



Relation of addiction genes to hypothalamic gene changes subserving genesis and gratification of a classic instinct, sodium appetite

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Sodium appetite is an instinct that involves avid specific intention. It is elicited by sodium deficiency, stress-evoked adrenocorticotrophic hormone (ACTH), and reproduction. Genome-wide microarrays in sodium-deficient mice or after ACTH infusion showed up-regulation of hypothalamic genes, including dopamine- and cAMP-regulated neuronal phosphoprotein 32 kDa (DARPP-32), dopamine receptors-1 and -2, α -2C-adrenoceptor, and striatally enriched protein tyrosine phosphatase (STEP). Both DARPP-32 and neural plasticity regulator activity-regulated cytoskeleton associated protein (ARC) were up-regulated in lateral hypothalamic orexinergic neurons by sodium deficiency. Administration of dopamine D1 (SCH23390) and D2 receptor (raclopride) antagonists reduced gratification of sodium appetite triggered by sodium deficiency. SCH23390 was specific, having no effect on osmotic-induced water drinking, whereas raclopride also reduced water intake. D1 receptor KO mice had normal sodium appetite, indicating compensatory regulation. Appetite was insensitive to SCH23390, confirming the absence of off-target effects. Bilateral microinjection of SCH23390 (100 nM in 200 nL) into rats' lateral hypothalamus greatly reduced sodium appetite. Gene set enrichment analysis in hypothalami of mice with sodium appetite showed significant enrichment of gene sets previously linked to addiction (opiates and cocaine). This finding of concerted gene regulation was attenuated on gratification with perplexingly rapid kinetics of only 10 min, anteceding significant absorption of salt from the gut. Salt appetite and hedonic liking of salt taste have evolved over >100 million y (e.g., being present in Metatheria). Drugs causing pleasure and addiction are comparatively recent and likely reflect usurping of evolutionary ancient systems with high survival value by the gratification of contemporary hedonic indulgences. Our findings outline a molecular logic for instinctive behavior encoded by the brain with possible important translational-medical implications.

The mammalian brain embodies many genetically determined neural organizations subserving specific behavioral patterns of high survival value, historically termed instincts. Sodium appetite, an exemplar, is generated by depletion of body salt content, adrenocorticotrophic hormone (ACTH) mimicking stress, and reproduction (1). This appetite is specific for sodium salts, paralleling thirst for water. Salt appetite may be gratified very rapidly by drinking saline solution, which is akin to a dehydrated animal satiating thirst (1). A water-depleted Saharan donkey, the camel, and the dog can repair near 10% of loss of body weight in 10 min (2, 3) as can rabbits, goats, and sheep (4). Severely sodium-depleted sheep will drink 2–3 L of 300 mmol NaHCO₃ solution in 5 min. Gratification, with complete loss of interest in salt (5), precedes significant absorption from the gut of fluid drunk. This process may take 10–15 min to be evident by commencing plasma concentration change (4, 6). The high survival value arising from evolution of this integrative physiological strategy is that animals

in the wild can drink and rapidly exit the source of salt, or water if thirsty, thus evading predation by carnivores.

Salt appetite is conspicuous in herbivores and omnivores, because sodium is very scarce in soil and vegetation in large regions of the planet (7). Carnivores obtain obligatory sodium intake with meat. Furthermore, response to salt taste is present in invertebrates, although salt appetite behavior is moot (1).

The hypothalamus is a major locus of organization of instinctive behaviors (8–10). Furthermore, sodium-deficient mice develop an avid, specific sodium appetite. Like other species, mice will rapidly gratify appetite over 10 min if presented with sodium solution (5, 11). Chronic administration of ACTH (5, 11) also produces a robust sodium appetite. Salt appetite during reproduction reflects the imperious need of sodium for the developing young in utero and during lactation.

We generated sodium appetite in mice by depletion or infusion of ACTH. Hypothalami and remainder of the brain were collected. A third group was offered 0.3 M NaCl to drink after induction of sodium appetite. Avid intake occurred over 10 min, and then, these gratified animals were euthanized. Using genome-wide coverage microarrays, the aim was to determine hypothalamic gene regulatory effects of sodium deficiency vs. control animals. Also, we determined if any gene expression changes evoked by sodium appetite were changed by rapid gratification of the appetite. Did the gestalt of neural inflow through 5th, 7th, 9th, and 10th cranial nerves with rapid drinking of 0.3M NaCl (1, 12, 13) modify any gene regulatory changes evoked by sodium appetite? Additionally, we compared gene expression in hypothalamus with the remainder of the brain.

We also used gene set enrichment analysis (GSEA) (14), a biocomputational method that interrogates regulation of groups of genes related to a particular physiological or pathological process. Gene sets in hypothalami of mice with sodium depletion and sodium appetite were significantly altered relative to sodium-replete controls. We found, unexpectedly, that genes regulated with sodium appetite were enriched for gene sets associated with

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Data deposition: The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession nos. GSE29451, GSM729057–GSM729100, and GSM729100).

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cocaine and opiate addiction (15), with up-regulation of enrichment by a combination of both methods of sodium appetite induction. No less surprising was the loss of enrichment for addiction gene sets on rapid gratification of sodium appetite. Based on the apparent presence of a specific gene regulatory program in the mammalian hypothalamus, we then focused on the lateral hypothalamus for verification of gene regulation in orexinergic neurons. Finally, moving to specific animal behavior, we were able to substantially reduce sodium appetite by systemic and hypothalamic local pharmacological manipulation.

Results

Genome-wide coverage microarrays (Fig. 1*A* and Fig. S1*A*) showed significant regulation of hypothalamic genes, including those genes previously identified as part of monoaminergic neurotransmission and gratification pathways like dopamine- and cAMP-regulated neuronal phosphoprotein 32 kDa (DARPP-32), proenkephalin, dopamine receptors-1 and -2, α -2C adrenoceptor, striatally enriched protein tyrosine phosphatase (STEP), and immediate early gene activity-regulated cytoskeleton associated protein (ARC) (Fig. 1*A*). Furthermore, by 10 min after gratification by drinking 0.3 M NaCl (Fig. 1*A*), we noted a perplexingly rapid loss of tight control of gene regulation. This finding means that up-regulated genes became less up-regulated, showing increased variation, and likewise, down-regulated genes became less down-regulated. Quantitative RT-PCR (qRT-PCR) confirmed these results (Fig. 1*B*).

To fully take advantage of our microarray results, we next used GSEA (14) to assess whether groups of functionally related genes were coregulated (i.e., enriched), suggesting a concerted regulation (Fig. 1*C* and Fig. S1*B*). Coregulations of gene sets that were implicated from previously conducted microarray studies of addiction (15) were shown for each condition, namely sodium deprivation-induced sodium appetite, as well as coregulation induced by ACTH. Moreover, their respective combination greatly increased significance levels. This unexpected result indicates that genes previously associated with addiction are shared between two different stimuli that give rise to the common motivation (salt appetite), whereas other gene sets, which are not shared between stimulatory modes, become diluted on combination. In contrast and as a confirmation of the specificity of the observed results, there was no significantly enriched set found in the remainder of the brain (SI Results). Thus, GSEA greatly increased the power of the microarray analysis method. Importantly, after gratification of sodium appetite, there was no more increase in enrichment on combination of groups, suggesting that gratification disrupts this closely coordinated gene regulatory program in the hypothalamus (Fig. 1*C*). Overall, this points to critical involvement of the observed gene regulatory program, including individually regulated genes, in generation of sodium appetite. This concept was confirmed by a detailed analysis of gratification-associated gene regulatory changes (Fig. 1*D*). However, it is likely that other regulated genes will follow a pattern, where return to the normal will be caused by full absorption of sodium drunk and rectification of blood and brain chemistry, which had originally been disturbed by induction of sodium appetite. In relation to the two methods used to produce sodium appetite, differential gene regulatory responses could be documented (Datasets S1, Tables S1–S3). Na⁺ deficiency-specific gene regulation comprised significantly more genes previously associated with addiction (7/100 vs. 1/100 with ACTH from addiction gene sets). Gene regulation specific for ACTH-evoked Na⁺ appetite was characterized by synaptic function-associated genes, which were not observed in Na⁺ deficiency.

We first focused additional analysis on protein expression of DARPP-32 in the hypothalamus using sodium-depleted rats. DARPP-32 was selected because of its known critical relevance in reward pathway dopaminergic signaling (16, 17), and the gene

was regulated robustly in both sodium appetite conditions as shown by microarray and confirmed by qPCR (Fig. 1*A* and *B*). In keeping with previous reports (16–18), we found DARPP-32 expression in suprachiasmatic and supraoptic neurons (Fig. S2). This labeling pattern was found in both sodium-depleted and control mice with no clearly appreciable difference. However, in sodium-depleted animals, we detected up-regulation of DARPP-32 in the periventricular nuclei (Fig. S2*A* and *B*) and also in the lateral hypothalamus (Fig. 2). These latter neurons coexpressed orexin (first known as hypocretin) (19), indicating that sodium deprivation leads to DARPP-32 up-regulation in this orexinergic/hypocretin subpopulation, which has key relevance to addiction-related behavior (17, 18) (Fig. 2). We also focused on the immediate-early gene *Arc* (activity-regulated cytoskeletal-associated protein), which is shown to function as a master regulator of protein-dependent synaptic and thus, neuronal plasticity. Extending our finding of regulated *Arc* hypothalamic gene expression (Fig. 1*A*), we detected ARC protein in lateral hypothalamic orexinergic neurons that also expressed DARPP-32 (Fig. 2*A–E*). This coexpression (Fig. 2*D*) suggests a possible neural plasticity mechanism, whereby ARC could function to prime lateral hypothalamic orexinergic neurons for subsequent activation of reward circuitry underlying gratification behavior (20). Interestingly, a previous study reported an increase of dendritic processes in the nucleus accumbens shell in sodium deficiency and also, a sensitization of sodium-depleted animals to amphetamine (21). Morphological alterations seem characteristic of ARC effects. Regulated expression of ARC in lateral hypothalamic orexinergic neurons prompted us to assess enrichment of gene sets associated with neural activity (22). However, we found them to not be enriched in each sodium appetite condition evaluated separately and in combination (Fig. S1*B* and Table S1). This finding suggests hypothalamic ARC regulation evoked by sodium appetite may be part of a neural plasticity mechanism somewhat specific for this instinct. Whether adaptation to a state of sodium deficiency or ACTH exposure, both evoking sodium appetite, can regulate other immediate early genes, particularly the long-term adaptation and drug addiction related Δ FOS-B, remains to be determined (23, 24).

Our experiments have led us to an exciting hypothesis, namely the existence of a hypothalamic gene regulatory program subtending sodium appetite. This mechanism would prime the animal with an intention to ingest salt to experience gratification. Therefore, we decided next to target reward pathways in rats and mice with deprivation-induced sodium appetite to address the question of in vivo relevance of the hypothalamic gene regulatory program for instinctive behavior. We used selective antagonists of dopamine receptors and metabotropic glutamate receptor 5 (mGlu5). This use was in light of their roles in reward and self-administration of drugs of abuse (25, 26) (Fig. 3 and Fig. S3*A* and *B*). We observed a dose-related decrease of gratification behavior on antagonism of both D1(5)-R and D2(3)-R (Fig. 3*A*). As for the role of mGlu5, known to be present on both D1 and D2 receptor-bearing neuronal populations, this role was substantiated in two rodent species using two different antagonists (Fig. S3*A* and *B*). Thus, D1(5)-R, D2(3)-R, and mGlu5 are functionally relevant in depletion-induced sodium appetite.

To address specificity of the affected instinct, control experiments were conducted to observe water intake after i.p. hypertonic saline injection into mice. Thirst, as a genetically programmed ingestive behavior, parallels salt appetite. In contrast to salt appetite, the D1(5)-R antagonist SCH23390, applied at identical dose, had no effect on osmotically evoked water intake. Antagonism of D2(3)-R as well as mGlu5 caused a moderate reduction of water intake (Fig. 3*B*). One explanation for these findings is that osmotically stimulated thirst is modulated to a greater degree by D2 receptor pathways (27, 28). Having established a selectivity of D1(5)-R antagonism for sodium appetite, we further investigated

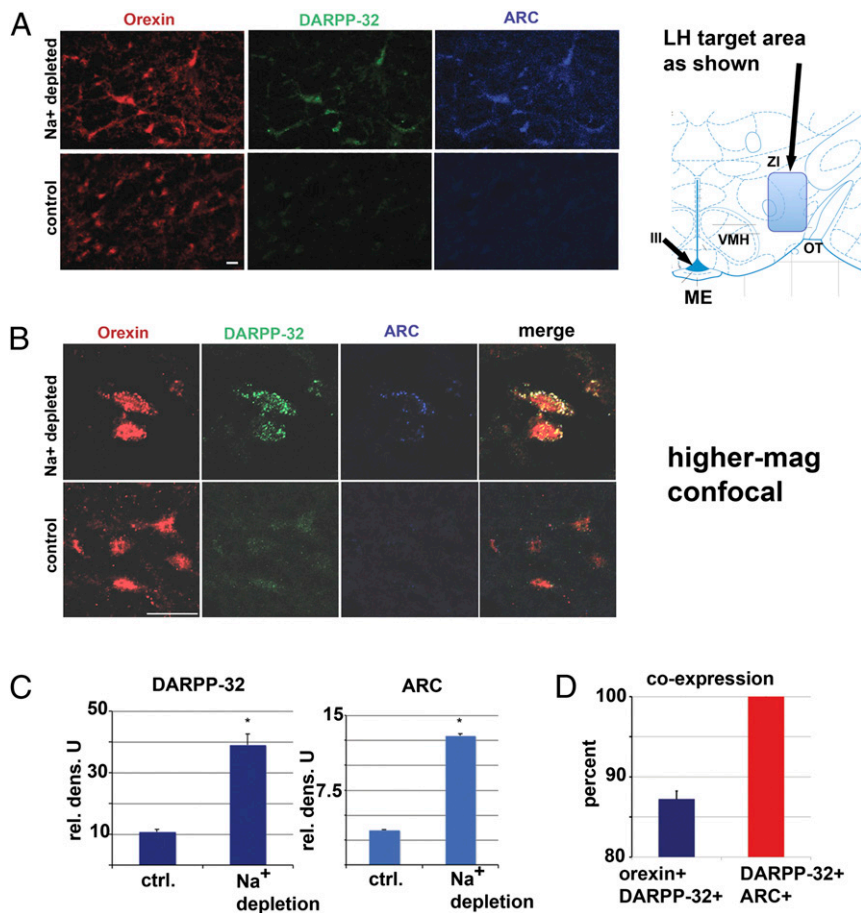


Fig. 2. Orexinergic lateral hypothalamic neurons up-regulate DARPP-32 and ARC in sodium-depleted rats. (*A Left*) Panels of micrographs show orexinergic labeling, colocalization with DARPP-32 immunoreactivity, and colocalization with ARC. Note the increased immunoreactivity of lateral hypothalamic neurons in sodium-depleted animals. (Scale bar: 30 μ m.) (*Right*) An anatomic orientation of the hypothalamus with the target area depicted in *A*. (*B*) Representative confocal micrographs showing partial subcellular colocalization of ARC and DARPP-32 in orexin-expressing lateral hypothalamic neurons. (Scale bar: 30 μ m.) (*C*) Morphometry (densitometry) of DARPP-32 and ARC expression in lateral hypothalamic orexinergic neurons. DARPP-32 and ARC are regulated in sodium depletion; differences are statistically significant at levels of $P < 0.01$ (*t* test; $n = 3$ animals per group and $n = 17$ – 40 orexin-expressing neurons per animal). (*D*) Morphometry of colabeling in lateral hypothalamic neurons. The blue bar indicates, based on data from three sodium-deprived rats ($n = 19$, 32 , and 33 lateral hypothalamic neurons positive for orexin), the percentage of colocalization of DARPP-32. The threshold was set at three times the average of nondepleted control animals. The red bar indicates the 100% colocalization of DARPP-32 with ARC in the orexin-DARPP-32-coexpressing neurons.

with SCH23390 (100 nM in 200 nL injection volume; $n = 4$). This injection led to a large reduction of sodium appetite at the level of complete elimination in two of four animals (Fig. 3*D*). Subsequent aCSF injection did not suppress robust sodium appetite (Fig. 3*B*). Thus, D1(5)-R signaling in the LH area is at least partially responsible for the significant effects on sodium appetite of SCH23390 on systemic application.

Discussion

An instinctive behavior pattern of which salt appetite is an exemplar reflects a genetically hard-wired neural organization naturally selected because of its high survival value. The constitution of the behavior pattern is a trinity—a closely integrated neural system. When strongly aroused, it incorporates an imperious sensation and a compelling specific intention. The subjective element of the trinity of components, the primordial emotion, coalesces all three physiological entities. There is a characteristic specificity. That is, a sexually aroused animal does not have an intention directed to salt intake, and a hungry animal does not have a specific intention to drink water.

The multiple gene changes that we are reporting here likely engender the behavior pattern that is aroused and executed, although our observations do not translate into understanding of

a general mechanism. The reversal of a substantial element of the gene changes within 10 min of gratification by drinking 0.3 M NaCl also suggests their involvement in the response to sodium deficiency, and the contemporaneous loss of appetite with gratification suggests that these gene expression changes are implicated in causation of the changed subjective state. It should be noted that it is not determined how rapidly the protein changes evoked by the initial gene expression are reversed by gratification. Additionally, no delineations have been attempted with regard to genes that may control other physiological organizations in sodium deficiency such as control of blood pressure.

In relation to the complexity of the underlying mechanisms, it can be recognized that many factors may be involved in the genesis of sodium appetite. Adrenal steroids and angiotensin are important in the mouse (1). However, with ruminants (e.g., sheep and cattle), sodium concentration in the CSF is a major causal factor, its role probably reflecting that the impact of a sodium-deficient environment falls primarily on the herbivores (1). Thus, differences between species reflect the different selection pressures operative in the evolution of the instinct. Furthermore, a quintet of steroid and peptide hormones is involved in the powerful sodium appetite of pregnancy and lactation. Thus, causation of sodium appetite is complex, and quantitative

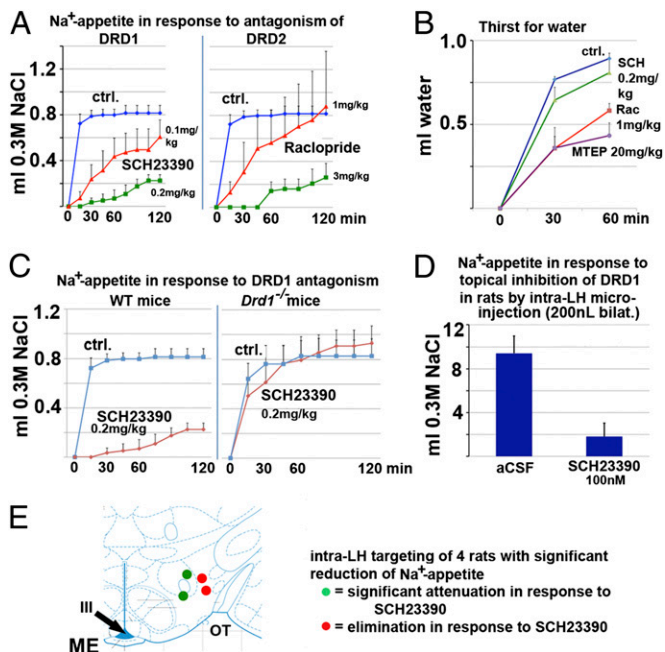


Fig. 3. Modulation of reward pathways in sodium appetite. **A** shows dose-dependent effects of specific inhibition of D1(5) and D2(3) receptors with specific compounds, SCH23390 and raclopride, on sodium appetite of sodium-depleted mice. Cumulative 0.3 M NaCl was assessed every 15 min. Note the striking effect of both compounds at higher doses and attenuation at lower doses, with raclopride-treated mice showing higher variation. **B** shows cumulative intake of water in response to osmotic dehydration in mice, the attenuating effects of raclopride (lower dose), mGlu5 antagonist, and MTEP, and the complete lack of effect of SCH23390 (higher dose). **C** illustrates normal depletion-evoked sodium appetite in *Drd1*^{-/-} mice; however, there is complete lack of effect of SCH23390 (again, higher dose) in these mice, reiterating the specificity and complete absence of off-target effects of this compound at 0.2 mg/kg. **(D)** The bar diagram shows averaged sodium appetite for four rats with intrahypothalamic catheters on injection of 200 nL aCSF and the striking reduction when they were infused with 200 nL SCH23390 at 100 nM; $P < 0.01$ (*t* test for paired samples) for this dataset (*Materials and Methods* and *Fig. S3*). **E** shows a neuroanatomic schematic, with red circles indicating the location of the injection needle in case of complete elimination of sodium appetite and green circles indicating attenuation (*Fig. 2A* and *Fig. S3B*).

delineation of the factors contemporaneously operating in the hypothalamus to contrive gene regulatory change in particular species is likely to be as complex as genesis of hunger.

However, the results reported here represent an important first step toward better understanding gene regulatory dynamics in the hypothalamus that subtend gratification (see also ref. 29). Going beyond, we showed here the functional relevance of dopamine and metabotropic glutamate receptor signaling for sodium appetite, and we identified a specific functional role for D1(5) receptors without effect on a related instinct, thirst for water.

Selective antagonism of D1(5) receptors by microinjection into the lateral hypothalamus virtually eliminated sodium appetite, thus reiterating the key role of this location as suggested by our DARPP-32-ARC immunohistochemistry. Our result concurs with a body of previous work showing the role of the mesolimbic dopaminergic system in sodium appetite generated by chronic administration of deoxycorticosterone acetate (30).

Phylogenetically, brain pathways linked to gratification of primal needs have evolved over much more than 100 million years. For example, salt appetite is highly developed in kangaroos. The data reported here suggest that human desire to use drugs such as cocaine and opiates reflects that, in recent human history, drugs of abuse have functionally exploited these phylogenetically an-

cient gratification mechanisms. This concept was proposed by Koob and Bloom (31, but also see ref. 32), and our data presented here give experimental validation of the idea. This lock into ancient pathways could constitute a partial explanation for the recalcitrance of drug addiction to therapeutic attempts aimed at abstinence. Animals or human addicts with nonsatisfied primal needs show altered responses to drugs of abuse (21, 33).

Beyond our finding of the hypothalamic up-regulation of DARPP-32 after sodium depletion, future studies need to more precisely address the molecular nature of the respective neural circuitry. This need translates into asking which circuits are differentially activated in sodium appetite and possibly, other instinctive needs beyond the established link between lateral hypothalamus orexinergic neurons and nucleus accumbens (34–36). To complement, one of our observations is concordant with a recent advance in addiction biology, namely that orexinergic lateral hypothalamic neurons are involved in addiction behaviors (34, 36). As early as 1964, Wolf (38) showed that bilateral lesions in the lateral hypothalamus greatly reduced the drinking response to sodium deficiency, although he commented that such lesions might damage structures nearby (e.g., zona incerta and median forebrain bundle). Another study reported it as a brain subregion with particularly dynamic gene regulation in cocaine-addicted rats (39).

Our experiments provide a foundation to directly address the question of which aggregate of gene regulatory mechanism underpins each specific instinct and its gratification pathways in the hypothalamus. It is attractive to view the hypothalamic gene regulatory mechanisms characterized here as a scaffold for instinctive behavior (as outlined above), including the compelling intention to specific gratification. Such specific gratification was termed the consummatory act by Lorenz (40). The fact that regulated ensembles of genes are associated with the changes of the subjective state represented by sodium appetite adds a molecular–genetic dimension to the hypothesis that the primordial emotions may have been the phylogenetic origin of consciousness (41, 42).

Materials and Methods

Animals. All animal studies were conducted with specific approval of the respective animal care and use committees of the Florey Neuroscience Institutes and Duke University.

Sodium Deprivation. Mice (C57/Bl6; male) were housed individually. After 7 d habituation to low Na food, they then had control measurements taken of body weight, food, water, and 0.3 M NaCl intake over the next 7 d. Then, the 0.3 M NaCl solution was removed, and mice were injected one time per day for 2 d with 1.2 mg furosemide i.p. (Aventis). On the third day, mice were euthanized, and a diencephalic tissue block incorporating all hypothalamus with minimal adjacent tissue (cuts being just rostral to optic chiasm, just caudal to mammillary body, and superiorly through thalamus) was collected into liquid nitrogen. The remainder of the brain served as control. Control mice had a diet with 0.5% NaCl.

Gratification. With gratification studies, on the third day, 0.3 M NaCl was returned to the mice. These mice drank avidly; ~10 min later, they were killed, and brain segments were collected.

For pharmacology, mice were treated as above, and then, on the third day, they were offered back the 0.3 M NaCl drinking solution; 0.3 M NaCl intake, water intake, food intake, and body weight were measured every 15 min for the following 1 h, and these measurements were followed by daily measurements (5 d). After this control experiment, the protocol was repeated; however, 10–20 min before the return of the 0.3 M NaCl drinking solution, the mice were injected with 0.1 mL saline s.c. ($n = 6$ mice), the D2/D3 antagonist raclopride at 1 or 3 mg/kg in 0.1 mL saline s.c. ($n = 6$ mice in each case), the D1/D5 antagonist SCH23390 at 0.1 or 0.2 mg/kg in 0.1 mL saline s.c. ($n = 6$ mice in each case), or the mGlu5 antagonist methyl-4-thiazolyl-ethylpyridine (MTEP) at 20 mg/kg i.p. in 0.1 mL ($n = 6$ mice). Again, intakes were measured every 15 min for the next 2 h, and then, measurements were taken daily.

Drd1a KO mice were used from our own colony (J.D.) (43).

Rats (male Sprague–Dawley rats; mean = 300 g at 12 wk of age) were Na⁺ deprived with low Na⁺ food (0.02%, TD90.228; Harlan) and deionized water

for 1 wk. On days 3–7 of that week, animals were injected with 5 mg/kg per d furosemide i.p. On day 7, two groups ($n = 6$ each) were formed and injected with mGlu5 inhibitor, fenobam (i.p., 25 mg/kg in 1 mL), or vehicle 20–30 min before access to 0.3 M NaCl. Rats were permitted to gratify with 0.3 M NaCl, and its intake was measured at 20 min.

ACTH Infusion. Female C57/B16 mice were offered water, 0.005 M NaCl, and low Na⁺ food (0.02% Na⁺). After 1 wk of daily control measurements of body weight, food, water, and 0.005 M NaCl, a miniosmotic pump (Alzet) was implanted s.c. to deliver ACTH (Ciba) at 2.8 μ g/d for 12 d. Control 0.005 M NaCl intake was 1.6 mL/d. On the last 4 d of ACTH infusion, the mean intake was 9.3 mL/d. After 12 d of ACTH infusion, mice were euthanized, and hypothalami and the remainder of the brain were removed as above. For the gratification group with ACTH, the same protocol as above was followed, except that 300 mM NaCl solution was available from the beginning of ACTH infusions until euthanasia.

RNA Isolation and Labeling, DNA Microarrays, RNA and Microarray Probe Preparation, and Hybridization. Total RNA was isolated from the hypothalamus and the remainder of the brain from each mouse using the TRIZOL-RNA-easy method, and RNA quality was assessed using spectrophotometry and microelectrophoresis; RNA was labeled, and DNA microarray analysis was conducted using the standard procedures as provided in detail in *SI Materials and Methods*.

GSEA. GSEA (8) was conducted using the standard procedures that are described in more detail in *SI Materials and Methods*.

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PCR. Evaluation of mRNA abundance of regulated genes, as suggested by microarray, was performed by qRT-PCR using commercially available primer sets (Openbiosystems), and the SYBR-green and $\Delta\Delta$ Ct methods for quantitative assessment (44) were used to perform a relative comparison between sodium appetite and the control group.

Immunohistochemistry. Standard methods were used (*SI Materials and Methods*).

Statistical Methods. Statistical methods for gene arrays and GSEA are described above. Mean and SEM of quantified outcome parameters were compared with their respective controls. Group comparisons were performed using Student unpaired t test or two-factor repeated measures ANOVA followed by Newman-Keuls test for multigroup comparison. Nonparametric statistical testing was performed using the Kruskal–Wallis ANOVA and posthoc Nemenyi test. Minimum significance was set at $P < 0.05$ for all testing.

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