The ethanol metabolite acetaldehyde induces water and salt intake via two distinct pathways in the central nervous system of rats

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- Abbreviated title: Acetaldehyde induces thirst
- Key words: ethanol, thirst, acetaldehyde, angiotensin II
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Abbreviations. ACD, acetaldehyde; ALDH, aldehyde dehydrogenase; AngII, angiotensin II; AP-5, D-2-amino-5-phosphonopentanoic acid; AT1R, angiotensin receptor type 1; AVP, vasopressin; Bic, (-)-bicuculline methiodide; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CVO, circumventricular organ; CY, cyanamide; DP, D-penicillamine; eGFP, enhanced green fluorescent protein; EtOH, ethanol; 4-MP, 4-methylpyrazole; NMDA, N-methyl-D-aspartic acid; i.c.v., intracerebroventricular; i.p., intraperitoneal; NS, isotonic normal saline; OVLT, organum vasculosum of the lamina terminalis; PVN, paraventricular nucleus; s.c., subcutaneous; SFO, subfornical organ; SON, supraoptic nucleus

Abstract

The sensation of thirst experienced after heavy alcohol drinking is widely regarded as a consequence of ethanol (EtOH)-induced diuresis, but EtOH in high doses actually induces anti-diuresis. The present study was designed to investigate the introduction mechanism of water and salt intake after heavy alcohol drinking, focusing on action of acetaldehyde, a metabolite of EtOH and a toxic substance, using rats. The aldehyde dehydrogenase (ALDH) inhibitor cyanamide was used to mimic the effect of prolonged acetaldehyde exposure because acetaldehyde is quickly degraded by ALDH. Systemic administration of a high-dose of EtOH at 2.5 g/kg induced water and salt intake with anti-diuresis. Cyanamide enhanced the fluid intake following EtOH and acetaldehyde administration. Systemic administration of acetaldehyde with cyanamide suppressed blood pressure and increased plasma renin activity. Blockade of central angiotensin receptor AT1R suppressed the acetaldehyde-induced fluid intake and c-Fos expression in the circumventricular organs (CVOs), which form part of dipsogenic mechanism in the brain. In addition, central administration of acetaldehyde together with cyanamide selectively induced water but not salt intake without changes in blood pressure. In electrophysiological recordings from slice preparations, acetaldehyde specifically excited angiotensin-sensitive neurons in the CVO. These results suggest that acetaldehyde evokes the thirst sensation following heavy alcohol drinking, by two distinct and previously unsuspected mechanisms, independent of diuresis. First acetaldehyde indirectly activates AT1R in the dipsogenic centers via the peripheral renin-angiotensin system following the depressor response and induces both water and salt intake. Secondly acetaldehyde directly activates neurons in the dipsogenic centers and induces only water intake.

1. Introduction

After drinking large amounts of alcohol, many people experience thirst (Penning et al., 2012). It is widely believed that the thirst sensation can be attributed to alcohol- or ethanol (EtOH)-induced diuresis (Wang et al., 1991a). EtOH has been reported to reduce vasopressin (AVP) release from the nerve terminals of the posterior pituitary, resulting in increased urine formation (Eisenhofer and Johnson, 1982; Wang et al., 1991b). However, although low doses induce diuresis, urine volume is reduced rather than increased by large doses of EtOH (Pohorecky, 1985). This suggests that diuresis is not always the most important underlying factor for thirst sensation after heavy-alcohol drinking. In the condition known as hangover, in which subjects experience nausea, vomiting and dizziness in addition to thirst, the symptoms are thought to be elicited by acetaldehyde, a metabolite of EtOH and a toxic substance (Penning et al., 2012). However, acetaldehyde is not usually considered to be the factor that causes thirst after alcohol drinking because acetaldehyde has no effect on AVP release from the posterior pituitary (Hashimoto et al., 1985).

Acetaldehyde dilates blood vessels and lowers blood pressure (Satoh et al., 2008). Such depressor responses induce renin secretion from the kidneys, resulting in an increase in plasma angiotensin II (AngII) concentration (Wagner et al., 2010). AngII activates neurons in the dipsogenic centers that are situated in the circumventricular organs (CVOs) of the brain, including the organum vasculosum of the lamina terminalis (OVLT) and the subfornical organ (SFO), increasing both water and salt intake (Fitzsimons, 1998; McKinley et al., 2003). In addition, *in vivo* and *in vitro* studies have reported direct effects of acetaldehyde on neurons in brain regions protected by the blood-brain barrier (Melis et al., 2009). The CVOs lack a blood-brain barrier so circulating acetaldehyde may act directly on the neurons in the CVOs and induce thirst sensation. Here, we hypothesized that acetaldehyde, a thirst-inducing factor after heavy alcohol drinking, acts in ways that are independent of diuresis.

While EtOH has both anesthetic and sedative effects that might mask the behavioral responses induced by EtOH, the anesthetic and sedative effects of acetaldehyde are very weak (Weight et al., 1991; Stubbs and Rubin, 1993). These differences confer a great advantage on acetaldehyde when investigating thirst-related behavior after heavy

alcohol drinking. For this reason we used acetaldehyde instead of EtOH in most of the experiments in the present study. Meanwhile, acetaldehyde is quickly degraded *in vivo* by aldehyde dehydrogenase (ALDH) (Isse et al., 2005). To avoid the problems associated with degradation and to maintain effective concentrations of acetaldehyde in the body similar to those after EtOH administration (or drinking) (Tsukamoto et al., 1989; Kinoshita et al., 2001), the ALDH inhibitor cyanamide was administered together with the acetaldehyde. In this study, we used a multidisciplinary approach combining behavioral, molecular biological and physiological approaches to support the hypothesis that acetaldehyde acts indirectly by activating AT1R in the dipsogenic centers and also activates neurons in the dipsogenic centers directly.

2. Methods

2.1. Animals

The study was conducted on male Wistar rats (7-10 weeks old for *in vivo* experiments and 3-4 weeks old for *in vitro* experiments). Rats of different ages were used intentionally for technical reasons to render the different types of experiments easier to conduct. AVP-enhanced green fluorescent protein (eGFP) Wistar transgenic rats were bred and maintained as described previously (Suzuki et al., 2009). All rats were housed in plastic cages under regular light/dark conditions (the lights were on from 8:00 AM to 8:00 PM). The temperature was maintained at 23 ± 1 °C. The rats had access to water and laboratory pellets *ad libitum*, except during experimental procedures. The handling and care of animals used in these experiments were in accordance with NIH recommendations for the humane use of animals. All experimental procedures were reviewed and approved by the appropriate animal experiment committees of Kyushu Dental University and University of Occupational and Environmental Health. All efforts were made to minimize animal suffering and to reduce the number of animals used. All AVP-eGFP rats were screened by polymerase chain reaction analysis of genomic DNA extracted from rat-ear biopsies (Suzuki et al., 2009).

2.2. Surgery and intracerebroventricular (i.c.v.) injections

A 24-gauge stainless-steel guide cannula was implanted into each rat under sodium pentobarbital anesthesia (60 mg/kg, i.p. injection) for i.c.v. injection of drugs, as described previously (Hirase et al., 2008; Miyahara et al., 2012). The cannulae were implanted into the lateral cerebral ventricles 0.7 mm caudal to the bregma, 1.4 mm to the left of the midline, and 2.0 mm below the dura. Test solutions were injected i.c.v. after a minimum of seven days following the surgery. Drugs were injected in a volume of 4 μl over a period of 30 s with a 25-μl Hamilton syringe (Hamilton, Reno, NV, USA) attached to polyethylene tube (PE10, Becton, Dickinson & Co., MD, USA). The positions of the tips of i.c.v. cannulae were verified by injecting methylene blue (Sigma-Aldrich, MO, USA) through the cannulae, followed by serial sectioning to confirm tip position.

2.3. Measurements of fluid intake using two-bottle test

The assessments of fluid intake were performed using two-bottle test, with one bottle filled with water and the other with 0.3 M NaCl solution. The amounts of water and salt intake were measured by subtracting the weight of each bottle (50 ml) at the end of each period from that at the beginning, (to the nearest 0.01 g). In both cases, the specific gravities of the solutions were considered one. The results were expressed as ml/100 g body weight. Animals were acclimated to the presence of the two-bottles for more than 3 days.

2.4. Measurement of urine volume

The measurement of urine volume was performed using a metabolic cage (3701D000, Tecniplast, Buguggiate, Italy). All animals were accustomed to being housed in the cages with water (and 0.3 M NaCl) bottles for more than 2 h for 3 d before testing. On the day of the experiment, they were moved to the cage with fluid solutions but without laboratory pellets 2 h before drug administration. Changes in urine volume after the relevant drug administration were measured either in the presence of or in the absence of the fluid solutions. Urine volume was expressed as ml/100 g body

weight.

2.5. Measurement of blood pressure

Rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). Blood pressure was monitored with a catheter filled with heparin (250 U/ml) in isotonic saline inserted into the right femoral artery. The catheter was connected to a pressure-transducer (Nihon Kohden, Tokyo, Japan). The blood pressure and heart rate were recorded with the PowerLab system (sampling rate: 200 Hz, ADInstruments, NSW, Australia). Changes in the mean blood pressure or mean heart rate were expressed as percentage changes every 2 min from the average values of the blood pressure or the heart rate for 6 min before the intraperitoneal (i.p.) or i.c.v. injections of acetaldehyde began.

2.6. Measurement of plasma renin activity, sodium level and osmolality

Rats were decapitated 45 min after i.p. administration of acetaldehyde and blood samples were collected from the trunk into EDTA-coated vials. Plasma was separated from the blood samples by centrifugation at 3,500 g for 15 min at 4 °C and stored in a deep freezer until measurements were made. Plasma renin activity was measured with a standard double antibody radioimmunoassay method (SRL, Tokyo, Japan). Plasma sodium level and osmolality were measured using a sodium ion electrode (SRL, Tokyo, Japan) and a Fiske osmometer (ONE-TEN, MA, USA), respectively.

2.7. Expression of AVP-eGFP

AVP-eGFP transgenic rats were perfused transcardially with 4% paraformaldehyde under sodium pentobarbital anesthesia 6 and 12 h after i.p. administrations of EtOH or isotonic normal saline (0.9% NaCl). In this experiment, we used a longer time protocol, which is different from the other experiments in this study, because it took several hours to synthesize proteins (Suzuki et al., 2009). The brains and pituitaries were carefully removed and divided into three blocks that included the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) in the hypothalamus, where AVP is synthesized in the

nuclei and transported to the posterior pituitary. The blocks were post-fixed in the same fixative for 48 h, following 20% sucrose for 48 h. The brain blocks were cut at 30 μm with a freezing microtome. The sections containing the SON, the PVN and posterior pituitary were observed by fluorescent microscopy (ECLIPSE E600, Nikon, Tokyo, Japan) with a GFP filter (Nikon, Tokyo, Japan) to establish AVP-eGFP expression. The images were photographed with a digital camera (DS-Fi1-L2, Nikon, Tokyo, Japan) and analyzed using the imaging software (NIS-Elements AR3.2, Nikon, Tokyo, Japan). The mean densities of eGFP fluorescence in the SON, the PVN and the posterior pituitary were expressed as a ratio with the controls.

2.8. Immunohistochemistry for c-Fos

Rats were perfused transcardially with 4% paraformaldehyde under sodium pentobarbital anesthesia 90 min after i.p. administrations of acetaldehyde or isotonic normal saline. Dissected brain blocks were post-fixed in the same fixative following immersion in 20% sucrose overnight. The brain blocks were sectioned serially at 40 μm in the coronal plane with a freezing microtome. Alternate free-floating sections containing the OVLT and the SFO were stained with rabbit polyclonal antibodies to c-Fos (diluted 1:2000; Lot#H0806, Santa Cruz Biotechnology, Dallas, USA), following the conventional protocols described in our previous studies (Inenaga et al., 2008; Asami et al., 2010). The use of the same procedures without incubation of the antibody for c-Fos on sections from two rats served as a negative control (data not shown). For quantification of c-Fos immunoreactive cells, one brain section from each nucleus that contained the greatest number of labeled cells was selected in the OVLT and SFO. The number of c-Fos immuno-positive cells was double-blind counted by two people (AK and SK).

2.9. Limitation of the number and interval of drug administration

The number of administrations with drugs including isotonic saline was limited to three times per rat, with at least a seven day interval between administrations. The treatments with the drugs were randomized.

2.10. In vivo experimental protocols

2.10.1. In vivo experimental protocol 1 - Systemic administration of EtOH in measurements of water and salt intake, urine volume, and expression of AVP-eGFP

To test the effects of EtOH on water and salt intake, urine volume, and expression of AVP-eGFP, EtOH (Kishida, Osaka, Japan) at 2.5 g/10 ml/kg (or 1.0 g/10 ml/kg) was administered i.p. either alone or together with the ALDH inhibitor cyanamide (Sigma-Aldrich, MO, USA) at 50 mg/10 ml/kg. As a control, isotonic normal saline was administered. Laboratory pellets were removed 2 h before the i.p. administration. Except for in the experiments involving the measurement of water and salt intake, the water and salt solution bottles were removed from the cages after the EtOH administration.

To test the involvement of acetaldehyde degraded from EtOH in fluid intake, the alcohol dehydrogenase inhibitor 4-methylpyrazole (Tokyokasei, Tokyo, Japan) (Closon et al., 2009) at 10 mg/ml/kg was administered i.p. 1 h before EtOH.

2.10.2. In vivo experimental protocol 2 - Systemic administration of acetaldehyde for measurement of water and salt intake, urine volume, blood pressure and plasma renin activity

To test the effects of acetaldehyde on water and salt intake, urine volume, blood pressure and plasma renin activity, acetaldehyde (Merck, Hohenbrunn, Germany) at 100 mg/10 ml/kg or isotonic normal saline was i.p. administered. Cyanamide at 50 mg/ml/kg or isotonic normal saline was administered i.p. 1 h before acetaldehyde or isotonic normal saline administration. Laboratory pellets were removed 2 h before and the water and 0.3 M NaCl bottles were removed 1 h before the acetaldehyde administration. In the experiments involving the measurement of water and salt intake, the water and 0.3 M NaCl solution bottles were returned to the cages.

To determine whether acetaldehyde induced fluid intake, the sequestering acetaldehyde agent D-penicillamine (Tokyokasei, Tokyo, Japan) (Orrico et al., 2013) at

50 mg/ml/kg was administered i.p. 1 h before the acetaldehyde administration.

2.10.3. In vivo experimental protocol 3 - Peripheral and central administrations of candesartan in testing AT1R involvement in acetaldehyde-induced water and salt intake and c-Fos immunoreactivity in the SFO and OVLT

To test the involvement of AT1R in acetaldehyde-induced water and salt intake, the AT1R antagonist candesartan was given subcutaneously (s.c.) and i.c.v. to rats. Candesartan (Tocris, MN, USA) was dissolved in 0.1 N Na_2CO_3 and further diluted in isotonic normal saline, with a final pH of 7.5-8.0. For s.c. administration, candesartan was given for 2 consecutive days before testing (1 mg/ml/kg/day), according to a previous study (Sanchez-Lemus et al., 2009). On the experimental days, the same dose of candesartan or the vehicle was administered s.c. 1 h before i.p. administration of acetaldehyde. For i.c.v. injection, candesartan (4 μg) or the vehicle was given in a volume of 4 μl, 15 min before i.p. administration of acetaldehyde. Other protocols were the same as those in *In vivo experimental protocol 2.*

2.10.4. In vivo experimental protocol 4 - Central administration of acetaldehyde and measurement of water and salt intake and blood pressure

To test the central effect of acetaldehyde on water and salt intake and blood pressure, acetaldehyde (3 μmol) was i.c.v. injected in a volume of 4 μl. The other protocols were the same as *In vivo experimental protocol 2.*

2.11. SFO slice preparations and extracellular recordings

To test the direct effect of acetaldehyde on neuronal activity in the SFO, SFO slice preparations were used. Rats were deeply anesthetized with sodium pentobarbital and decapitated. SFO slices with thicknesses of 300 μm were prepared in a cold slice-cutting solution (in mM; Sucrose 211, KCl 3, $NaHCO_3$ 26, glucose 10, $MgSO_4$ 1.3, KH_2PO_4 1.24, $CaCl_2$ 2.1), and preincubated in the bathing and perfusion solution (in mM; NaCl 124, KCl 3, $NaHCO_3$ 26, glucose 10, $MgSO_4$ 1.3, KH_2PO_4 1.24, $CaCl_2$ 0.75, which was

oxygenated with 95% O_2 and 5% CO_2) at room temperature for at least 1 h before electrophysiological recording (Miyahara et al., 2012).

Extracellular multi-unit recordings (MEA Multichannel Systems, Reutlingen, Germany) were made from the SFO as described in our previous studies (Miyahara et al., 2012). This recording system gives stable and long-term recordings, compared with conventional extracellular recordings using a single glass micropipette. Frequently, continuous and reliable recordings could be obtained for 4-5 h. Each slice from which recordings were made was submerged in a recording chamber containing an array of microelectrodes with a volume of 1 ml at room temperature (20-23°C). The perfusion flow rate was set to 1 ml/min. Acetaldehyde at 1-100 μM, AngII at 0.1 μM and cyanamide at 2 mM were applied to the perfusion medium. Synaptic blockade involved the inclusion of the non-NMDA (N-methyl-D-aspartic acid) receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Sigma-Aldrich, MO, USA) at 3 μM, the NMDA receptor antagonist D-2-amino-5-phosphonopentanoic acid (AP-5, Tocris, MN, USA) at 50 μM and the $GABA_A$ receptor antagonist (-)-bicuculline methiodide (Sigma-Aldrich, MO, USA) at 10 μM in the perfusion solution. The data were stored on a computer hard disk drive through the PowerLab system (sampling rate: 10 kHz). In offline analysis, the amplitude ranges were set to discriminate neural activity as multi-units using a window discriminator and with amplitude and interspike interval histograms (Spike Histogram, AD Instruments, NSW, Australia). The average discharge rate for the 5 min before the drug administration was used as the control. To evaluate the responses the averaged discharge rate for 1 min after the application of the stimulus was compared with the control. If the average discharge rate was greater or lesser than the control value by 20% or more, the unit was considered sensitive to the applied chemicals.

2.12. Statistical analysis

The numerical data are presented as the means ± SEM, and n represents the number of animals or units. Statistical analysis was performed using GraphPad Prism software (GraphPad, San Diego, CA, USA). Two-way repeated measures ANOVA with time and treatment as independent variables followed by the Bonferroni *post hoc* test and

unpaired two-tailed Student's *t*-tests were used to compare different groups. Fisher's exact probability test was used to investigate the relationship between two dichotomous variables. Statistical significance was described as $p < 0.05$, 0.01 and 0.001.

3. Results

3.1. Systemic administration of EtOH induces water and salt intake

The intakes of water and salt (0.3 M NaCl) solutions after i.p. administration of EtOH at 1.0 g/kg or 2.5 g/kg were compared with those following i.p. isotonic normal saline administration (NS, n = 7). Water and salt intake was not changed by EtOH at 1.0 g/kg (n = 7, $F_{(1, 12)} = 0.18$, $p = 0.6810$ for water intake vs NS; $F_{(1, 12)} = 0.48$, $p = 0.5008$ for salt intake vs NS). EtOH at 2.5 g/kg markedly increased water intake 3-5 h following its administration (EtOH, n = 8, $p < 0.001$; Fig. 1A). Two-way repeated measures ANOVA revealed that EtOH at 2.5 g/kg showed a slight but significant increase in salt intake compared to NS ($F_{(1, 13)} = 7.06$, $p = 0.0197$), although the Bonferroni *post hoc* test revealed no individual differences. When the ALDH inhibitor cyanamide (50 mg/kg) was administered with EtOH at 2.5 g/kg (EtOH + CY, n = 7), both water and salt intake after 1-3 h ($p < 0.05$ for both vs NS) and 3-5 h ($p < 0.05$ for water intake vs NS; $p < 0.01$ for salt intake vs NS) were significantly increased. In addition, two-way repeated measures ANOVA revealed that EtOH + CY showed a significant increase in water intake ($F_{(1, 11)} = 6.02$, $p = 0.0321$) although the Bonferroni *post hoc* test revealed no individual differences, and showed a significant increase in salt intake after 1-3 h ($p < 0.05$) and 3-5 h ($p < 0.01$), compared to CY (n = 6). When EtOH at 1.0 g/kg was administered with cyanamide (n = 7), water ($F_{(1, 14)} = 4.03$, $p = 0.0644$) and salt intake ($F_{(1, 14)} = 3.52$, $p = 0.0817$) was slightly but not significantly increased compared with NS. When evaluating the total volume of fluid intake over 7 h, water intake was significantly increased compared with NS ($p < 0.01$, 0.33 ± 0.13 ml/100 g for NS; 1.22 ± 0.24 ml/100 g for EtOH at 1.0 g/kg). Additionally, the alcohol dehydrogenase inhibitor 4-methylpyrazole, which was administered 1 h before EtOH, significantly suppressed EtOH at 2.5 g/kg and EtOH (2.5 g/kg) + CY induced water intake (Supplemental Fig. 1, n = 8). Salt intake induced by EtOH and EtOH + CY was

seemed to be suppressed by 4-methylpyrazole but not significantly. These results suggest that the EtOH metabolite acetaldehyde was responsible for the fluid intake.

(Fig. 1, here)

Thirst sensation is thought to be caused by alcohol- or EtOH-induced diuresis (Dopico et al., 1995). Unexpectedly, the urine volume did not increase but significantly decreased during the 3-5 h after EtOH (n = 7, $p < 0.001$) or EtOH + CY (n = 7, $p < 0.05$) compared with NS (n = 7) and after EtOH + CY ($p < 0.001$) compared with CY (n = 7, Fig. 1C). To determine the reason that urine volume decreased after EtOH administration, expression of AVP-eGFP was investigated in the SON, PVN and posterior pituitary following EtOH at 2.5 g/kg (Fig. 2). The expression levels increased in the SON ($p < 0.01$, unpaired *t*-test) and PVN ($p < 0.001$, unpaired *t*-test) and decreased in the posterior pituitary 6 h ($p < 0.01$, unpaired *t*-test) (n = 6, Fig. 2A) and tended to recover to the control level 12 h after EtOH administration (n = 4, Fig. 2B) compared with NS (n = 6 for 6 h; n = 3 for 12 h). The decreased urine volume induced by EtOH administration may be explained by increased production of AVP in the SON and PVN and increased release of AVP from the posterior pituitary, suggesting that, as a result, the body fluid increases and thirst sensation is suppressed.

(Fig. 2, here)

3.2. Systemic administration of acetaldehyde induces both water and salt intake without change in urine volume

As shown in Fig. 1A, EtOH alone and EtOH with cyanamide induced significant thirst responses at 3-5 h and at 1-3 h, respectively, for the first time. The delayed responses were considered to be due to the anesthetic and sedative effect of EtOH (Stubbs and Rubin, 1993). Next, we tested the effects of acetaldehyde, suggested as a thirst inducing factor in this study and with very weak anesthetic and sedative effects, on drinking behavior. Acetaldehyde is quickly degraded by ALDH (Isse et al., 2005). To suppress the degradation of acetaldehyde and to keep an effective level of acetaldehyde in circulation, cyanamide at 50 mg/kg was administered i.p. 1 h prior to acetaldehyde (100 mg/kg). While acetaldehyde alone slightly but significantly increased salt intake at

0-1 h (NS + ACD, n = 7, $p < 0.05$), the pre-application of cyanamide with acetaldehyde significantly enhanced both water and salt intake at 0-1 h ($p < 0.001$ for both) and at 1-3 h ($p < 0.01$ for water intake) (CY + ACD, n = 8), compared with NS + NS (n = 7) (Fig. 1B). Cyanamide alone also led to small but significant increases in water intake (two-way ANOVA, $F_{(1, 13)} = 5.32$, $p = 0.0382$) and salt intake at 0-1 h ($p < 0.05$) (CY + NS, n = 8) in this experimental protocol, suggesting that cyanamide suppresses the degradation of intrinsically produced acetaldehyde and subsequently helps maintain an effective concentration of acetaldehyde (Ostrovsky, 1986; Gill et al., 1992) (see Discussion). In the experiments described below, cyanamide was administered i.p. 1 h before acetaldehyde administration (unless otherwise noted).

To test whether acetaldehyde induced water and salt intake, the sequestering acetaldehyde agent D-penicillamine at 10 mg/kg was administered with cyanamide 1 h before acetaldehyde (Supplemental Fig. 2, DP + CY +ACD, n = 8). D-Penicillamine significantly suppressed both water ($p < 0.001$) and salt ($p < 0.001$) intake at 0-1 h.

No change in urine volume was found after i.p. administration of acetaldehyde with the pre-administration of cyanamide (CY + ACD, n = 6), compared with both NS + NS (n = 6) and CY + NS (n = 6), different from the EtOH results. When the animals were allowed to drink both water and salt solutions (Drinking + CY + ACD, n = 6; 2.10 ± 0.67 ml/100 g body weight at 0-1 h, 1.83 ± 1.05 ml/100 g body weight at 1-3 h, 0.46 ± 0.59 ml/100 g body weight at 3-5 h), the urine volume was increased for the first time at 1-3 h ($p < 0.001$ vs CY + ACD; Fig. 1D). Together with the results of EtOH administration, these results indicate that water and salt intake induced by EtOH or acetaldehyde administration is not a consequence of diuresis, and diuresis is a consequence of fluid intake; however, there is a discrepancy that has to be solved between the results from the EtOH and acetaldehyde studies.

3.3. Systemic administration of acetaldehyde induces a depressor response and increases plasma renin activity without changing plasma sodium level

It has been reported that acetaldehyde induces a depressor response (Satoh et al., 2008). Physiological changes, such as the depressor response, are thought to activate the peripheral renin-angiotensin system (Fitzsimons, 1998). We thus investigated whether

the changes in blood pressure that followed acetaldehyde administration occurred in anaesthetized rats. Mean blood pressure was significantly reduced by i.p. administration of acetaldehyde associated with a significant increase in heart rate (CY + ACD, n = 7; Fig. 3A and B) for both NS + NS (n = 7) and CY + NS (n = 7). We next investigated the changes in plasma renin activity that occurred 45 min after i.p. administration of acetaldehyde in conscious rats. Fig. 3C shows that acetaldehyde (CY + ACD, n = 8) significantly increased plasma renin activity ($p < 0.01$, unpaired *t*-test). Plasma sodium concentration, another trigger that activates the peripheral renin-angiotensin system, was unchanged (Fig. 3D) for both NS + NS (n = 7) and CY + NS (n = 7). These results suggest that renin secretion is triggered by lowering of blood pressure rather than the lowering of plasma sodium concentration.

(Fig. 3, here)

Thirst sensation is caused by hyperosmotic stimulation, as well as activation of the renin-angiotensin system (Fitzsimons, 1998). To assess whether osmotic change was responsible for the acetaldehyde-induced drinking responses, plasma osmolality was measured in the same samples as used in the measurements of plasma renin activity and plasma sodium concentration. Fig. 3E showed that no change in plasma osmolality occurred after acetaldehyde administration. This result suggests that the acetaldehyde-induced changes in fluid intake observed in the present study were not the result of changes in plasma osmolality.

3.4. Systemic administration of acetaldehyde induces fluid intakes via central AT1R

We next tested whether AngII was involved in acetaldehyde-induced fluid intake. To answer this question, we made s.c. and i.c.v. injections of the AT1R antagonist candesartan. To evaluate the effect of candesartan, we compared the fluid intake amounts at the time point of 0-1 h, where the most marked changes were observed. As shown in Fig. 3F, s.c. administration of candesartan (Can_{sc}) at 1 mg/kg (n = 11) significantly suppressed both the acetaldehyde-induced water ($p < 0.001$, unpaired *t*-test) and salt intake for the first hour ($p < 0.05$, unpaired *t*-test), compared with the effect of acetaldehyde administration without candesartan (n = 12). The AngII-induced

water and salt intake is thought to be a consequence of the activation of AT1R in the central nervous system (Fitzsimons, 1998). We thus tested this possibility via i.c.v. injection of candesartan. Fig. 3G shows that candesartan (Can_{icv}, n = 12) significantly suppressed both the acetaldehyde-induced water ($p < 0.001$, unpaired *t*-test) and salt intake ($p < 0.05$, unpaired *t*-test), when compared with acetaldehyde administration without candesartan (n = 12). S.C. or i.c.v. injection (n = 5/group) of candesartan in the absence of acetaldehyde had no effects on water and salt intake (data not shown). These results suggest that the angiotensin receptors related to the acetaldehyde-induced fluid intake are located in the central nervous system.

3.5. Acetaldehyde activates neurons in dipsogenic areas of the brain

c-Fos expression is a commonly used marker of neural activity (Asami et al., 2010). To test whether dipsogenic centers in the brain were involved in the acetaldehyde-induced drinking responses, changes in the number of c-Fos immuno-positive neurons in the OVLT and SFO were evaluated with a similar stimulation protocol to that in drinking behavior experiments. The number of c-Fos immuno-positive neurons in the two nuclei was significantly increased 90 min after i.p. administration of acetaldehyde (CY + veh_{icv} + ACD, n = 6) compared with both NS + veh_{icv} + NS (n = 4) and CY + veh_{icv} + NS (n = 4) (both, $p < 0.05$ for the OVLT; both, $p < 0.01$ for the SFO, unpaired *t*-test; Fig. 4). The increased number of c-Fos immuno-positive neurons was significantly suppressed by i.c.v. injection of candesartan (CY + Can_{icv} + ACD, n = 6) compared with CY + veh_{icv} + ACD ($p < 0.01$ for the OVLT; $p < 0.001$ for the SFO, unpaired *t*-test). These results suggest that the dipsogenic centers of the brain, such as the OVLT and SFO, are involved in the acetaldehyde-induced fluid intake by activating AT1R receptors in these regions. In addition, the number of c-Fos immuno-positive neurons in the SFO significantly increased due to CY administration (CY + veh_{icv} + NS) compared with NS + veh_{icv} + NS, indicating that the effect of endogenous acetaldehyde on neuronal activity occurred in the SFO.

(Fig. 4, here)

3.6. Central administration of acetaldehyde induces water intake but has no effects on

blood pressure

Acetaldehyde may directly affect neurons in the dipsogenic centers because it has been found to do so in other brain regions (Melis et al., 2009). To test the central effects of acetaldehyde on fluid intake, we performed i.c.v. injections of acetaldehyde (ACD_{icv}). The concentration of acetaldehyde used (3 μmol/4 μl) was based on a previous study (McLaughlin et al., 2008). Acetaldehyde with cyanamide (CY + ACD_{icv}, n = 7) significantly increased water intake compared with NS + NS_{icv} (i.c.v. injection of isotonic saline) (n = 7) and CY + NS_{icv} (n = 7), but, surprisingly, had no effects on salt intake (Fig. 5A). Acetaldehyde alone (NS + ACD_{icv}, n = 6) did not affect water or salt intake. This is considered to be due to the quick degradation of acetaldehyde by ALDH, which is richly present in the circumventricular wall (Zimatkin et al., 1992). The effect of i.c.v. injection of acetaldehyde on blood pressure was also investigated under pentobarbital anesthesia. In contrast to i.p. administration of acetaldehyde (Fig. 3A), the i.c.v. injection of acetaldehyde (CY + ACD_{icv}, n = 7) had no effect on blood pressure when compared to both NS + NS_{icv} (n = 6) and CY + NS_{icv} (n = 7) (Fig. 5B). These results suggest that central stimulation by acetaldehyde evokes water intake but not salt intake by a mechanism that does not involve the renin-angiotensin pathway.

(Fig. 5, here)

3.7. Direct activation of SFO neurons by acetaldehyde in the slice preparation

The direct action of acetaldehyde on the neurons of the SFO was tested in 37 units from 12 brain slices using extracellular multi-unit recordings. Application of acetaldehyde while recording from the SFO units showed either excitation (Fig. 6A) or inhibition (Fig. 6B). In each case, the effect was dose-dependent (Fig. 6C). The threshold concentration for the action of acetaldehyde was approximately 30 μM in both cases. In earlier studies on SFO slice preparations we found that the synaptic inputs to SFO neurons responsible for changing their activity depended upon glutamatergic and GABAergic input (Inenaga et al., 1995; Xu et al., 2000). We thus tested the effects of synaptic blockades with the non-NMDA receptor antagonist CNQX at 3 μM, the NMDA receptor antagonist AP-5 at 50 μM and the $GABA_A$ receptor antagonist

bicuculline at 10 μM on the acetaldehyde-induced responses. Acetaldehyde responses were maintained in all units tested under the synaptic blockade, as shown in Figs. 6A (unit for excitation, n = 7) and B (unit for inhibition, n = 5), suggesting that acetaldehyde affected SFO neurons directly rather than indirectly though synaptic inputs. In addition, we tested whether the acetaldehyde responses were influenced by cyanamide. The aim of this experiment was to investigate whether acetaldehyde was degraded by ALDH present in the SFO (Zimatkin et al., 1992) and thus affected the neural responses by this mechanism. The concentration of 2 mM cyanamide used in the present experiment was based on that used in an earlier *in vitro* study (Srivastava et al., 1998). Application of cyanamide at 2 mM made no obvious change to the acetaldehyde responses (Fig. 6C), suggesting that the excitatory and inhibitory responses found in the present study are induced directly by acetaldehyde. We then tested the relationship between acetaldehyde responses and AngII responses. All 11 units that were excited by acetaldehyde at 100 μM were also excited by AngII at 0.1 μM (Fig. 6A). In addition, 3 of the 9 units that showed inhibition after acetaldehyde exhibited excitation after AngII at 0.1 μM; other units showed no responses (Fig. 6B). No units were inhibited by AngII at 0.1 μM. Fisher's exact probability test showed a significant association in unit responses of the SFO between acetaldehyde and AngII ($p < 0.01$), suggesting that SFO neurons excited by acetaldehyde were selectively responsive to AngII while those showing inhibitory responses were less responsive to AngII. These results suggest that acetaldehyde has specific effects on different subpopulations of SFO neurons.

(Fig. 6, here)

4. Discussion

Although the sensation of thirst after heavy alcohol drinking has been popularly proposed to be caused by ethanol-induced diuresis, this study reports for the first time that the EtOH metabolite acetaldehyde evokes water and salt intake, possibly via thirst sensation due to the activation of dipsogenic centers in the brain via two distinct mechanisms, an indirect mechanism involving the activation of AT1R and a direct effect on cells in the circumventricular regions, both of which are independent of diuresis.

4.1. EtOH and acetaldehyde elicit water and salt intake, independent of diuresis

EtOH is metabolized to acetaldehyde, and the acetaldehyde is further metabolized by ALDH. The ALDH inhibitor cyanamide, which is used to encourage abstinence from alcohol in alcoholics, induces an accumulation and a prolonged effect of acetaldehyde in the body if a substantial amount of EtOH is present (Jones et al., 1988). Administration of EtOH significantly increased water intake in the present study, which is consistent with a previous study (Fitts and Hoon, 1993). Water and salt intake induced by EtOH was enhanced by co-administration of cyanamide. Acetaldehyde given with cyanamide significantly increased both water and salt intake after its administration while the administration of acetaldehyde alone had no obvious effect on the fluid intake, even if given by i.c.v. injection. This result may be explained if acetaldehyde given alone is rapidly degraded with a half-life of several minutes *in vivo* (Isse et al., 2005). After alcohol drinking, a certain amount of acetaldehyde is accumulated and sustained in the body for several hours (Tsukamoto et al., 1989). In addition, this study showed that the EtOH- and acetaldehyde-induced fluid intakes were suppressed by the alcohol dehydrogenase inhibitor 4-methylpyrazole and the sequestering acetaldehyde agent D-penicillamine, respectively. Such observations suggest that the acetaldehyde accumulated after heavy alcohol drinking elicits water and salt intake. Administration of cyanamide on its own elicited small but significant increases in water and salt intake (Fig. 1B), an increased c-Fos immunoreactivity in the SFO (Fig. 4B), and a slight increase in water intake (Fig. 6A). This result may be because acetaldehyde continues to be synthesized endogenously in the body, and its degradation is inhibited by cyanamide (Ostrovsky, 1986; Gill et al., 1992). The data in Fig. 1B were obtained under *in vivo experimental protocol-2,* where rats were not allowed to drink fluid solutions for 1 h after administration of cyanamide (50 mg/ml/kg), and then, two-bottle test was started. However, we failed to find an effect of cyanamide in the other experimental protocol (*in vivo experimental protocol-1*; Fig. 1A), where rats were allowed to drink fluid solutions, and two-bottle test was started just after the administration of cyanamide (50 mg/10 ml/kg). Thus, the different results may be caused by the different timing of the cyanamide administrations and the different volumes of the cyanamide solutions.

Although we are unable satisfactorily to explain the different results of the cyanamide effects, they may occur because the influence of endogenous acetaldehyde is not significant.

Previous studies in both animals and humans have suggested that the dipsogenic response after alcohol administration or drinking is caused by EtOH-induced diuresis, which may be a consequence either of a suppressed release of AVP (Dopico et al., 1995) or a suppression of AVP V2 receptor activation (Taivainen et al., 1995). This behavior may be an over simplification because it has also been shown that, while EtOH-induced diuresis occurs after administration of low doses of EtOH, the administration of larger doses of EtOH gives an anti-diuretic response (Pohorecky, 1985). We found in the present study that urine volume decreased due to EtOH administration at doses high enough to induce water intake. The same dose levels increased expression of AVP-eGFP synthesized in the SON and PVN but decreased it in the pituitary of AVP-eGFP transgenic rats, suggesting that AVP was released from the posterior pituitary by EtOH loading so that urine volume decreased (Suzuki et al., 2009). EtOH produces acute vasodilatory action (Kawano et al., 1992) and hypovolemia (Fitts and Hoon, 1993), which could increase the AVP and AngII levels in the plasma. While it remains to be determined the way in which EtOH influences AVP expression and whether it really increases AVP in the plasma, such observations suggest that the increased water and salt intake that follows loading with EtOH (or acetaldehyde) is not a consequence of diuresis.

4.2. Acetaldehyde indirectly activates central AT1R via the peripheral renin-angiotensin system

Activation of AT1R in the dipsogenic regions of the brain evoke both water and salt intake (Gutman et al., 1989; Fitzsimons, 1998). We found a significant increase in plasma renin activity after the administration of acetaldehyde (Fig. 3C). Physiological stimulation of renin secretion from the juxtaglomerular cells of the kidney follows hypovolemia or a decrease in blood pressure and plasma sodium concentration (Fitzsimons, 1998). Hypovolemia is detected by vascular stretch receptors in various parts of the circulation (Fitzsimons, 1998). Thus, atrial blood pressure falls in

hypovolemia can influence the stretch receptors. It has been known that atrial blood pressure is reduced after acute EtOH intake (Kodavali and Townsend, 2006). Like EtOH, acetaldehyde dilates blood vessels and elicits hypotensive action (Satoh et al., 2008). In addition, acetaldehyde increases vascular permeability, which can accelerate fluid extravasation and induce hypovolemia (Haorah et al., 2005). The increased vascular permeability can also elicit hypotensive action (Bjorkqvist et al., 2013). The present study clearly showed that acetaldehyde induced depressor response (Fig. 3A) although presently it was difficult to discriminate whether the response was mediated through the mechanism of hypovolemia, but did not change sodium concentration in the plasma (Fig. 3D). The same protocol enhanced both water and salt intake (Fig. 1B). Furthermore, systemic and central administration of the AT1R antagonist candesartan significantly suppressed acetaldehyde-induced fluid intake (Figs. 3F and 3G). The central administration of candesartan suppressed the increased number of c-Fos immuno-positive neurons by acetaldehyde in the SFO and OVLT (Fig. 4). Together these findings suggest that acetaldehyde induces a depressor response that is followed by renin secretion and in turn elicits fluid intake via activation of central AT1R. On the other hand, hypovolemia occurs not only with decrease in blood pressure, but also without changes in blood pressure (Flynn and Stricker, 2003; Smith et al., 2007). Therefore, it is remained to clarify whether hypovolemia without changes in blood pressure is another factor for renin secretion in the administration of EtOH and acetaldehyde.

4.3. Acetaldehyde directly influences neurons in the CVOs

Penetration of acetaldehyde into the central nervous system from blood is restricted by the high ALDH activity at the blood-brain barrier (Hipolito et al., 2007), while its penetration into the cerebrospinal fluid is relatively high (Poso et al., 1981). Mounting evidence on alcoholism suggests that acetaldehyde affects neurons even protected by the blood-brain barrier (for review, (Melis et al., 2009)). Meanwhile, CVOs, such as the SFO and OVLT, lack a blood-brain barrier, and neurons there contact the cerebrospinal fluid (Johnson and Gross, 1993). In the electrophysiological recordings from SFO slice preparations, the threshold concentration of acetaldehyde (approximately 30 μM) was

comparable to the plasma concentration after EtOH loading (Tsukamoto et al., 1989; Kinoshita et al., 2002), although it was higher than that in the other brain regions (Diana et al., 2008). These findings suggest that neurons in CVOs might be directly affected if they were exposed to much higher concentrations of acetaldehyde than those found in other brain regions. Because CVOs lack blood-brain barriers, there is a real possibility that they might encounter high concentrations of acetaldehyde.

4.4. Acetaldehyde directly activates neurons of the AT1R-rich subpopulation in the SFO and induces water intake

As discussed in *4.2*, AT1R in the specific brain regions may be involved in the mechanism of acetaldehyde-induced fluid intake. Recently, it has been reported that restricted activation of AT1R-rich neurons in the SFO selectively enhanced water intake but not salt intake (Oka et al., 2015). If this is so, salt intake seen after EtOH and acetaldehyde administration in the present study is probably mediated through AT1R in regions of the brain other than the SFO, that are involved in sodium appetite (Geerling and Loewy, 2008). The present study showed that i.c.v. injection of acetaldehyde induced only water intake, but not salt intake, and had no effects on blood pressure, suggesting that the response is independent of the indirect one via the acetaldehyde-activated renin-angiotensin system already discussed. The electrophysiological results showed that SFO neurons with excitatory responses to acetaldehyde were selectively responsive to AngII, while those with inhibitory responses to acetaldehyde were less responsive to AngII. If these results are considered alongside the results of Oka et al. (Oka et al., 2015), it seems likely that a specific subpopulation of AT1R-rich neurons in the SFO is activated by acetaldehyde as well as by AngII and that activation of this subpopulation selectively elicits water intake.

5. Conclusion

The present study demonstrates that acetaldehyde evokes water and salt intake through both indirect and direct neuronal activation in the dipsogenic centers, independent of diuresis. Because acetaldehyde is toxic (Penning et al., 2012), it might be expected that acetaldehyde would be speedily diluted and excreted from the body.

Thus, fluid intake induced by acetaldehyde may have an important pharmaco-physiological role. Further, because indirect activation by acetaldehyde can be initiated by a depressor response (Fig. 3A), an increase in the total body fluid induced by both water and salt intake may be an appropriate physiological response to allow for blood pressure recovery. Pure water intake induces a greater diuresis than electrolyte solution (Perez-Idarraga and Aragon-Vargas, 2014). Thus, a possible physiological role of the direct effect of acetaldehyde may be to excrete urine with acetaldehyde from the body. Such acetaldehyde-induced responses may be involved in the experience of thirst after heavy alcohol drinking in humans.

Acknowledgements

We wish to acknowledge valuable discussion with Dr. T. Isse (University of Occupational & Environmental Health, Kitakyushu, Japan) and technical assistance of Drs. S. Kuramitsu, S. Kanda, T. Kondo, K. Tanaka and T. Yamaguchi and Ms. A. Kuroki (Kyushu Dental University, Kitakyushu, Japan). This work was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (JSPS KAKENHI 23592743 (K.I.) and 26462819 (K.I.)).

Figure legends

Figure 1. Enhancement of ethanol (EtOH) and acetaldehyde (ACD)-induced fluid intake by the aldehyde dehydrogenase inhibitor cyanamide (CY), independent of diuresis. A, Fluid intake after EtOH administration (n = 7-8/group). EtOH at 2.5 g/kg (EtOH) showed a significant increase in water intake 3-5 h after administration, compared to the isotonic normal saline group (NS). Water and salt intake due to EtOH was significantly enhanced by the combination of CY at 50 mg/kg (EtOH + CY) at 1-3 h and 3-5 h compared with NS. In addition, two-way repeated measures ANOVA revealed that EtOH + CY showed a significant increase in water intake ($F_{(1, 11)} = 6.02$, $p = 0.0321$) although the Bonferroni *post hoc* test revealed no individual differences and a significant increase of salt intake after 1-3 h and 3-5 h compared with CY (n = 6). **B**, Fluid intake after ACD administration (n = 7-8/group). ACD at 100 mg/kg with 1 h pre-application of CY at 50 mg/kg (CY + ACD) significantly increased water and salt intake at 0-1 h and 1-3 h compared with NS (1 h pre-application of isotonic normal saline) + NS and at 0-1 h compared with CY + NS while ACD (NS + ACD) alone slightly but significantly increased salt intake at 0-1 h. Note that CY (CY + NS) alone showed small but significant increases in water intake (two-way ANOVA, $F_{(1, 13)} = 5.32$, $p = 0.0382$) and salt intake at 0-1 h. **C**, Urine volume after EtOH administration (n = 7/group). The urine volume was significantly decreased 3-5 h after EtOH and EtOH + CY. Note that the gradual increase in the urine volume found even in the control groups (NS and CY) is due to i.p. loading of solutions of 10 ml/kg. **D**, Urine volume after ACD administration (n = 6/group). No change in the urine volume was found after ACD administration (CY + ACD). When the animals were allowed to drink (Drinking + CY + ACD), the urine volume was significantly increased compared with CY + ACD. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs NS or NS + NS, # $p < 0.05$ and ## $p < 0.01$ vs CY or CY + NS, +++ $p < 0.001$ vs CY + ACD, in the Bonferroni *post hoc* test following two-way ANOVA. Data are presented as the mean ± SEM.

Figure 2. The expression levels of enhanced green fluorescent protein (eGFP) in the supraoptic nucleus (SON), paraventricular nucleus (PVN) and posterior pituitary

(PP) in the vasopressin (AVP)-eGFP transgenic rats after i.p. administration of ethanol (EtOH). A, AVP-eGFP expression 6 h after the administration of isotonic normal saline (NS, n = 6) and EtOH (2.5 g/kg, n = 6). The expression levels were increased in the SON and PVN but decreased in the PP in the AVP-eGFP transgenic rats, suggesting that AVP was released from the PP by EtOH loading. **B**, AVP-eGFP expression 12 h after the administration of NS (n = 3) and EtOH (n = 4). The expression levels had almost recovered to the control level in all regions. ** $p < 0.01$ and *** $p < 0.001$ vs NS in unpaired *t*-test. Data are presented as the means ± SEM. Scale bar: 200 μm for the SON and PVN; 500 μm for the PP.

Figure 3. Systemic administration of acetaldehyde (ACD) induces depressor response, increases plasma renin activity and induces fluid intakes via central AT1R. A and **B**, Depressor response (**A**) and increase of heart rate (**B**) after ACD administration (CY + ACD, n = 7) under anesthesia, compared with the administration of isotonic normal saline groups (NS + NS and CY + NS, n = 7/group). ACD at 100 mg/kg was i.p. administered 1 h after cyanamide (CY) at 50 mg/kg. ACD or NS was applied at the arrow. **C**, **D** and **E**, ACD increased plasma renin activity (**C**) without changing plasma sodium concentration (**D**) and osmolality (**E**). Blood samples were obtained by decapitation 45 min after i.p. administration of ACD (CY + ACD, n = 8) or NS (NS + NS and CY + NS, n = 7/group). **F**, Decreases in ACD-induced water and salt intake for 1 h by subcutaneous (s.c.) administration of candesartan (Can_{sc}) at 1 mg/kg (n = 11) compared with the vehicle (n = 12). **G**, Decreases in the ACD-induced water and salt intake for 1 h due to intracerebroventricular (i.c.v.) injection of Can_{icv} at 4 μg/4 μl (n = 11) compared with the vehicle (n = 10). In **A** and **B**, * $p < 0.05$ vs NS + NS and ## $p < 0.01$ and ### $p < 0.001$ vs CY + NS from the Bonferroni *post hoc* test following two-way ANOVA. In **C**, **$p < 0.01$ vs NS + NS and ## $p < 0.01$ vs CY + NS, from the unpaired *t*–test. In **F** and **G**, * $p < 0.05$ and *** $p < 0.001$ vs CY + ACD, from the unpaired *t*-test. Data are presented as the means ± SEM.

Figure 4. c-Fos expression in the organum vasculosum of the lamina terminalis (OVLT) and the subfornical organ (SFO) by acetaldehyde (ACD) administration and suppression by the AT1R antagonist candesartan. A, Representative views of c-

Fos immunoreactivity in the OVLT and SFO 90 min after isotonic normal saline (NS + veh_{icv} + NS), cyanamide (CY + veh_{icv} + NS), ACD (CY + veh_{icv} + ACD) and ACD with candesartan (CY + Can_{icv} + ACD). Can at 4 μg/4 μl or the vehicle (veh) was i.c.v. injected 15 min before ACD or NS. **B**, Can_{icv} suppresses c-Fos expression in the OVLT and SFO induced by ACD. NS + veh_{icv} + NS (n = 4), CY + veh_{icv} + NS (n = 4), CY + veh_{icv} + ACD (n = 6) and CY + Can_{icv} + ACD (n = 6). * $p < 0.05$ and ** $p < 0.01$ vs NS + veh_{icv} + NS, # $p < 0.05$ and ## $p < 0.01$ vs CY + veh_{icv} + NS, ++ $p < 0.01$ and +++ $p < 0.001$ vs CY + veh_{icv} + ACD, in unpaired *t*-test. Data are presented as the mean ± SEM.

Figure 5. Intracerebroventricular (i.c.v.) injection of acetaldehyde (ACD_{icv}) induces water intake but not salt intake without effects on blood pressure. A, Water intake, but not salt intake, was significantly increased for 1 h by CY + ACD_{icv} (n = 7), compared with i.c.v. injection of isotonic normal saline (NS_{icv}) (both NS + NS_{icv} and CY + NS_{icv}, n = 7/group) or ACD (NS + ACD_{icv}, n = 6). ACD at 3 μmol/4 μl or NS was i.c.v. injected 1 h after cyanamide (CY) at 50 mg/kg or NS. ** $p < 0.01$ vs NS + NS_{icv}, ++ $p < 0.01$ vs NS + ACD_{icv} and # $p < 0.05$ vs CY + NS_{icv}, in unpaired *t*-test. n.s. indicates a non-significant relationship. **B**, ACD_{icv} had no effect on the mean blood pressure. The arrow indicates i.c.v. injections of NS (NS + NS_{icv} and CY + NS_{icv}, n = 7/group) or ACD (CY + ACD_{icv}, n = 7). For comparison, the result of depressor response with an intraperitoneal administration of ACD (CY + ACD_{ip}), shown in Fig. 3A, is described in this figure with a faint color. Data are presented as the means ± SEM.

Figure 6. Acetaldehyde (ACD) directly affects neurons in the subfornical organ (SFO). A and **B**, Extracellular recordings from slice preparations of the SFO. Units in **A** and **B** show excitation and inhibition, respectively, by ACD at 100 μM. The responses persisted after pharmacological synaptic blockade (n = 7 for excitation, n = 4 for inhibition). For synaptic blockades, the non-NMDA receptor antagonist CNQX at 3 μM, the NMDA receptor antagonist AP-5 at 50 μM and the $GABA_A$ receptor antagonist bicuculline (Bic) at 10 μM were applied to slice preparations. AngII at 0.1 μM excited a unit in **A** but not in **B**. **C**, Dose-response curves of ACD-induced responses with or without cyanamide (CY) at 2 mM. The curves were obtained from continuous unit recordings, which were performed at 3 or 4 different concentrations. n shows the

number of units. Data are presented as the means ± SEM.

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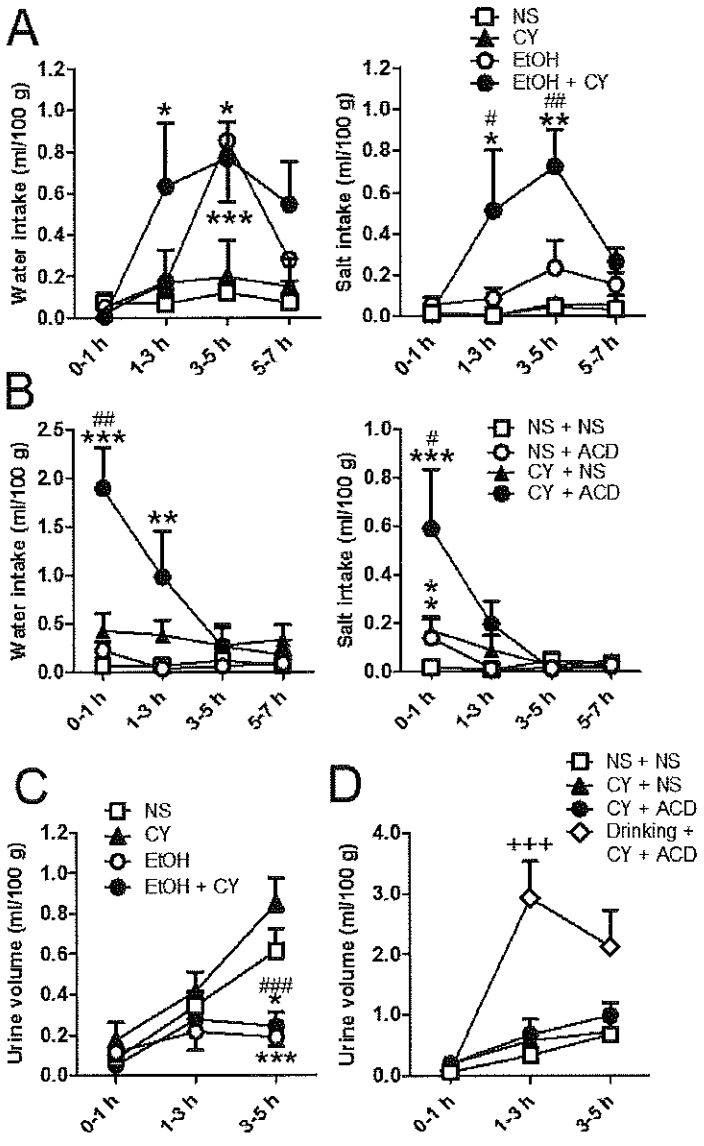

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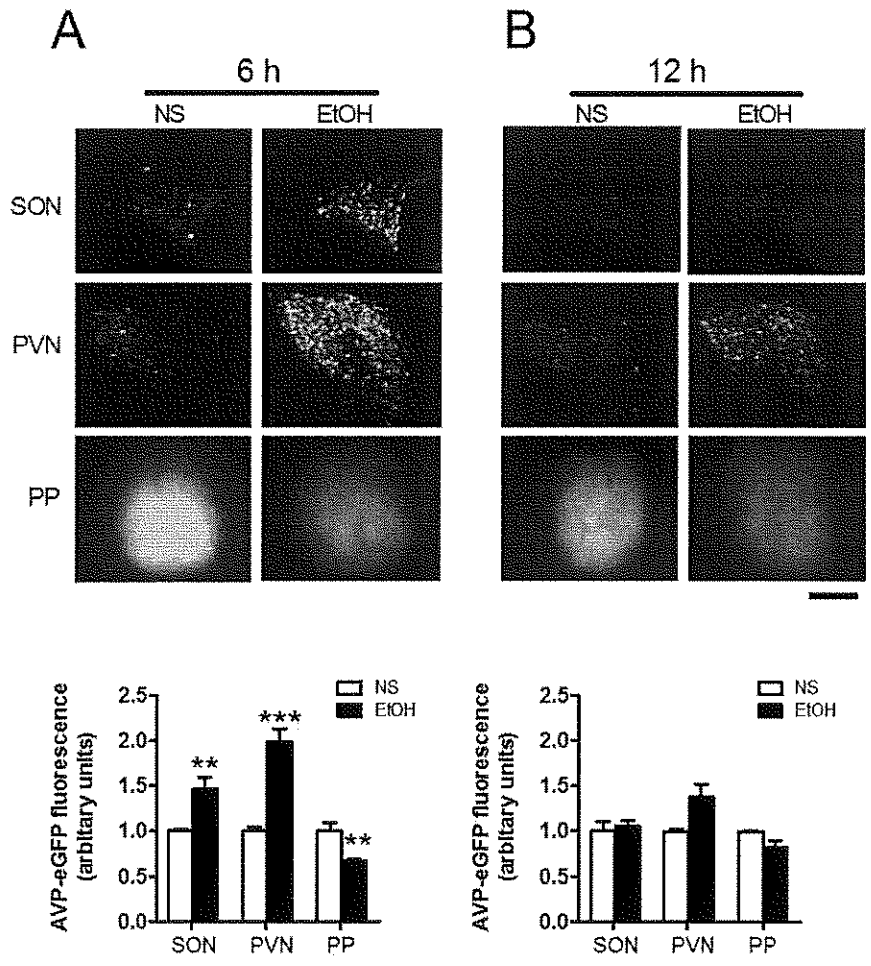

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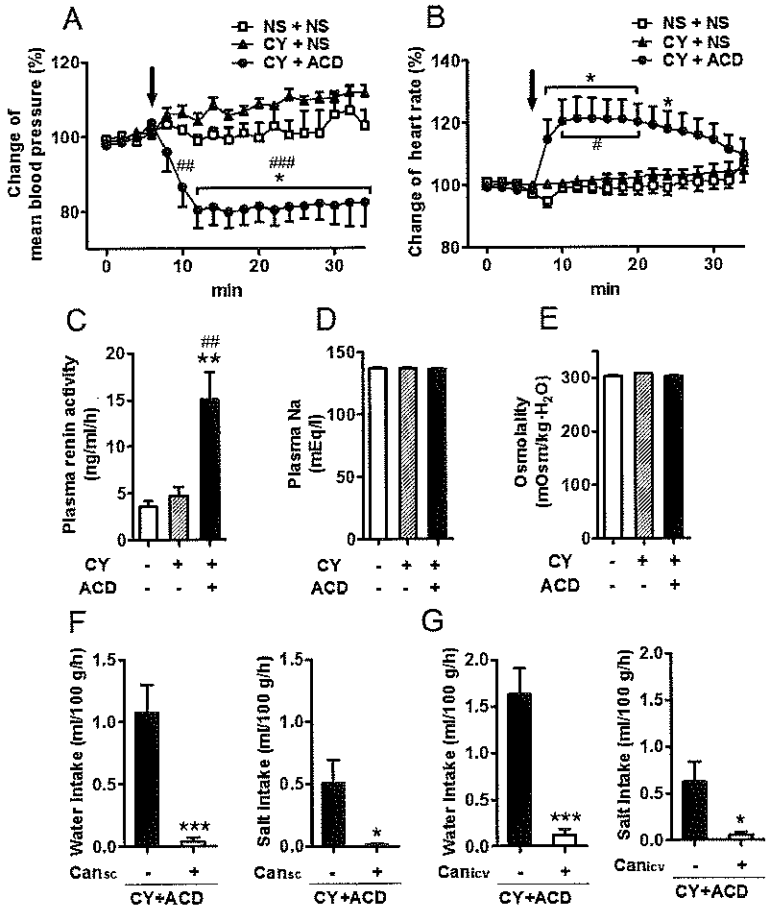

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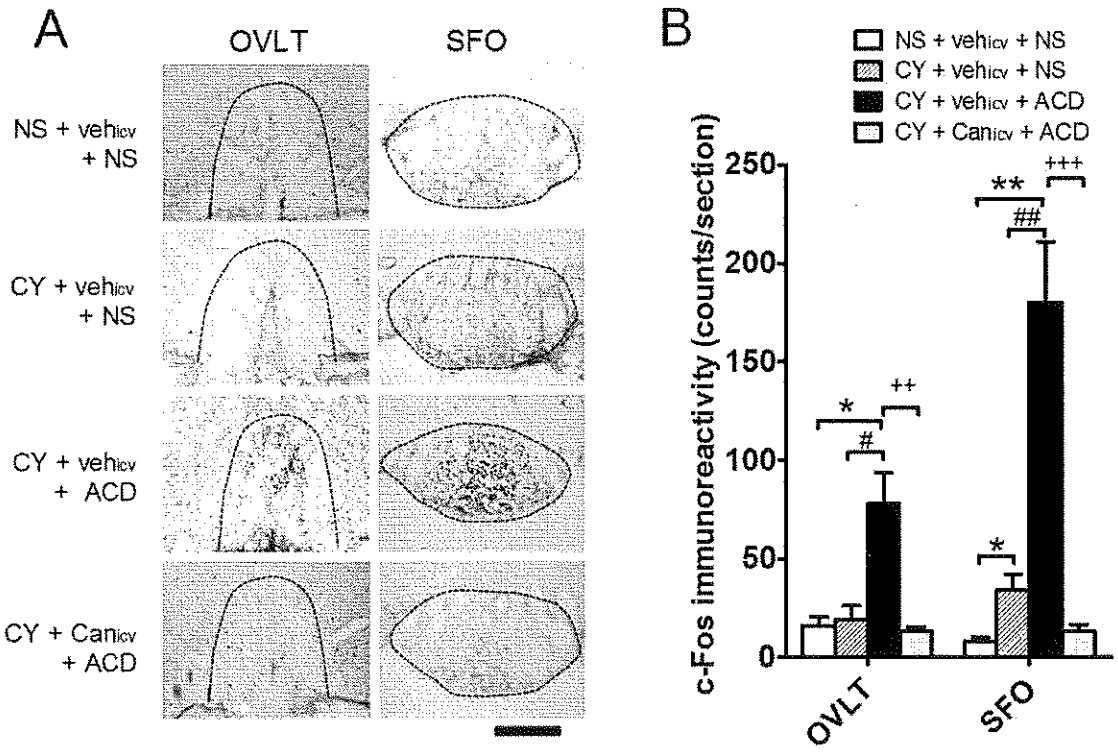

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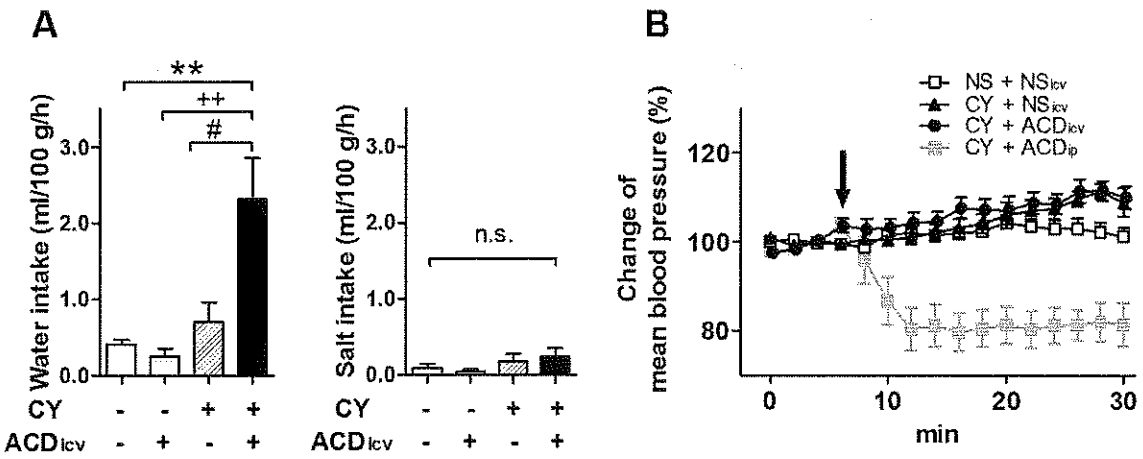

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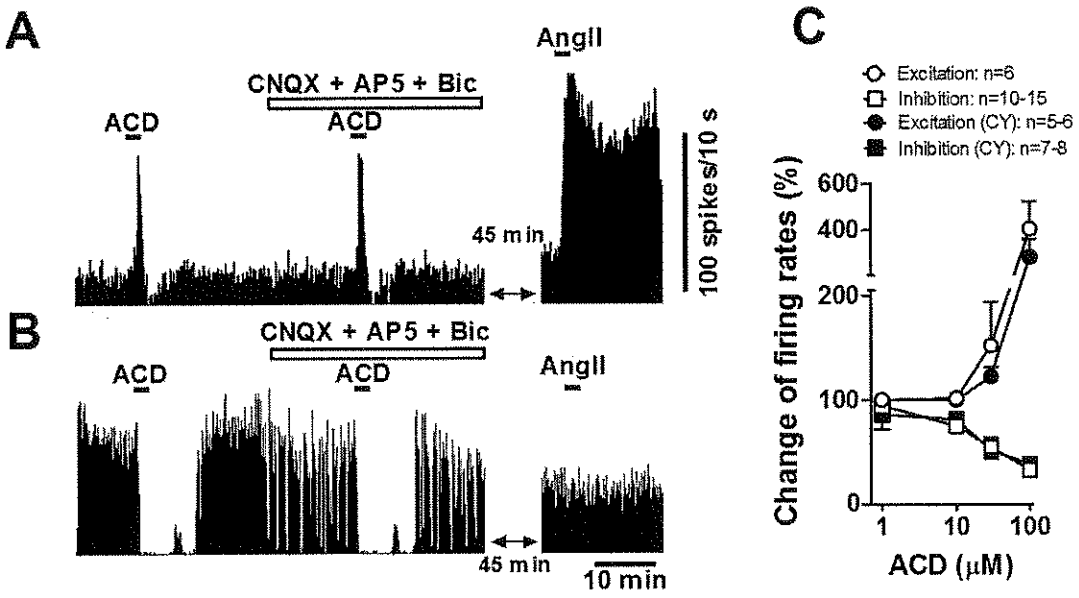


Figure-6(Inenaga)

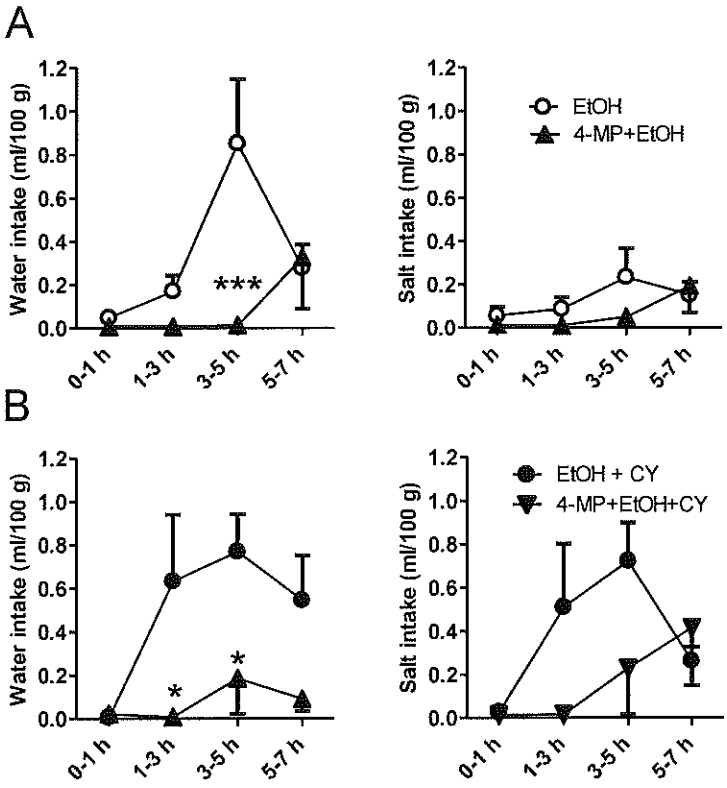
Supplemental figures

Supplemental Figure 1. Suppression of ethanol (EtOH)-induced water and 0.3 M NaCl intake by the alcohol dehydrogenase inhibitor 4-methylpyrazole (4-MP)

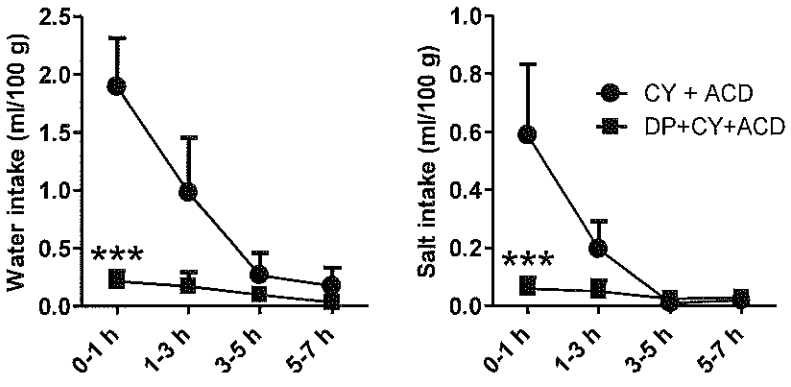
A, Suppression of water intake after EtOH administration at 2.5 g/kg (EtOH) by 4-MP (4-MP + EtOH) (n = 8/group). The water intake following EtOH administration was significantly suppressed by 4-MP at 3-5 h. **B**, The suppression of water intake after the combination of EtOH at 2.5 g/kg and cyanamide at 50 mg/kg (EtOH + CY) by 4-MP (4-MP + EtOH + CY) (n = 8/group). The water intake by EtOH + CY was significantly suppressed by 4-MP at 1-3 and 3-5 h. 4-MP at 10 mg/ml/kg was administered 1 h before EtOH or EtOH + CY. * $p < 0.05$ vs EtOH + CY and *** $p < 0.001$ vs EtOH from the Bonferroni *post hoc* test following two-way ANOVA. Data are presented as the means ± SEM.

Supplemental Figure 2. Suppression of acetaldehyde (ACD)-induced water and 0.3 M NaCl intake by the sequestering acetaldehyde agent D-penicillamine (DP)

Suppression of water and salt intakes after ACD at 100 mg/kg (CY + ACD) by DP (DP + CY + ACD) (n = 8/group). CY at 50 mg/kg and DP at 50 mg/kg were administered i.p. 1 h before ACD. The water and salt intakes following CY + ACD were significantly suppressed by DP at 0-1 h. *** $p < 0.001$ vs CY + ACD from the Bonferroni *post hoc* test following two-way ANOVA. Data are presented as the means ± SEM.



Supplemental Figure 1



Supplemental Figure 2