Supporting Information

Liedtke et al. 10.1073/pnas.1109199108

SI Results

In regards to gene set enrichment analysis, there was no significantly enriched set detected in the remainder of the brain (i.e., 0 of the 54 gene sets queried showed a P value < 0.1). Specifically, the most significantly enriched set was neuroactive ligand receptor interaction, comprising 75 genes. Its normalized enrichment score was −1.26, indicating some moderate enrichment, but its nominal P value was 0.192. The second gene set, MAPK pathway, consists of 65 genes, and it had a normalized enrichment score of −0.938 and a nominal P value of 0.54. All addiction-related sets were significantly lower in ranking. It has to be considered that other locations within the central nervous system, such as in the ventral tegmentum, amygdala, and medial prefrontal cortex, probably have important involvement in sodium appetite but are diluted out by the large noninvolved brain areas when using the remainder of the brain as a control for hypothalamus.

With regard to sodium appetite and modulation of emotion and behavior, an interesting set of findings has been made by Morris et al. (1), Na et al. (2), Morris et al. (3), Na et al. (4), Grippo et al. (5), and Morris et al. (6). They showed that nongratiﬁed salt appetite is associated with anhedonia, a key component of major depression in humans (1–6). This particular impact of sodium appetite on mood is paralleled by the depressed mood in addicted humans and experimental animals on drug withdrawal.

The serum- and glucocorticoid-dependent kinase SGK1, which was characterized by Wärtgnes et al. (7) as expressed in various brain areas and found up-regulated in its expression by dehydration, had a tendency to up-regulation in our microarrays. By comparing normalised gene expression for SGK1 for control vs. furosemide-depleted hypothalamic samples, the P value was 0.006 for the isolated comparison, but taking into account (as for all data shown here) the context of the genome-wide analysis, the P value was 0.31. With these data, SGK1 is more likely to be regulated than not regulated, and it ranked 173 for genes exclusively regulated in furosemide/Na⁺ depletion-induced sodium appetite. Aforementioned up-regulation of SGK1 in the brain (7) did not include study of the hypothalamus, and it was conducted in response to dehydration, not in response to conditions that evoke sodium appetite. In regards to the functional role of SGK1 for mineral appetite, Sgk1 was found necessary for mineralocorticoid-induced sodium appetite evoked by deoxycorticosterone acetate (DOCA) in SgkJ null mice (7, 8). This concept is not contradicted by our data. A gene that is necessary for a certain homeostatic behavior need not necessarily be regulated by the cue that evokes the respective behavior.

Another interesting aspect is dependence of sodium appetite on peripheral afferents. Complete peripheral denervation abrogates sodium appetite, which was shown decades ago. Whether dopaminergic transmission is of critical relevance in the peripheral taste system for sodium taste is currently unknown, representing an interesting subject for future studies. However, we wish to stress that we did identify one critical local relay for sodium appetite in the lateral hypothalamus by our injection studies.

SI Materials and Methods

DNA Microarrays. Mouse oligonucleotide arrays were printed at the Duke Microarray Facility using the Operon’s Mouse Genome Oligo Set (3.0), which contains 31,769 70-mer probes representing 24,878 genes and 32,829 transcripts (Operon). Arrays were printed using an Omnimgrid 300 arrayer by Genomic Solutions on Corning UltraGap amino-saline coated glass slides (Fisher Scientific).

RNA and Microarray Probe Preparation and Hybridization. Total RNA (1 μg) from each sample (mouse hypothalamus or remainder of the brain) and the reference (Universal Mouse Reference RNA; Stratagene) were used in probe preparation. The puriﬁed antisense RNA was ﬂuorescently labeled with Cy3 (reference) and Cy5 (sample; GE Healthcare). Sample and reference RNAs were pooled, mixed with 1× hybridization buffer (50% formamide, 5× SSC, and 0.1% SDS), COT-1 DNA, and poly-dA to limit non-specific binding, and heated to 95 °C for 2 min. This mixture was pipetted onto a microarray slide using a MetaImager hybridization chamber (BioMicro Systems) and hybridized overnight at 42 °C on an automated Maui Hybridization Station (BioMicro Systems). The array was then washed at increasing stringencies and scanned on a GenePix 4000B microarray scanner (Axon Instruments). Detailed protocols are available on the Duke Microarray Facility website (http://www.genome.duke.edu/cores/microarray/services/spotted-arrays/protocols/).

Microarray Analysis. The resulting microarray data were analyzed using the Duke Microarray Database analysis platform, a locally installed version of the Stanford Microarray Database (9, 10). Background-subtracted and normalized data were averaged for each of greater than or equal to four animals within the three groups. Regulated genes were identiﬁed by comparison of normalized values.

Within-array Loess normalization was performed using the Limma package (11) to remove any bias that the intensity has on log-fold change. Next, normalization was carried out between arrays using variance stabilization in that both the red and green channels on the arrays are aligned to each other based on the assumption that most of the genes are constant across experiments (12). Spots not reaching the quality criterion were removed from additional analysis. Quality was determined in three ways. (i) There was insuﬃcient red or green intensity to produce a reliable ratio. (ii) The consistency of the ratio throughout the spot was low. (iii) Other machine-annotated flags indicated that the spot was not reliably read. After filtering, if a particular spot was found only to be present on a small number of arrays (<15), then that spot was filtered for all arrays. To be sure that the experiment consistently measured diﬀerences between groups, we performed a principle component analysis. This procedure allowed us to separate the experiments by both brain region (hypothalamus vs. whole brain without hypothalamus) and treatment [adrenocorticotropic hormone (ACTH)-induced sodium appetite, sodium deprivation plus furosemide, and control]. Spots passing these criteria were ranked and analyzed for statistically signiﬁcant diﬀerences using a linear model (13).

Gene Set Enrichment Analysis. Gene set enrichment analysis (GSEA) (14) was used to test the hypothesis that addiction-related genes were regulated by sodium appetite. GSEA takes as input a set of microarray data, a set of comparisons to make between groups in that experiment, and a series of gene sets. Each of those gene sets, often pathways, represents a hypothesis of which genes are regulated in the data. The function of GSEA is to test each of those hypotheses against the data in a statistically rigorous manner. The processed and normalized microarray data were altered by hand to conform to the .gct format. After being imported into the GSEA, a comparison was made, using a t test, between the expression of genes under the control conditions and the expression of genes in mice with sodium appetite. We tested these gene expression changes against lists of genes that


Fig. S1. Gene expression analysis using microarrays. A depicts the heat map, as shown in Fig. 1A, with spelled-out abbreviations for gene symbols and maximum log-fold change. B shows the original enrichment plots of GSEA analysis. Depicted here are enrichment plots for the gene sets for opioids, cocaine, alcohol, and neural activity (1, 2), and the location of each gene in the set is represented against the ranking of all genes (red to blue) by a black vertical line (below the running score is the green line). The location of the gene set members is used to calculate the enrichment score, which is defined as the maximum/minimum distance of the running score (green line) from baseline. Note the different scales of the y axis for different sets: opioids minimum = −0.5, cocaine minimum = −0.4, alcohol minimum = −0.35, and neural activity minimum = −0.2. The even distribution in the activity set is conspicuous, indicating lack of enrichment, as is the robustly enriched feature (right-shifted to overexpressed genes) in the addiction-related subsets. These four subsets depict the combined data from both methods of induction of sodium appetite.

Fig. S2. DARPP-32 expression in the periventricular nucleus of a sodium-depleted rat is shown. A shows the anatomic orientation, including anatomical hallmarks of this section of the hypothalamus, shown in the micrograph. B depicts representative examples of up-regulated DARPP-32 expression in the periventricular nucleus in a sodium-depleted animal. Note the positive label in the ependymal cells as well. This expression pattern was not detected in control animals; counts were 18, 14, and 23 labeled periventricular cells in three animals vs. 0, 0, and 0 (three animals) in sodium depletion vs. control. (Scale bar: 30 μm.) III indicates the third ventricle for orientation. C depicts lack of regulation of DARPP-32 immunoreactivity in the suprachiasmatic (SCN) and supraoptic (SON) nucleus of two representative animals. (Scale bar: 30 μm.)
Fig. S3. Pharmacological antagonism attenuates gratification behavior of sodium appetite. (A) This figure shows results of specific inhibition of metabotropic glutamate receptor 5 receptors in mouse and rat. Left shows the time course of gratification behavior in sodium-depleted mice as significantly attenuated by the specific metabotropic glutamate receptor 5 receptor antagonist, MTEP, applied at 20 mg/kg (n = 5 mice per group). Differences to vehicle-treated mice are statistically significant. Right shows the robust effects of fenobam, an mGlu5 inhibitor, strongly reducing gratification behavior in rats with induced sodium appetite. Fenobam was used at 25 mg/kg fenobam (1, 2). Upper Right is the amount of 0.3 M NaCl consumed within 20 min; in Lower Right, the latency to first drinking is shown. Note that, in the rat model (similar to mouse models), ≥90% of 0.3 M NaCl is consumed within the first 20 min after 0.3 M NaCl is made available (n = 6 rats per group; P < 0.001, t test). (B) This diagram shows the attenuation of sodium appetite by intra-lateral hypothalamus (LH) microinjection [D(1S)-R-specific antagonist SCH23390 at 100 nM in 200 nL injection volume] in individual rats 1–4. Schematics (B Right) depicting the location of the injection needle within the lateral hypothalamus are shown. The diagram with red dots for the verified injection site depicts the two rats that had complete elimination of sodium appetite with SCH23390. Note that the size of a calculated sphere with 362 μm radius correlating to an injection volume of 200 nL is shown in light red. The schematic in green depicts the injection sites for rats 1 and 4, with the same rendering of 200 nL injection volume. The micrograph on the right is of an injection site with a fluorescently labeled dextran tracer. (Scale bar: 400 μm.)

**Table S1. Top 10 enriched gene sets**

<table>
<thead>
<tr>
<th>Name</th>
<th>Size</th>
<th>ES</th>
<th>NES</th>
<th>NOM P value</th>
<th>FDR q value</th>
<th>FWER P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opioids</td>
<td>72</td>
<td>-0.481</td>
<td>-1.826</td>
<td>0.007</td>
<td>0.011</td>
<td>0.010</td>
</tr>
<tr>
<td>Cocaine</td>
<td>108</td>
<td>-0.417</td>
<td>-1.635</td>
<td>0.028</td>
<td>0.062</td>
<td>0.099</td>
</tr>
<tr>
<td>Cytokine–cytokine receptor interaction</td>
<td>81</td>
<td>-0.393</td>
<td>-1.537</td>
<td>0.038</td>
<td>0.112</td>
<td>0.217</td>
</tr>
<tr>
<td>Alcohol</td>
<td>207</td>
<td>-0.344</td>
<td>-1.472</td>
<td>0.070</td>
<td>0.162</td>
<td>0.306</td>
</tr>
</tbody>
</table>

Values for pooled data based on both sodium appetite conditions are shown (increased over single sodium appetite condition); also, note the enrichment parameters for gene set neural activity, which indicate relative lack of enrichment. The neural activity gene set is also in Fig. 1 and *SI Material and Methods*. Addiction gene subsets are highlighted in turquoise. ES, enrichment score; FDR, false discovery rate; NES, normalized enrichment score; NOM, nominal; FWER, family-wise error rate.

**Dataset S1, Table S1. Top 600 regulated genes for both conditions of sodium appetite combined**

**Dataset S1, Table S2. Top 100 regulated genes for sodium depletion**

This dataset shows genes specifically regulated for Na⁺ depletion. Fold regulation is shown as log₂. The top 100 regulated genes are shown, which are exclusively regulated in one condition, not regulated in the other, and not regulated in the combined data set. Turquoise highlighting indicates members of the addiction gene set.

**Dataset S1, Table S3. Top 100 regulated genes for sodium appetite induced by ACTH**

This dataset shows genes specifically regulated for ACTH-evoked Na⁺ appetite. Fold regulation is shown as log₂. The top 100 regulated genes are shown, which are exclusively regulated in one condition, not regulated in the other, and not regulated in the combined data set. Turquoise highlighting indicates members of the addiction gene set, orange indicates synaptic function-related genes, and green indicates other genes of interest. Regarding the latter, note the up-regulation of salt-inducible kinase 2 (SIK2), as well as a functional subunit of the glucocorticoid receptor, suggesting the possibility of a feed-forward dysregulation underlying the effects of chronic ACTH administration. Specifically, we report expression and regulation for ACTH-mediated SIK2 overexpression in the central nervous system. SIK2 refers to the concept of a cellular sodium sensor that can also regulate chromatin (1–4).