Salt Appetite Is Reduced by a Single Experience of Drinking Hypertonic Saline in the Adult Rat

Michael P. Greenwood¹, Mingkwan Greenwood¹, Julian F. R. Paton², David Murphy¹,³

¹ School of Clinical Sciences, University of Bristol, Bristol, England, ² School of Physiology and Pharmacology, University of Bristol, Bristol, England, ³ Department of Physiology, University of Malaya, Kuala Lumpur, Malaysia

Abstract

Salt appetite, the primordial instinct to favorably ingest salty substances, represents a vital evolutionary important drive to successfully maintain body fluid and electrolyte homeostasis. This innate instinct was shown here in Sprague-Dawley rats by increased ingestion of isotonic saline (IS) over water in fluid intake tests. However, this appetitive stimulus was fundamentally transformed into a powerfully aversive one by increasing the salt content of drinking fluid from IS to hypertonic saline (2% w/v NaCl, HS) in intake tests. Rats ingested HS similar to IS when given no choice in one-bottle tests and previous studies have indicated that this may modify salt appetite. We thus investigated if a single 24 h experience of ingesting IS or HS, dehydration (DH) or 4% high salt food (HSD) altered salt preference. Here we show that 24 h of ingesting IS and HS solutions, but not DH or HSD, robustly transformed salt appetite in rats when tested 7 days and 35 days later. Using two-bottle tests rats previously exposed to IS preferred neither IS or water, whereas rats exposed to HS showed aversion to IS. Responses to sweet solutions (1% sucrose) were not different in two-bottle tests with water, suggesting that salt was the primary aversive taste pathway recruited in this model. Inducing thirst by subcutaneous administration of angiotensin II did not overcome this salt aversion. We hypothesised that this behavior results from altered gene expression in brain structures important in thirst and salt appetite. Thus we also report here lasting changes in mRNAs for markers of neuronal activity, peptide hormones and neuronal plasticity in supraoptic and paraventricular nuclei of the hypothalamus following rehydration after both DH and HS. These results indicate that a single experience of drinking HS is a memorable one, with long-term changes in gene expression accompanying this aversion to salty solutions.

Introduction

The precise regulation of salt and water balance is essential for survival and good health, and when threatened, osmotic stability is aggressively defended. Integrated neuroendocrine and behavioural mechanisms function to control the excretion and consumption of water and salt in order to maintain the optimal bodily composition required for good health [1]. However, as Cordain and colleagues have pointed out [2], “the evolutionary collision of our ancient genome with the nutritional qualities of recently introduced foods may underlie many of the chronic diseases of Western civilisation”. Indeed, contemporary foodstuffs are rich in salt, and habitual high salt intake is one of the unfavourable population-wide risk factors for epidemic cardiovascular disease, including hypertension [3,4]. Interestingly, the neural organisation subserving the instinct of salt craving, which in evolutionary terms has a high survival value, appear to be plastic with respect to chronically high salt intake, resulting in enduring pathogenic changes in salt appetite and consumption [5].

The neuroendocrine reflexes regulating salt and water balance are centred on the hypothalmo-neurohypophysial system (HNS). The HNS consists of the large peptidergic magnocellular neurons (MCNs) of the supraoptic nucleus (SON) and paraventricular nucleus (PVN), and more medial sub-division of smaller parvocellular neurons in the PVN [1]. The rise in plasma osmolality that follows high salt ingestion is detected by intrinsic MCN mechanisms [6] and by specialised osmoreceptive neurons in the circumventricular organs such as the subfornical organ, which provide excitatory inputs to shape the firing activity of MCNs for hormone secretion [7] and parvocellular neurons for activation of the sympathetic nervous system [3].

Behavioural mechanisms regulating body fluid homeostasis are determined by the ancient primal instincts of thirst and salt appetite, which are thought to be evolutionary progenitors to more complex forms of consciousness [8]. Extracellular fluid hyperosmolality, hypovolemia and the exogenous application of angiotensin II (AngII) can all stimulate the sensation of thirst, which is the powerful behavioural driver to seek and consume water [9]. In contrast, sodium loss elicits an increased motivation to find and ingest salt thus increasing salt appetite [5,9]. To replenish body sodium animals show an exaggerated preference for palatable hypertonic to isotonic saline (IS) solutions and a willingness to ingest hypertonic saline (HS) solutions that would normally be avoided [10]. One of the primary gustatory nerves, chorda
salt appetite [17–20]. This capacity to ingest salty substances is vital for survival, but any lasting effects of this experience, in terms of neuronal plasticity and/or behaviour are not known.

The ingestion of high salt diets (HSD) during gestational and early post-natal phases of development increase salt intake in adult rats [21, 22]. However, maintenance on HSD after weaning has been reported not to have any long-term influences on salt intake in adult rats [23, 24]. Behavioural studies have shown that salt appetite can be enhanced, as indicated by increased HSD intake, by successive rounds of sodium depletion in rats and these changes are thought to be associated with changes in neuronal plasticity in forebrain nuclei implicated in salt appetite regulation [25,26]. It is known that eating salty foods generates a short-term shift in preference from saline solutions to water in rats [24], but this behaviour does not persist when normal food is returned in the adult [23]. The involuntary ingestion of IS or HS solutions produces a similar shift in salt preference as animals make a conscious decision to drink water as opposed to salty solutions to quench their thirst [10]. This behavioural response resembles conditioned taste aversion (CTA), though few have sought to investigate how long this behaviour persists. Taste aversion to salt solutions can be formed in adult rats by pairing the salty taste with subsequent LiCl induced sickness [27]. Such studies have demonstrated strong and persistent aversions to normally highly palatable salt solutions in adult rats [14,27].

All told we hypothesised that acute high salt intake may alter salt appetite in adult rats. Hence, we investigated whether involuntary ingestion of saline solutions could alter salt appetite and consumption in adult rats by testing the long-term effects of a 24 h of IS and HS intake on subsequent dietary preference. We found that a single exposure to HS sensitises the animals to drinking salty but not sweet solutions in choice tests for at least 5 weeks after this drinking experience. Finally, we identified persistent changes in gene expression in the SON and PVN after 24 h of IS (or HS) intake that maybe responsible for mediating long-term changes in neuronal activity, plasticity and behaviour.

Methods

Animals

Male Sprague-Dawley (SD) rats were purchased from commercial suppliers (Harlan laboratories) and housed in the animal facilities at the University of Bristol. Rats with starting weights ranging between 275–325 g were maintained under a 14:10 light dark cycle (lights on at 5 am) for at least 1 week prior to experimentation. Rats were singly housed at an ambient temperature of approximately 21°C and food and tap water were supplied ad libitum, unless stated. All salt solutions were prepared in tap water by adding the stated percentage by mass (w/v) NaCl (Melford Laboratories, UK). The HSD was custom made (International Product Supplies Limited, UK) to precisely match the formula of the standard laboratory chow (5LFS; 0.6% w/w NaCl) but contained 4% w/w NaCl. Animal experiments were performed between 0900 h and 1100 h with the exception of AngII studies that were conducted between 1600 h and 1900 h. All experiments were performed under a Home Office UK licence held under, and in strict accordance with, the provision of the UK Animals (Scientific Procedures) Act (1986); they had also been approved by the University of Bristol Ethical Review Committee.

Experimental series 1 - Salt intake in SD rats

The ingestion of IS (0.9% NaCl) and HS (2% NaCl) solutions were evaluated in one-bottle or two-bottle tests. In one-bottle tests fluid intake was recorded for 3 d before and 7 d after the addition of salt to drinking water. For two-bottle choice tests animals were given access to two-bottles of tap water for 7 d prior to experimentation to adapt to drinking modality. On day 8, rats were simultaneously presented with one-bottle of water and one-bottle of IS, or one-bottle of water and one-bottle of HS, and fluid intake was recorded after 24 h. The preference scores, calculated by salty solution intake/total fluid intake, indicate the degree of preference for, or aversion to one-bottle compared to the other.

Experimental series 2 – Effect of IS and HS ingestion on future two-bottle choice tests

Rats were randomly allocated into control water exposed (WEx), isotonic saline exposed (ISEx), hypertonic saline exposed (HSEx), dehydrated exposed (no fluid; DHEx) and high salt diet exposed (4% w/w salt chow plus tap water; HSDEx) groups. On day 1, drinking water was replaced with one-bottle of IS or HS for the ISEx and HSEx groups, respectively. Water was removed from the DHEx group, whereas rats in the HSDEx group were given HSD in place of standard laboratory chow for 24 h. The WEx and HSEx group had free access to water throughout the 24 h experimental period. With the exception of the DHEx group, fluid intake was recorded for the 24 h treatment period and for the subsequent 24 h recovery period. Rats then were presented with two-bottles of water and standard laboratory chow ad libitum for 7 d. On day 9, 24 h two-bottle choice tests were performed, comparing intakes of water and IS for all experimental groups. An additional two-bottle choice test with water and IS was conducted for the WEx, ISEx and HSEx groups on day 35.

Experimental series 3 – Salt sensitivity testing

To establish the threshold of aversion to salt solutions, a series of two-bottle choice tests were performed after initially confirming aversion to IS with descending percentage solutions 0.7%, 0.5%, 0.3%, 0.2%, 0.1%, 0.05%, 0.025%, 0.0125% and 0.00625% of salt, over the course of 19 d. Two groups of animals were used for this study, WEx and HSEx rats, with the protocol beginning on day 9 and ending on day 28 post treatment. The two-bottle choice test with water and IS was repeated on day 30 to confirm maintained aversion to IS in the HSEx group. On day 32 two-bottle choice tests were performed with water vs 1% w/v sucrose (Sigma) followed on day 34 by two-bottle choice tests with water vs 1% sucrose prepared in IS. Two-bottles of water were returned to the animals for 24 h between each choice test. All two-bottle choice tests were performed for 24 h with the positions of the bottles being alternated for each test to avoid position preference.

Experimental series 4 – The effect of AngII on IS and water intake

The effects of AngII on IS and water intake were investigated in the HSEx group compared to WEx rats. On day 9 of the protocol from experimental series 2, rats received a single SC injection of 200 μg/kg body weight of AngII (Sigma). This dose of AngII and...
route of administration has previously been shown to induce thirst [25]. The animals were placed back in their home cages with one-bottle of water, one-bottle of IS and food ad libitum. All animals began drinking within minutes of AngII administration. Fluid intake was measured 1 h after AngII administration where IS was removed and replaced with water. This protocol was repeated, firstly with one-bottle containing IS, and secondly with one-bottle of water with three intervening recovery days between treatments.

Experimental series 5 – The effects of hyperosmotic stress and rehydration on gene expression

To induce hyperosmotic stress, water was removed for 24 h or replaced by HS in drinking water for 24 h and rats were sacrificed. In a separate experiment, rats were randomly assigned into 5 experimental groups. Two groups of rats were DH for 24 h and water was returned (rehydration; RH) for 1 d or 7 d before sacrifice. Two additional groups had their water replaced with HS for 24 h and access to water for 1 d or 7 d before being killed. The control groups had access food and water ad libitum throughout the experiment. All rats were humanely killed by striking of the cranium, brains frozen on dry ice, and stored at −80°C.

RNA extraction and cDNA synthesis

A 1 mm micropunch (Fine Scientific Tools) was used to collect SON and PVN samples from 60 micron coronal sections in a cryostat. Sections were mounted on glass slides, stained with toluidine blue, and visualised on a light microscope until MCNs were evident. Using the optic chiasm (SON) or MCNs medial to the third ventricle (PVN) for reference, samples were punched from frozen brain slices and dispensed into 1.5 ml tubes stored on dry ice within the cryostat. Bilateral punches were collected from 8 and 12 consecutive 60 micron sections for PVN and SON, respectively. Total RNA was extracted from micropunch samples by combining Qiazol Reagent with Qiagens RNeasy kit protocols (Qiagen). The micropunch samples were removed from dry ice and rapidly resuspended, by vortexing, in 1 ml Qiazol reagent. Following Qiazol phase separation with chloroform, 350 μl of the upper aqueous phase was removed, mixed with 70% ethanol and applied to RNeasy columns. The remaining steps were performed as recommended by the manufacturer. For cdNA synthesis 200 ng of total RNA was treated with Genomic DNA wipeout and reverse transcribed using the Quantitect reverse transcription kit (Qiagen).

Real-time quantitative PCR analysis

The steady-state RNA levels in the SON and PVN were assessed by qPCR. All of the genes chosen for analysis have previously been shown to be regulated by high salt intake. Primers for c-Fos (5’-AGCATGGGCTCCCCTGTCA-3’ and 5’-GAGA-CCAGAGTGGGCTGCA-3’), orphan nuclear receptor Nr4a1 (5’-CTGCGACTGGGTCCTGGGTC-3’ and 5’-TGTCAGGT-GGTTCACGCGT-3’), Activity-regulated cytoskeleton-associated protein (Arc) (5’-GGCCGAAAGGAACCTTACCT-3’ and 5’-CTGAGAGGGGACCTATGCTG-3’), heteronuclear arginine vasopressin (hnAVP) (5’-GAGGCAAGAGGGCCACATC-3’ and 5’-CTCTCCTAGCCCATGACCCCTT-3’), mature AVP (5’-TGCCCTGCTACTTCCAGAACTGC-3’ and 5’-AGGGGAGACACTGTGCTCAGCTG-3’), heteronuclear oxytocin (hnOT) (5’-TGACGAGAGGGGGGCTAGC-3’ and 5’-TGCAAGAGAAATGGGTCAGT-3’), mature OT (5’-TGCCCCAGTC-3’ and 5’-TCCAGGTCTAGCGCAGCCC-3’), heteronuclear corticotropin releasing hormone (hnCRH) (5’-GGGGGAAATAGCTTAAACCTG-3’ and 5’-CTAAATGAGAATCGTTTTG-GC-3’), mature CRH (5’-CTCTCTGGATCTCACCTTCCAC-3’ and 5’-TGCCAATGGCATATCGTGCTG-3’), and ribosomal protein RPL19 (5’-GCGTCTGCAGCATGAGTA-3’ and 5’-TGGCATTGGCGATTTCGTTG-3’) were synthesised by Eurofins MWG Operon. The optimisation and validation of primers was performed using standard ABI

Figure 1. Mean fluid intake in two-bottle and one-bottle salt intake tests in naïve rats. Intakes of IS (A) and HS (B) compared to water in 24 h two-bottle choice tests in naïve rats. C, mean preference scores for IS and HS solutions in 24 h two-bottle salt choice tests with water. One-bottle intake tests with IS (D) and HS (E) in naïve rats. Error bars +SEM, n = 6–10 animals per group. *p<0.05, **p<0.01, ***p<0.001. HS, hypertonic saline; IS, isotonic saline; W, water.

doi:10.1371/journal.pone.0104802.g001

Salt Appetite Is Reduced after Drinking Hypertonic Saline

PLOS ONE | www.plosone.org 3 August 2014 | Volume 9 | Issue 8 | e104802
protocols. The qPCRs were carried out in duplicate in 25 μl reaction volumes using 12.5 μl 2X SYBR green master mix buffer (Roche), optimum concentrations of primers, and first strand cDNA template. The reactions were performed using an ABI 7500 Sequence Detection System (ABI, Warrington, UK), with universal cycling conditions. For relative quantification of gene expression the 2^(-ΔΔCT) method was employed [28]. The internal control gene used for these analyses was the housekeeping gene RPL19.

Statistical analysis

Statistical differences between two experimental groups were evaluated using independent sample unpaired Student’s t tests. One way ANOVA and Tukey’s post-hoc test were used to determine the differences between more than two groups with only a single influencing factor. For 7 d one-bottle IS and HS studies, a repeated measures ANOVA with Bonferroni post-hoc test has been used. P<0.05 was considered significant. Results are expressed as mean ±SEM of the number of replicates indicated in the figure legend.

Results

Salt intake in SD rats

In two-bottle choice tests, rats showed a strong preference for IS, ingesting significantly more compared with water (Fig. 1A). Offering HS in place of IS reversed this effect, with rats preferring to drink water (Fig. 1B). As expected, the preference score for salt significantly decreased in rats presented with HS compared to IS solutions in two-bottle choice tests (Fig. 1C). When fluid intake tests were performed with only one-bottle of IS, rats immediately (24 h) ingested significantly greater volumes of this salty solution compared with earlier water intake measures (Fig. 1D). This significant increase in fluid intake was maintained for the 7 d IS drinking test. The strong aversion for HS, as observed in two-bottle choice tests, was overcome in a one-bottle HS test (Fig. 1E). The mean intake of HS was comparable to that of earlier water intake on day 1 and 2, from which HS intake steadily increased from 3–7 d.

Long-term changes in salt preference after ingestion of IS and HS solutions

Fluid intake was first measured over a 24 h period in control WEx, ISEx, HSEx, DHx and HSDx groups (Fig. 2Ai). Compared to WEx rats, fluid intake was significantly higher in the ISEx and HSEx groups. Water intake during the 24 h recovery period was significantly higher for all experimental groups (Fig. 2Aii), with the exception of the HSEx group. The effects of these treatments on salt appetite were then investigated 7 d later using two-bottle IS vs. water choice tests (Fig. 2B). As before (Fig. 1A), WEx animals showed a strong preference for IS compared to water. There were no significant differences in IS preference in
DHEx or HSDEx treated rats compared to WEx controls. Interestingly, prior exposure to 24 h ingestion of either IS or HS solutions in the ISEx and HSEx groups respectively resulted in significant alterations in drinking behaviour. The ISEx group displayed no preference for either water or IS. In contrast, the HSEx group showed a strong preference for water over IS. Both the ISEx and HSEx rats exhibited significantly lower salt preference scores compared to control WEx rats (Fig. 2C). After a further 28 d, the two-bottle test was repeated on these same animals (Fig. 2D). We were surprised to see that, 35 days after exposure, the ISEx and HSEx groups still showed no preference for the normally very palatable IS, rather, as at 7 d, the ISEx group showed no preference, whereas the HSEx group preferred water. However, only HSEx rats exhibited significantly lower salt preference scores compared to control WEx rats (Fig. 2E).

A loss in preference for salty by not sweet solutions after ingestion of HS

To determine the threshold of aversion to salt elicited by HS, rats were presented with a descending series of salt solutions (0.7%, 0.5%, 0.3%, 0.2%, 0.1%, 0.05%, 0.025%, 0.0125% and 0.00625%) in 24 h two-bottle choice tests with water (Fig. 3A). The control WEx rats preferred the salty solution at all salt percentages tested, as indicated by mean scores >0.5. The opposite behaviour was observed for the HSEx group, which preferred water over salt solutions, as indicated by preference scores <0.5. The paired comparisons of preference scores in HSEx and WEx rats showed that HSEx rats maintained a significantly lower preference for salt solutions as low as 0.0125%. A strong aversion to IS was still evident after 30 d in HSEx compared to WEx rats (Fig. 3B). Both the WEx and HSEx rats preferred 1% sucrose solution in the absence (Fig. 3C) or presence (Fig. 3D) of IS compared to water consumption. Interestingly, the preference score significantly dropped in HSEx compared to WEx rats after addition of salt to the sucrose solution (Fig. 3E).

The effect of AngII on IS and water intake

To test whether the salt aversion in the HSEx group could be overcome by an agent known to provoke a profound thirst and salt appetite, a 1 h two-bottle choice test was performed after SC injection of AngII in WEx and HSEx rats (Fig. 4). As expected, AngII robustly increased total fluid intake in both groups (Fig. 4A). However, the HSEx group ingested significantly more water (Fig. 4Aii) and significantly less IS (Fig. 4Aiii) compared to WEx controls, resulting in a lower salt preference score (Fig. 4B). In one-bottle tests, no differences in water (Fig. 4C) or IS (Fig. 4D) intake were observed in when WEx or HSEx rats were given AngII.
Proiling the effects of hyperosmotic stress and rehydration on gene expression in the SON and PVN

We used qPCR to ask if different salt ingestion paradigms altered gene expression in the SON and PVN. First we showed that both 1 d DH and HS induced the expression of hnAVP (Fig. 5A) and hnOT (Fig. 5B) in both the SON and the PVN (except 1 d DH hnOT). In contrast, mature AVP and OT mRNA expression was similar to control animals with only AVP mRNA expression being signiﬁcantly increased in SONs of 1 d HS rats (Fig. 5C, D). These two paradigms did not signiﬁcantly alter hnCRH or mature CRH transcripts in the PVN compared to control animals (Fig. 5E). We observed higher c-Fos (Fig. 5F) and Nr4a1 (Fig. 5G) mRNA levels in the SON and PVN of DH and HS rats, but no change in Arc (Fig. 5H) mRNA expression, compared with control animals.

In a separate experiment, we analysed the expression of the same transcripts after 1 d and 7 d RH in 1 d DHEx or 1 d HSEx compared to control (euhydrated) animals (Fig. 6). There were no signiﬁcant differences in hnAVP (Fig. 6A) or hnOT (Fig. 6B) RNA expression in either the SON or PVN following either 1 d or 7 d RH. However, the expression of mature AVP transcripts were signiﬁcantly higher in PVN, but not SON, of 7 d RH following DHEx and 1 d and 7 d RH following HSEx compared to control animals, suggesting long-term alterations in the expression of this gene (Fig. 6C). OT mRNA expression was signiﬁcantly higher in SON, but not PVN, of 1 d and 7 d RH following DHEx and 1 d following HSEx compared to control again supporting long-term changes in gene expression (Fig. 6D). As in 1 d DH and 1 d HS animals, no signiﬁcant differences in hnCRH or mature CRH transcripts were detected in the PVN after 1 d or 7 d RH (Fig. 6E). In contrast, c-Fos mRNA expression was signiﬁcantly lower in SON and PVN of DHEx and HSEx animals after 1 d RH (Fig. 6F) and in the PVN of 7 d DHEx animals. Nr4a1 mRNA levels were also signiﬁcantly lower than controls at both RH time-points in both the SON and PVN (Fig. 6G). Interestingly, Arc mRNA expression was signiﬁcantly lower at both time-points in SON but only for DHEx and not HSEx animals compared to control, with no signiﬁcant changes in PVN (Fig. 6H).

Discussion

In this study, we have shown that 24 h drinking of IS or HS solutions is sufﬁcient to produce long-term changes in salt appetite in SD rats. The two-bottle choice test has been widely used to investigate the drinking behaviour of rodents in a range of experimental models [26,29–31]. We performed two-bottle choice tests in naı¨ve rats to conﬁrm previous reports of a preference for IS over water, and an aversive response to HS (Fig. 1A–C) [15]. However, when denied a choice (involuntary drinking), naı¨ve rats consumed large volumes of both IS and HS (Fig. 1D, E). The increase in HS intake likely results from a suppression of the taste response to HS, overriding the normal aversive behaviour, making salty solutions more palatable [13,32]. This capacity to drink HS in an involuntary situation is vital for survival.

Although many studies have looked at the effects of sodium deprivation on salt taste responses [11,13,14], few have sought to address the impact of acute sodium overload on chronic salt taste sensitivity [16,24]. Rats prefer to drink water over salt solutions when dietary sodium intake (ﬂuid or food) is high, or immediately after HS or DH [10], but to our knowledge no lasting effects of such stressors on salt taste sensitivity have been reported. We know from behavioural experiments that multiple rounds of sodium depletion enhance HS ingestion in acute and chronic tests, suggesting that such experiences are remembered [25,26]. Thus we asked, does prior ingestion of ﬂuid high in salt (or food) alter the conscious decision of rats to actively seek out salty solutions in later choice tests? Accordingly, naıve rats were primed for 1 d by involuntary exposure to IS, HS, DH or HSD. Performing two-bottle choice tests 7 d post-treatment enabled rats to re-establish ﬂuid and electrolyte homeostasis. While no changes in drinking behaviour were observed in DHEx or HSDEx groups, ISEx and HSEx groups showed signiﬁcantly lower preferences for IS

Figure 4. The effect of AngII on IS and water intake. A, total ﬂuid intakes in WEx and HSEx rats in 1 h two-bottle choice tests with water and IS after AngII administration. Water intake (Aii) and IS intake (Aiii) in WEx and HSEx rats after AngII administration. B, salt preference score in WEx and HSEx rats after AngII administration. One-bottle tests with IS (C) and water (D) following AngII administration. Values are means ±SEM of n = 10 animals per group. *p<0.05. HSEx, hypertonic saline exposed; IS, isotonic saline; WEx, water exposed.

doi:10.1371/journal.pone.0104802.g004
This salt aversion is incredibly sensitive, with animals showing a strong preference for water over salt solutions as low as 0.0125% (Fig. 3A). Remarkably, the drinking of HS is such a memorable experience that animals continued to avoid normally palatable solutions of salt for the 35 d experimental period (Fig. 2D, E). It is not known how long this aversive behaviour to IS persists after drinking HS, or indeed if it is reversible. Further, salt aversion cannot be overcome by AngII administration (Fig. 4).

We were surprised that behavioural differences were confined to groups that ingested salty solutions. The observed changes in drinking behaviour may underlie distinct differences in mechanism provoking thirst in DH and HSD exposed compared to salty solution exposed animals. Unlike other experimental groups, rats exposed to HSD had access to water throughout the exposure period, so could immediately correct body electrolytes by altering water intake, as observed by increased water intake. In the absence of drinking fluid (DH), extracellular and intracellular fluid volumes decrease as water and sodium are inevitably lost in sweat and urine, leading to hypovolemia [10,33]. In contrast, intake of HS solution increases body sodium content, causing an increase in the

**Figure 5. The effects of 1 d DH and 1 d HS exposure on gene expression in rat SON and PVN.** Relative mRNA expression of hnAVP, hnOT, AVP, OT, hnCRH, CRH, c-Fos, Nr4a1 and Arc was investigated by qPCR in the SON and PVN of control, 1 d DH and 1 d HS rats. Values are means ±SEM of n = 5–6 animals per group. *p<0.05, **p<0.01, ***p<0.001. 1 d DH, 1 day dehydrated; 1 d HS, 1 day hypertonic saline.

doi:10.1371/journal.pone.0104802.g005
extracellular and a decrease in intracellular fluid volumes [33]. Therefore, unlike rats exposed to salty solutions [16], DH animals also display enhanced salt appetite, which occurs shortly after the initial phase of rehydration to replace body sodium [10]. No such enhancement in salt appetite is seen immediately after IS or HS exposure [16], clearly highlighting behaviour differences between these experimental paradigms in relation to salt appetite.

Although such salt avoidance characteristics are observed in Fischer 344 rats [12,34], we were surprised that SD rats displayed aversive behaviours to a range of salt concentrations, which are normally very palatable for this strain [35]. We propose that simply drinking HS elicits a conditioned taste aversion (CTA) to IS and hypotonic saline solutions. The generation of CTA involves ingestion of a novel substance (conditioning), in our case HS, followed by malaise (unconditioned stimulus) so the animal

---

**Figure 6. Gene expression changes in rat SON and PVN 1 d and 7 d after DHEx or HSEx compared to control animals.** Relative mRNA expression of hnAVP, hnOT, AVP, OT, hnCRH, CRH, c-Fos, Nr4a1 and Arc was investigated by qPCR in the SON and PVN of rats RH (1 d and 7 d) after DHEx and HSEx. Values are means ±SEM of n = 6 animals per group. *p<0.05, **p<0.01, ***p<0.001. DH, dehydrated; DHEx dehydrated exposed; HS, hypertonic saline; HSEx, hypertonic saline exposed; RH, Rehydration.

doi:10.1371/journal.pone.0104802.g006
remembers the taste of the ingested substance [27]. This is commonly achieved by administering LiCl shortly after the conditioned stimulus to generate LiCl induced sickness [27]. In agreement with our findings, these protocols produce a shift in preference for water that extends to salt solutions as low as 0.003 M [14,30]. We can only speculate that the ingestion of HS may produce gastrointestinal discomfort leading to formation of the unconditioned stimulus. One argument against this hypothesis comes from HS intake in rats with open or closed gastric fistulas [36]. In this study, Davis et al. found that post-ingestional stimulation is the principal component in the control of water and IS intake, whereas the orosensory response appears to be controlling intake of HS. Thus, taste could be the important factor governing decision subsequent to HS consumption.

Previous studies have also shown that temperature [37] and indeed taste of fluid [38] can lead to CTA. To acquire a strong CTA to salt, the taste information is conveyed via one of the primary gustatory nerves, the CT, which is highly sensitive to salt, with increased firing rate as salt concentrations increase [39]. A decrease in CT nerve firing in sodium-deprived rats is thought to be a necessary response to increase salt intake [11,40]. Transection of the CT in rats, as well as other mammals, diminishes salt avoidance, supporting the importance of this nerve in increasing salt appetite [12,41]. HS drinking for 1 d will undoubtedly have increased the firing rate of the CT. However, once established, CT to salt no longer requires the CT, as aversive behaviour to salt solutions are retained following nerve transaction [12], suggesting long-term changes in the brain.

We reasoned that these modifications to salt appetite may be attributed to changes in neuronal plasticity, similar to that described following sodium depletion [25,26]. Thus, we asked whether gene expression is altered in the hypothalamus as a consequence of HSEx. We and others have reported changes in gene expression in the SON and PVN following hypo- and hyperosmotic stress [42–44]. Indeed, as expected 1 d DH and 1 d HS significantly increased mRNA expression of immediate early genes c-Fos and Nr4a1 and hRNA for neurohypophysial hormones AVP and OT (with exception of 1 d DH in the SON and PVN (Fig. 5), consistent with previous studies [45,46].

Access to water after DHEx and HSEx has been shown to rapidly reduce Fos and Nr4a1 protein staining in the MGNs of the SON and PVN [45,47]. At the RNA level, we observed significantly lower c-Fos and Nr4a1 mRNAs, by comparison with euhydrated naïve controls following RH, particularly after 7 d (Fig. 6). The higher mRNA expression of AVP and OT transcripts during RH (Fig. 6) are supported by previously reported increases in these transcripts following recovery from DHEx and HSEx [17,48]. This is intriguing as both of AVP and OT peptides have been shown to be important in the regulation of salt appetite [49,50]. Interestingly, another immediate early gene, Arc, was similarly down regulated in the SON during RH. This gene has been shown to function as a master regulator of protein-dependent synaptic and thus, neuronal plasticity [51]. In agreement, microarray analysis of hypothalamic genes regulated by sodium deficiency identified Arc as being significantly upregulated, implying that Arc regulation may be important for increasing salt appetite [52]. This lends support to the concept that lower Arc expression in the present study may lead to reduced salt appetite.

The changes in gene expression observed in the SON and PVN would imply that mRNAs may also be altered in additional brain centres important for salt appetite regulation. Likely future targets include the circumventricular organs such as the subfornical organ (SFO) and organum vasculosum of the lamina terminalis (OVLT). Of particular interest is the SFO, which provides excitatory inputs to shape the firing activity of MCNs for hormone secretion [7]. Being directly exposed to the chemical environment of the circulation, the SFO is able to sense sodium concentration in the body fluid through specialised osmoreceptive neurons, and is thus thought to be the principle site of regulation of salt intake behaviour [53]. In agreement, damage to SFO results in an attenuation of induced salt appetite by AngII [34]. Furthermore, Hiyama et al. identified specific Na+ channels in the SFO that are important in sensing sodium and thus appear to be instrumental in regulating salt intake behaviour [55].

In summary, we have shown that drinking IS and HS solutions in SD rats cause a strong aversion to normally palatable saline solutions in two-bottle choice tests. We propose that this behaviour underlies the successful formation of CTA to salt solutions creating a new, exciting, and simple model to investigate salt appetite behaviours. The apparent destruction of the ancient evolutionary behaviour to naturally seek out and favourably ingest salty substances in our model is intriguing. Thus, increasing our understanding of the mechanisms responsible is of importance. We report here, lasting changes in mRNAs for markers of neuronal activity, peptide hormones and neuronal plasticity following RH though these effects were predominantly observed after both DHEx and HSEx. These data also imply that extreme care should be taken when planning experiments involving multiple episodes of HS exposure as the present findings clearly suggest that intake of HS solutions can alter salt appetite in SD rats.

Author Contributions
Conceived and designed the experiments: MPG MG JFPR DM. Performed the experiments: MPG MG. Analyzed the data: MPG MG. Contributed to the writing of the manuscript: MPG DM.

References
1. Antunes-Rodrigues J, de Castro M, Elias LL, Valenca MM, McCann SM (2004) Neuropeptide control of body fluid metabolism. Physiol Rev 84: 169–208.
2. Cordain L, Eaton SB, Sebastian A, Mann N, Lindeberg S, et al. (2005) Origins and evolution of the Western diet: health implications for the 21st century. Am J Clin Nutr 81: 341–354.
3. Osborn JW, Fink GD, Sved AF, Toney GM, Raizada MK (2007) Circulating angiotensin II and dietary salt: converging signals for neurogenic hypertension. Curr Hypertens Rep 9: 220–235.
4. Stamen J (1997) The INTERSALT Study: background, methods, findings, and implications. Am J Clin Nutr 65: 626S–642S.
5. Morris MJ, Na ES, Johnson AK (2008) Salt craving: the psychobiology of increased sodium intake. Physiol Behav 94: 709–721.
6. Bourque CW (2008) Central mechanisms of osmosensation and systemic osmoregulation. Nat Rev Neurosci 9: 519–531.
7. McKinley MJ, Mathai ML, McAllen RM, McCleary RC, Miselin RR, et al. (2004) Vasopressin secretion: osmotic and hormonal regulation by the lamina terminalis. J Neuroendocrinol 16: 340–347.
8. Denton DA, McKinley MJ, Farrell M, Egan GF (2009) The role of primordial emotions in the evolutionary origin of consciousness. Conscious Cogn 18: 500–514.
9. Johnson AK (2007) The sensory psychobiology of thirst and salt appetite. Med Sci Sports Exerc 39: 1388–1400.
10. De Luca LA Jr, Pereira-Derderian DT, Vendramini RC, David RB, Menani JV (2010) Water deprivation-induced sodium appetite. Physiol Behav 100: 535–544.
11. Garcia JM, Curtis KS, Contreras RJ (2008) Behavioral and electrophysiological taste responses change after brief or prolonged dietary sodium deprivation. Am J Physiol Regul Integr Comp Physiol 295: R1754–1761.
12. Sullivan SI, Tracy CJ, Bernstein IL (1996) Retention of conditioned taste aversion to NaCl after chorda tympani transection in Fisher 344 and Wistar rats. Physiol Behav 60: 65–69.
13. Curtis KS, Krause EG, Contreras RJ (2001) Altered NaCl taste responses precede increased NaCl ingestion during Na+ deprivation. Physiol Behav 72: 743–749.
14. Lu B, Yan J, Yang X (2009) Effects of sodium depletion on detection thresholds for salty taste in rats. Physiol Behav 97: 463–469.
15. Rowland NE, Morian KR, Nicholson TM, Salisbury JJ (1995) Preference for NaCl solutions in sham drinking Sprague-Dawley rats: water deprivation, sodium depletion, and angiotensin II. Physiol Behav 57: 753–757.

16. Deverport LD (1975) Aversion to a palatable saline solution in rats: interactions of physiology and experience. J Comp Physiol Psychol 83: 98–105.

17. Amaia F, Tanaka M, Hayashi S, Tanaka Y, Ibata Y (2001) Hypothalamo-pituitary-adrenal axis sensitization after chronic salt loading. Neuroendocrinology 73: 185–193.

18. Greenwood M, Bordieri L, Greenwood MP, Rosso Molo M, Colomboari DS, et al. (2014) Transcription Factor CREB1L1 Regulates Vasopressin Gene Expression in the Rat Hypothalamus. J Neurosci 34: 3810–3820.

19. Lichtman SL, Young WS III (1987) Vasopressin, oxytocin, dynorphin, enkephalin and corticotrophin-releasing factor mRNA stimulation in the rat. J Physiol 394: 23–39.

20. Ueta Y, Levy A, Lichtman SL (2002) Gene expression in the supraoptic nucleus. Microsc Res Tech 56: 158–163.

21. Contreras RJ, Kostin T (1983) Prenatal and early postnatal sodium chloride intake modifies the solution preferences of adult rats. J Nutr 113: 1051–1062.

22. Curtis KS, Krause EG, Wong DL, Contreras RJ (2004) Gestational and early postnatal dietary NaCl levels affect NaCl intake, but not stimulated water intake, by adult rats. Am J Physiol Regul Integr Comp Physiol 286: R1043–1050.

23. Milikoff EE, Bernstein IL (1983) The influence of age and experience on salt preference of the rat. Dev Psychobiol 16: 353–394.

24. Prieha TW, Mooney KJ, Bernard RA (1991) High dietary sodium enhances gustatory nerve activity and behavioral responses to NaCl. Am J Physiol 261: R52–58.

25. Fregly MJ, Lockley OE, Rowland NE (1987) Water versus NaCl intake by rats administered certain digoxins acutely. Brain Res Bull 18: 245–251.

26. Na ES, Morris MJ, Johnson RF, Belz TG, Johnson AK (2007) The neural substrates of enhanced salt appetite after repeated sodium depletions. Brain research 1171: 104–110.

27. Yamamoto T, Shimura T, Sako N, Yasoshima Y, Sakai N (1994) Some critical factors involved in formation of conditioned taste aversion to sodium chloride in rats. Chem Senses 19: 209–217.

28. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402–408.

29. Curtis KS, Davis LM, Johnson AL, Therrien KL, Contreras RJ (2004) Sex differences in behavioral taste responses to and ingestion of sucrose and NaCl solutions by rats. Physiol Behav 80: 657–664.

30. Lu B, Yan J, Yang X, Li J, Chen K (2012) Involvement of brain ANG II in acute sodium depletion induced salty taste changes. Regul Pept 179: 15–22.

31. Korb E, Finkbeiner S (2011) Arc in synaptic plasticity: from gene to behavior. Trends Neurosci 34: 591–598.

32. Van der Velden E, Watanabe E, Okado H, Noda M (2004) The subfornical organ is a specialized sodium channel, and the subfornical organ is a specialized sodium channel, and the subfornical organ is a specialized sodium channel. J Physiol 551: 915–918.

33. Davis JD, Smith GP, McCann DP (2002) The control of water and sodium chloride intake by postgestational and orosensory stimulation in water-deprived rats. Physiol Behav 75: 7–14.

34. Midkiff EE, Bernstein IL (1983) The influence of age and experience on salt preference. J Comp Physiol Psychol 83: 98–105.

35. Fregly MJ, Rowland NE (1992) Comparison of preference thresholds for NaCl solutions in rats of the Sprague-Dawley and Long-Evans strains. Physiol Behav 51: 423–427.

36. Smith PL, Smith JC, Hospt TA (2010) Interactions of temperature and taste in conditioned aversions. Physiol Behav 99: 324–333.

37. Smith JC, Finkbeiner S, Fisher E (1999) Profound conditioned taste aversion induced by oral consumption of 2-deoxy-D-glucose. Physiol Behav 68: 221–226.

38. Frank M (1973) An analysis of hamster afferent taste nerve response functions. J Gen Physiol 61: 381–418.

39. Bernstein IL, Taylor EM (1992) Amiloride sensitivity of the chorda tympani response to sodium chloride in sodium-depleted Wistar rats. Behav Neurosci 106: 125–127.

40. Golden GJ, Ishiwatari Y, Theodorides ML, Bachmanov AA (2011) Effect of chorda tympani nerve transection on salt taste perception in mice. Chem Senses 36: 811–819.

41. Frank M (1973) An analysis of hamster afferent taste nerve response functions. J Comp Physiol Psychol 83: 98–105.

42. Contreras RJ, Kosten T (1983) Prenatal and early postnatal sodium chloride intake modifies the solution preferences of adult rats. J Nutr 113: 1051–1062.

43. Curtis KS, Krause EG, Wong DL, Contreras RJ (2004) Gestational and early postnatal dietary NaCl levels affect NaCl intake, but not stimulated water intake, by adult rats. Am J Physiol Regul Integr Comp Physiol 286: R1043–1050.

44. Milikoff EE, Bernstein IL (1983) The influence of age and experience on salt preference of the rat. Dev Psychobiol 16: 353–394.

45. Prieha TW, Mooney KJ, Bernard RA (1991) High dietary sodium enhances gustatory nerve activity and behavioral responses to NaCl. Am J Physiol 261: R52–58.

46. Fregly MJ, Lockley OE, Rowland NE (1987) Water versus NaCl intake by rats administered certain digoxins acutely. Brain Res Bull 18: 245–251.

47. Na ES, Morris MJ, Johnson RF, Belz TG, Johnson AK (2007) The neural substrates of enhanced salt appetite after repeated sodium depletions. Brain research 1171: 104–110.

48. Yamamoto T, Shimura T, Sako N, Yasoshima Y, Sakai N (1994) Some critical factors involved in formation of conditioned taste aversion to sodium chloride in rats. Chem Senses 19: 209–217.

49. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402–408.

50. Curtis KS, Davis LM, Johnson AL, Therrien KL, Contreras RJ (2004) Sex differences in behavioral taste responses to and ingestion of sucrose and NaCl solutions by rats. Physiol Behav 80: 657–664.

51. Lu B, Yan J, Yang X, Li J, Chen K (2012) Involvement of brain ANG II in acute sodium depletion induced salty taste changes. Regul Pept 179: 15–22.

52. Flynn FW, Culver B, Newton SV (2003) Salt intake by normoosmotic and spontaneously hypertensive rats: two-bottle and lick rate analyses. Physiology & Behavior 78: 689–696.

53. Oka Y, Busun K, von Buchholtz I, Ryba NJ, Zuker CS (2013) High salt recruits averse taste pathways. Nature 494: 472–475.

54. McKinley MJ, Johnson AK (2004) The physiological regulation of thirst and fluid intake. News Physiol Sci 19: 1–6.

55. Milikoff EE, Fitts DA, Simpson JB, Bernstein IL, Krause EG (1983) Absence of sodium chloride preference in Fischer-344 rats. Am J Physiol 249: R438–442.

56. Fregly MJ, Rowland NE (1992) Comparison of preference thresholds for NaCl solutions in rats of the Sprague-Dawley and Long-Evans strains. Physiol Behav 51: 915–918.

57. Davis JD, Smith GP, McCann DP (2002) The control of water and sodium chloride intake by postgestational and orosensory stimulation in water-deprived rats. Physiol Behav 75: 7–14.