,18,27,29,36,48). The results we have obtained in Agt-/- mice show that angiotensin peptides are not necessary in the brain (or elsewhere) for central osmoregulatory thirst mechanisms to function adequately. These mice ingested as much water in excess of baseline intakes as did WT-type mice in response to i.p. injections of hypertonic saline. As well, they appeared to ingest appropriate volumes of water in response to periods of water deprivation. As a consequence of a developmental disorder of their renal concentrating mechanisms (21),
Agt-/- mice lose greater volumes of urine than do wild-type C57BL6 mice. Accordingly, we observed that Agt-/- mice deprived of water for 7 hours, incurred approximately the same loss of body weight as did WT-mice deprived of water for 24 h. When water was returned to the mice, Agt-/- mice drank amounts of water during the next hour that were still more than the intakes of the WT-type mice that had been deprived of water for 24 h.

If angiotensinergic mechanisms within the CNS are unnecessary for osmotically stimulated drinking mechanisms, how then can the reported inhibitory effects that angiotensin antagonists exert on osmotically stimulated thirst (5,27) be explained? It is possible that centrally administered losartan may be blocking another type of receptor that influences thirst and is independent of the brain angiotensin system, i.e. losartan, at the doses used, is not a specific angiotensin receptor antagonist. Another possibility is that a non-angiotensin ligand that could bind to AT₁ receptors may be released upon stimulation of central osmoreceptors. Evidence of a non-angiotensin ligand partially purified from sheep hypothalamus that can bind to AT₁ and AT₂ receptors, shows the feasibility of this suggestion. It is also possible that the AT₁ receptor could be activated without a ligand, for instance by physical changes. There are data showing that the AT₁ receptor in mouse myocytes can be activated by mechanical stretch independent of angiotensin peptides (51). This mechanical activation of the AT₁ receptor is blocked by an AT₁ antagonist, candesartan. Thus, a possible explanation of our results is that activation of the AT₁ receptor in osmoregulatory brain regions is caused by direct mechanical changes (cell shrinkage) associated with osmotically stimulated movement of fluids (osmosis), without the involvement of angiotensin II. While the results reported here show that brain angiotensin is not essential for adequate osmoregulatory thirst and fluid intake, the engagement of compensatory redundant mechanisms is often advanced as an explanation for a function being maintained in an animal with a deleted gene. This possibility cannot be discounted here.

Unlike the appropriate water drinking that occurred in Agt-/- mice in response to systemic hypertonicity or dehydration, we observed that hypovolemic thirst resulting from subcutaneous polyethylene glycol treatment was severely inhibited in Agt-/- mice. Subcutaneous administration of PEG causes sequestration of extracellular fluid under the
skin, with a reduction in blood and extracellular fluid volumes (14,45). In mice, this treatment has been shown previously to stimulate water drinking (20,37,38) and sodium appetite (37). We observed that while water drinking and intake of 0.3 mol/l NaCl solution increased in PEG treated WT-mice during 8 h following treatment, there was no increase in water or salt intake in Agt-/- mice compared to controls with PEG treatment. This result implicates an angiotensin mechanism, either peripheral or central, in hypovolemic thirst and salt appetite subsequent to PEG administration. It suggests also that neural signals from low or high pressure baroreceptors do not mediate the PEG-induced water and salt intake, unless such signals act together in concert with angiotensin. This is because change in baroreceptor signals would be expected to be even greater in Agt-/- than in WT-mice after PEG treatment, yet water and sodium intakes in Agt-/- were similar to the control saline injected Agt-/- mice.

This result contrasts with that of Crews and Rowland who observed that hypovolemic thirst was not blocked by losartan (100 mg/kg) treatment in mice (7). A possible explanation for this disparity in results is that PEG treatment caused a greater non-specific debilitation in Agt-/- mice compared to wild-types treated with PEG. Arterial pressure is already 20 mm Hg lower in the Agt-/- mice than in wild-type mice (1,22,48) and could be expected to have fallen much further with PEG treatment in the null mice because they lack a functioning renin-angiotensin system to support arterial pressure. Clearly there was a deleterious effect of PEG treatment in Agt-/- mice at some stage, because both water and NaCl intakes fell significantly below baseline in the subsequent 24 h, and 3 of the 5 Agt-/- mice died 24-48h after treatment. Notwithstanding these observations, evidence of Agt-/- mice being behaviourally debilitated during the first 8 h following PEG administration was not apparent. Their intake of food over this period, although small, was not significantly different than that of saline injected controls and appeared similar to that of WT-mice during the initial 8 h following PEG treatment. As well, intakes of water and sodium were similar to those observed during the baseline day, levels that could have been expected if the dipsogenic signalling pathway for hypovolemic thirst had been disrupted in Agt-/- mice. Therefore, the possibility remains that angiotensin, acting either peripherally or centrally, has a significant role in mediating
hypovolemic thirst in mice as it does in the rat. Similarly, our results show that sodium intake stimulated by PEG-induced hypovolemia in WT-mice is also dependent on angiotensin II, either of peripheral or central origin. Contrasting with some earlier studies (7,20,38), we were able to observe increased sodium chloride intake in WT-mice following PEG administration. The reasons for this difference are not clear, however there is one other report of increased intake of NaCl solution (0.15 mol/l) in mice following PEG Administration (37).

In regard to c-fos expression in the brains of Agt-/- and WT-mice, baseline levels of Fos-IR tended to be low in osmoregulatory regions of the brain such as the lamina terminalis and hypothalamic SON and PVN. However, there was a tendency for some Agt-/- mice to exhibit marginally more Fos-IR in some of these regions. This probably reflects the high water turnover in Agt-/- mice (a consequence of their partial nephrogenic diabetes insipidus) with some mice being killed for brain immunohistochemistry at a time when their water drinking had not totally replenished current losses in urine. A marked increase of baseline Fos-IR was observed in the commissural and medial parts of the NTS and also the area postrema in Agt-/- mice compared with WT-mice. This is not surprising in view of the lower arterial blood pressure that has been reported to occur in Agt-/- mice (1,22,48), the importance of these two regions in cardiovascular control mechanisms (9), and previous reports of increased Fos expression in these regions caused by hypotensive stimuli (4,34).

The pattern of Fos immunoreactivity observed in osmoregulatory regions (lamina terminalis, SON, PVN) of the brains of Agt-/- mice in response to hypertonicity was similar in Agt-/- and WT-mice, consistent with the comparable osmoregulatory drinking responses observed in the two strains of mice. Although we did not measure blood levels of vasopressin, its secretion in response to hypertonicity probably occurred in Agt-/- as well as WT-mice because similar increases in Fos expression were observed in the magnocellular regions of the hypothalamic SON and PVN of both groups of mice with the hypertonic stimulus.
Interestingly, we did observe differences in Fos-IR in the lamina terminalis of Agt-/- mice subjected to PEG treatment in comparison with that in WT-mice. Neither the SFO or OVLT showed any increase in Fos-IR in response to this hypovolemic stimulus in Agt-/- mice, whereas such hypovolemia resulted in a large increase in the number of cells (presumably neurons) expressing Fos-IR in these circumventricular organs in WT-mice. These data show that activation of neurons in the SFO and OVLT of WT-mice in response to hypovolemia is probably caused by the action of circulating angiotensin II that would increase in the circulation due to hypovolemic stimulation of the renin-angiotensin system by PEG treatment in WT-mice, but not in Agt-/- mice. Our data are consistent with observations of Crews and Rowland (7) showing that peripheral administration of the AT₁ receptor antagonist losartan prevents c-fos expression in the SFO and OVLT of PEG-treated C57BL6 mice. By contrast, the number of neurons exhibiting Fos-IR in response to hypovolemia increased in the SON of both Agt-/- and WT-mice. Hypovolemia resulting from PEG treatment increases vasopressin secretion (42) and it is likely that a significant proportion of the Fos-IR in the SON is localised to vasopressin-containing magnocellular neurons. The Fos-IR observed in these magnocellular neurons in Agt-/- mice shows that activation of such neurons by hypovolemia may be independent of angiotensin mechanisms (either peripheral or central). Most likely, ascending neural pathways from either or both low and high pressure receptors in the thorax, that are relayed to the hypothalamus via the medulla oblongata, instigate hypovolemia-stimulated release of vasopressin from the neurohypohysial axons of magnocellular neurons.

PERSPECTIVES AND SIGNIFICANCE

Adequate water intake following hypertonic stimuli observed in mice totally lacking angiotensin peptides shows that angiotensinergic neural pathways are not essential for adequate osmoregulatory thirst. By contrast, angiotensin, either peripherally or centrally generated, is probably essential for the thirst and salt appetite that occurs in response to hypovolemia in mice.
Our results question whether there is any role at all for angiotensin as a signalling molecule for osmoregulatory mechanisms within the CNS. If there is not, it is difficult to explain why centrally administered AT₁ antagonists inhibit drinking stimulated by intracerebroventricular (ICV) injection of hypertonic saline in several species that include mice. As mentioned above, the explanation could be that either AT₁ antagonists are non-specific, blocking another receptor, or they block the action of a non-angiotensin molecule that could act at the AT₁ receptor. Another possibility is that drinking stimulated by ICV hypertonic saline is not comparable to normal osmotic thirst. We speculate that the dipsogenic response to ICV hypertonic NaCl does not use the physiological neural pathway that is activated by systemic hypertonicity.
Acknowledgements

We thank Prof. P. Meneton for kindly providing Agt+/- mice. We thank Melinda Goga for expert immunohistochemistry.

Grants

This work was supported by grants from the National Health and Medical Research Council of Australia (NHMRC), Project Grant 300023, Robert J Jr and Helen C Kleberg Foundation, Harold D and Leila Mathers Trust and the Search Foundation. DJC is the recipient of an NHMRC Senior Research Fellowship (Grant ID 395508), BJO the recipient of an NHMRC Principal Research Fellowship (ID 384221, and MJMcK the recipient of an NHMRC Senior Principal Research Fellowship (ID454369).

Disclosures

No disclosures
REFERENCES


30. McKinley MJ, Mathai ML, Mcallen RM, McClear RC, Miseslis RR, Pennington GL, Vivas L, Wade JD, Oldfield BJ. Vasopressin secretion:


Legends to Figures

Fig. 1. *Ad libitum* intakes of water and food during 24 hours in wild-type (open circles, n = 6) and Agt-/- mice (black circles, n = 6). Both cumulative (lower panel) and non-cumulative intakes (upper panel) are shown. In the upper panel, the non-cumulative intakes are shown at the midpoint of each period of observation. Significant differences from the wild-type value at the corresponding time are indicated by * p < 0.05, **p < 0.01. In the lower panel, only the final total cumulative intakes during the 24 h were tested statistically. # indicates p < 0.0001 (paired t-test).

Fig. 2. Water intake during 1 hour following i.p. injection of isotonic 0.15 mol/l NaCl, hypertonic 0.4 mol/l NaCl or 0.8 mol/l NaCl in wild-type (WT, open bars, n = 8) or Agt-/- mice (black bars, n = 8). Letters a-e indicate statistically significant differences (p < 0.05). a = greater than 0.15M NaCl in WT; b = greater than 0.4M NaCl in WT; c = greater than 0.15M NaCl in Agt-/-; d = greater than 0.4M NaCl in Agt-/-; e = greater than WT/0.8 M NaCl.

Fig. 3. Cumulative intakes of water, 0.3 mol/l NaCl and food following subcutaneous injection of 30% polyethylene glycol (PEG) or isotonic normal saline (NS) in wild-type (n = 5) or Agt-/- (n = 5) mice. A significant difference between wild-type and Agt-/- mice in the non-cumulative intakes for the particular two hour period ending at that time point is indicated by * = p < 0.05, ** = p < 0.01. # indicates a statistical difference (p < 0.01, Mann Whitney test) in the total cumulative intake over the 8h observation period.
Fig. 4. Daily intakes of water, NaCl and food during the 2 days before and 2 days after subcutaneous injection of either normal saline (NS, open bars, n = 5) or polyethylene glycol PEG, diagonal hatching, n = 5) in wild-type and Agt-/- mice. Significant difference from the corresponding values for first 2 days is indicated by * = p < 0.05, ** = p < 0.01.

Fig. 5. Photomicrographs of the effect of no treatment (Cont; A,B), i.p. injection of 0.8 mol/l NaCl (HTS; C,D) or s.c. 30% polyethylene glycol (PEG; E,F) on Fos-immunoreactivity (seen as black dots) in the supraoptic nucleus of wild-type (WT) or Agt-/- mice. Bar = 100µm

Fig. 6. Photomicrographs of the effect of no treatment (Cont; A,D), i.p. injection of 0.8 mol/l NaCl (HTS; B,E) or s.c. 30% polyethylene glycol (PEG; C,F) on Fos-immunoreactivity (seen as black dots) in the hypothalamic paraventricular nucleus of wild-type (WT) or Agt-/- mice. Abbreviations; LM, lateral magnocellular subnucleus; MM, medial magnocellular subnucleus; MP, medial parvocellular subnucleus. Bar = 150µm

Fig. 7. Photomicrographs of the effect of no treatment (Cont; A,B), i.p. injection of 0.8 mol/l NaCl (HTS; C,D) or s.c. 30% polyethylene glycol (PEG; E,F) on Fos-immunoreactivity (seen as black dots) in the organum vasculosum of the lamina terminalis (OVLT) of wild-type (WT) or Agt-/- mice. Note that Fos immunoreactivity
was not confined to the dorsal part of the OVLT in mice, unlike rats treated with hypertonic saline that express *c-fos* mainly in the dorsal cap of the OVLT (30). Bar = 100µm

Fig. 8. Photomicrographs of the effect of no treatment (Cont; A,B), i.p. injection of 0.8 mol/l NaCl (HTS; C,D) or s.c. 30% polyethylene glycol (PEG; E,F) on Fos-immunoreactivity (seen as black dots) in the subfornical organ of wild-type (WT) or Agt-/- mice. Bar = 100µm

Fig. 9. Photomicrographs of the effect of no treatment (Cont; A,B), i.p. injection of 0.8 mol/l NaCl (HTS; C,D) or s.c. 30% polyethylene glycol (PEG; E,F) on Fos-immunoreactivity (seen as black dots) in the ventral part of the median preoptic nucleus (arrowed) of wild-type (WT) or Agt-/- mice. Bar = 100µm.
Table 1. Number of neurons expressing Fos-immunoreactivity (Fos positive cells per section) in selected osmoregulatory brain regions with no treatment, i.p. injection of normal 0.15 mol/l NaCl (n sal), hypertonic 0.8 mol/l NaCl or subcutaneous injection of polyethylene glycol (PEG). N = 4 or 5 in each group. Values are mean ± standard error of the mean; statistical evaluation by two factor analysis of variance and subsequent Newman-Keuls test for multiple comparisons. The IP n. sal. WT Group was not included in the statistical analysis.

<table>
<thead>
<tr>
<th></th>
<th>No treatment</th>
<th>IP n sal.</th>
<th>IP Hypertonic NaCl</th>
<th>Subcut. PEG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT Agt-/-</td>
<td>WT Agt-/-</td>
<td>WT Agt-/-</td>
<td>WT Agt-/-</td>
</tr>
<tr>
<td>SON</td>
<td>2 ± 1</td>
<td>10 ± 1</td>
<td>2 ± 1</td>
<td>64 ± 6a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>79 ± 5a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>38 ± 14a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>38 ± 7a</td>
</tr>
<tr>
<td>PVN</td>
<td>9 ± 3</td>
<td>25 ± 8</td>
<td>42 ± 14</td>
<td>121 ± 6a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>132 ± 22a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>61 ± 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>47 ± 9</td>
</tr>
<tr>
<td>OVLT</td>
<td>5 ± 3</td>
<td>17 ± 3</td>
<td>5 ± 1</td>
<td>82 ± 16a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>72 ± 11a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100 ± 9a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>19 ± 5b</td>
</tr>
<tr>
<td>SFO</td>
<td>0 ± 0</td>
<td>1 ± 1</td>
<td>0 ± 0</td>
<td>38 ± 4a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>35 ± 6a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30 ± 4a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4 ± 1b</td>
</tr>
<tr>
<td>MnPO</td>
<td>10 ± 6</td>
<td>15 ± 4</td>
<td>10 ± 3</td>
<td>49 ± 6a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>66 ± 10a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>19 ± 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>29 ± 5</td>
</tr>
</tbody>
</table>

*aSignificant difference (p < 0.01) from “No treatment” value for the same genotype,

bSignificant difference (p < 0.01) between WT and Agt-/- groups for a particular treatment.
Wild-type

Day 1 Day 2 Day 3 Day 4
0.0 2.5 5.0 7.5 10.0 12.5 15.0

NS PEG

Day 1 Day 2 Day 3 Day 4
0.0 2.5 5.0 7.5 10.0 12.5 15.0

NS PEG

PEG or NS

Day 1 Day 2 Day 3 Day 4
0.0 2.5 5.0 7.5 10.0 12.5 15.0

PEG or NS

**

Day 1 Day 2 Day 3 Day 4
0.0 2.5 5.0 7.5 10.0 12.5 15.0

PEG or NS

McKinley et al, Figure 4
Fig. 5. Photomicrographs of the effect of no treatment (Cont; A,B), i.p. injection of 0.8 mol/l NaCl (HTS; C,D) or s.c. 30% polyethylene glycol (PEG; E,F) on Fos-immunoreactivity (seen as black dots) in the supraoptic nucleus of wild-type (WT) or Agt-/- mice. Bar = 100µm
Fig. 6. Photomicrographs of the effect of no treatment (Cont; A,D), i.p. injection of 0.8 mol/l NaCl (HTSD; B,E) or s.c. 30% polyethylene glycol (PEG; C,F) on Fos-immunoreactivity (seen as black dots) in the hypothalamic paraventricular nucleus of wild-type (WT) or Agt-/- mice. Abbreviations; LM, lateral magnocellular subnucleus; MM, medial magnocellular subnucleus; MP, medial parvocellular subnucleus. Bar = 150µm
289x260mm (96 x 96 DPI)
Fig. 7. Photomicrographs of the effect of no treatment (Cont; A,B), i.p. injection of 0.8 mol/l NaCl (HTS; C,D) or s.c. 30% polyethylene glycol (PEG; E,F) on Fos-immunoreactivity (seen as black dots) in the organum vasculosum of the lamina terminalis (OVLT) of wild-type (WT) or Agt-/- mice. Note that Fos immunoreactivity was not confined to the dorsal part of the OVLT in mice, unlike rats treated with hypertonic saline that express c-fos mainly in the dorsal cap of the OVLT (30). Bar = 100µm
Fig. 8. Photomicrographs of the effect of no treatment (Cont; A,B), i.p. injection of 0.8 mol/l NaCl (HTS; C,D) or s.c. 30% polyethylene glycol (PEG; E,F) on Fos-immunoreactivity (seen as black dots) in the subfornical organ of wild-type (WT) or Agt-/- mice. Bar = 100µm
289x137mm (96 x 96 DPI)
Fig. 9. Photomicrographs of the effect of no treatment (Cont; A,B), i.p. injection of 0.8 mol/l NaCl (HTS; C,D) or s.c. 30% polyethylene glycol (PEG; E,F) on Fos-immunoreactivity (seen as black dots) in the ventral part of the median preoptic nucleus (arrowed) of wild-type (WT) or Agt-/- mice. Bar = 100µm.