Original article

Glut4 expression defines an insulin-sensitive hypothalamic neuronal population

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ABSTRACT

Insulin signaling in the CNS modulates satiety and glucose metabolism, but insulin target neurons are poorly defined. We have previously shown that ablation of insulin receptors (InsR) in Glut4-expressing tissues results in systemic abnormalities of insulin action. We propose that Glut4 neurons constitute an insulin-sensitive neuronal subset. We determined their gene expression profiles using flow-sorted hypothalamic Glut4 neurons. Gene ontology analyses demonstrated that Glut4 neurons are enriched in olfacto-sensory receptors, M2 acetylcholine receptors, and pathways required for the acquisition of insulin sensitivity. Following genetic ablation of InsR, transcriptome profiling of Glut4 neurons demonstrated impairment of the insulin, peptide hormone, and cAMP signaling pathways, with a striking upregulation of anion homeostasis pathway. Accordingly, hypothalamic InsR-deficient Glut4 neurons showed reduced firing activity. The molecular signature of Glut4 neurons is consistent with a role for this neural population in the integration of olfacto-sensory cues with hormone signaling to regulate peripheral metabolism.

1. INTRODUCTION

Insulin acts through the insulin receptor (InsR) in the CNS to regulate food intake [1], counterregulatory response to hypoglycemia [2], and leptin sensitivity [3]. However, anatomic and functional mapping of insulin's CNS action is not nearly as detailed as those of other hormones [4]. This is partly due to the fact that InsR are thought to be widespread among neurons, not to mention glial, and non-neural cells [5]. Glucose utilization is thought to occur largely in an insulin-independent manner in the CNS [6]. Nonetheless, a sub-population of widely scattered neurons possesses the insulin-responsive glucose transporter, Glut4 [7]. While the function of this transporter in glucose metabolism continues to be a matter of speculation, it's a fact that removing InsR from Glut4 neurons has a profound detrimental effect on peripheral metabolism. Thus mice lacking InsR in Glut4-expressing tissues develop type 2 diabetes [7], highlighting the contribution of CNS insulin resistance to the pathophysiology of diabetes [8].

In this study, we set out to investigate the nature of Glut4 neurons in the hypothalamus, a key regulatory site for appetite and glucose homeostasis [9]. As a first step toward characterizing the physiological function(s) of Glut4 neurons, we determined their gene expression profiles. To this end, we generated transgenic mice with chemically defined Glut4 neurons [7]. We flow-sorted Glut4 neurons and compared their gene expression profile with that of non-Glut4 neurons using a previously reported protocol [10]. Interestingly, we found pathways critical for sensory perception, receptor signaling, and response to nutrient are significantly enriched in the Glut4 neurons,

consistent with the hypothesis that Glut4 neurons have critical roles in metabolic regulation.

2. MATERIAL AND METHOD

2.1. Mice

$Gt(Rosa)26Sor^{tm9(CAG-tdTomato)Hze}$ mice were from the Jackson Laboratories. GIRKO mice were generated as previously described [7]. The Columbia University Animal Care and Utilization Committee approved all procedures. Normal chow diet (NCD) had 62.1% calories from carbohydrates, 24.6% from protein and 13.2% from fat (PicoLab rodent diet 20, 5053; Purina Mills).

2.2. Flow cytometry, gene profiling, and quantification of neurons

We dissociated mediobasal hypothalami from 3-week-old mice with transgenes (*Glut4-Cre* [line 535]; *Rosa-Tomato*; *InsR lox* allele) with papain dissociation kit (Worthington Biochemical). We gated live neurons to collect Rfp-positive neurons. We performed microarray for gene profiling using a procedure described previously [10]. We used Partek Genomic Suite and R for pathway analysis and heatmap generation. We analyzed the FACS data using Flowjo software. We quantified immunofluorescence using Image J software.

2.3. Electrophysiological studies

For patch-clamp recording in acute slices, we sectioned 350-μm-thickn coronal brain sections in a cold sucrose-based cutting solution containing (in mM): 195 sucrose, 10 NaCl, 25 NaHCO$_3$, 25 glucose, 2.5 KCl, 1.25 NaH$_2$PO$_4$, 2 Na pyruvate, 0.5 CaCl$_2$, and 7 MgCl$_2$ (pH 7.3)

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using a vibratome (Leica VT1000S). Slices were incubated in a sub-merged chamber at room temperature for at least 90 min before recordings, perfused with recording solution containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH$_2$PO$_4$, 25 NaHCO$_3$, 2 CaCl$_2$, 1 MgCl$_2$, 25 glucose (pH 7.3) and continuously bubbled with 95% O$_2$ and 5% CO$_2$. We selected cells using an upright microscope fitted with fluorescence optics (Nikon) and patched under IR-DIC optics. We acquired patch-clamp recordings with a MultiClamp 700B amplifier and Clampex data acquisition software (Axon Instruments) at room temperature. We digitized data at 10 kHz with a 2 kHz low-pass filter. We fabricated a glass pipette of 5—8 MΩ and filled it with electrode solution (in mM): 130 K gluconate, 10 KCl, 5 HEPES, 5 MgCl$_2$, 0.06 CaCl$_2$, 0.6 EGTA, 2 MgATP, 0.2 Na$_2$GTP, and 20 Phosphocreatine. We adjusted osmolarity to 310 mOsm and pH to 7.3 with KOH. We assessed electrical access in the whole-cell recording mode using a small-voltage step pulse before and at the end of the current-clamp recordings. We discarded recordings in which the series resistance had been >30 MΩ. Resting membrane potential and spontaneous action potentials were recorded in the current-clamp mode with no current injection. Detection and measurements of action potentials were performed using MiniAnalysis software (Synaptosoft).

2.4. Statistical analyses

We analyzed data with Student's *t*-test or 1way or 2way ANOVA using GraphPad Prism and Partek Genomic Suite software. We used the customary threshold of $p < 0.05$ to declare statistical significance.

3. RESULTS

3.1. Glut4 neurons are enriched in olfacto-sensory receptors and hormone signaling pathways

We took an unbiased approach to characterize Glut4 neurons. We generated *Glut4-Cre: Rosa26-Tomato* mice, in which Glut4 neurons were labeled by Cre-mediated activation of red fluorescent protein (Rfp). We used fluorescence-activated cell sorting (FACS) to collect Rfp-labeled Glut4 neurons and non-fluorescent control neurons from basal hypothalami of transgenic mice. We compared transcriptomes of Glut4 and control neurons by microarray analysis. Gene ontology (GO) enrichment analysis demonstrated that the Glut4 neuron transcriptome was enriched in olfacto-sensory systems and G-protein coupled receptors. Other classes of enriched genes could be subsumed under these classifications. In addition, we found enrichment in genes required for nutrient sensing and glucose utilization, consistent with the notion that these neurons have metabolic control activity (Table 1). Indeed, GO ANOVA analysis indicated an overrepresentation of genes involved in the maturation of metabolically active cells (adipocytes). This class included transcriptional regulators such as Ppar-γ, Tcf7l2, and others that could be subsumed under the classification of histone modification genes, which was also enriched in Glut4 neurons. GO pathway analysis revealed enrichment in genes required for O-glycan biosynthesis and ribosome biogenesis (Table 1).

In an attempt to assign Glut4 neurons to a specific class of neurons, we carried out a separate analysis of genes that are essential for neuronal activity, including neurotransmitter receptors and ion channels. A heatmap rendering shows that most neurotransmitter receptors have variable levels of enrichment in Glut4 neurons (Figure 1A). We analyzed the data by grouping the major sub-classes of neurotransmitter receptors (Figure 1B—I). Overall, we found no enrichment of specific neurotransmitter subsets, indicating that Glut4 neurons likely include different neuronal subtypes (Figure 1B—I). However, we found a significant enrichment of the M2 subtype muscarinic receptor, along with

Gene ontology enrichment

Function	Enrichment score	*p*-Value	%Genes in group represented
Olfactory receptor activity	17.3227	3.00E-08	4.07031
Sensory perception of smell	16.485	6.93E-08	3.95328
Sensory perception of chemical stimulus	15.3567	2.14E-07	3.75313
Sensory perception	11.6154	9.03E-06	3.20382
G-protein coupled receptor activity	10.9879	1.69E-05	3.00578
Neurological system process	10.0768	4.20E-05	2.94638
Transmembrane signaling receptor activity	9.06199	0.000115992	2.74177
G-protein coupled receptor signaling pathway	8.72103	0.000163119	2.7376
Signaling receptor activity	7.98683	0.000339911	2.62781
Cellular response to nutrient	7.31929	0.000662631	27.2727
Regulation of transcription by glucose	5.55087	0.00388407	33.3333

Gene ontology ANOVA

GO description	# of probes	GO ID	*p*-Value (attribute)
Fat cell differentiation	63	45444	0.00140471
Phospholipid homeostasis	8	55091	0.00212119
Sulfonylurea receptor binding	2	17098	0.00325252
Calcium- and calmodulin-responsive adenylate cyclase activity	5	8294	0.00384257
Positive regulation of histone modification	41	31058	0.00548655
Negative regulation of small GTPase mediated signal transduction	30	51058	0.00609217
Mitochondrial respiratory chain	5	5746	0.00635338
Pyrimidine ribonucleoside binding	4	32551	0.00722503
Aging	76	7568	0.00741073
Carnitine transport	4	15879	0.00883626

Pathway enrichment

Pathway name	# of probesets	Pathway ID	*p*-Value (attribute)
Mucin type O-Glycan biosynthesis	25	Kegg pathway 48	0.00864224
Ribosome biogenesis in eukaryotes	75	Kegg pathway 100	0.0275843
Protein export	19	Kegg pathway 22	0.0642122
Regulation of autophagy	28	Kegg pathway 9	0.0965468

Table 1: Gene ontology and pathway analysis of Glut4 neurons.
Top categories in each group of gene ontology analyses performed using RNA profiling data from flow-sorted Glut4 neurons.

a less impressive increase of dopamine D2 receptor (Figure 1G). The expression of ion channels showed no particular pattern of enrichment in Glut4 neurons (Figure 1J). We analyzed the expression of major sub-classes of ion channels (Figure 1K—N), but they revealed no significant change.

3.2. Characterization of InsR-deficient Glut4 neurons

Our hypothesis was that Glut4 neurons are important to mediate CNS insulin signaling. To test this possibility, we interrogated the gene expression profiles of InsR-deficient Glut4 neurons using the same method described above (exon microarray hybridization of flow-sorted hypothalamic neurons), and performed electrophysiological studies.

We used Tomato fluorescence to identify Glut4 neurons in WT and GIRKO mice bearing either two/one (*i.e. Insr*$^{lox/+}$ or *Insr*$^{+/-}$) or no intact *InsR* allele (*Insr*$^{Δ/Δ}$ or *Insr*$^{Δ/-}$). We collected Tomato-positive (i.e. Glut4 neurons) and Tomato-negative (i.e. non-Glut4 cells) fractions from both WT and GIRKO mice (Figure 2A). Using DNA extracted post-sort from two independent sample sets (Figure 2B), we confirmed that Glut4 neurons from GIRKO mice had minimum intact *InsR* allele. We compared gene expression profiles from sorted neurons (Figure 2C). Gratifyingly, gene set enrichment analysis of the array results showed that *InsR* was one of the most decreased mRNA (Supplemental Table 1), and that impaired expression of genes involved in the cellular responses to insulin topped the list of altered pathways, demonstrating the robustness of the method (Table 2).
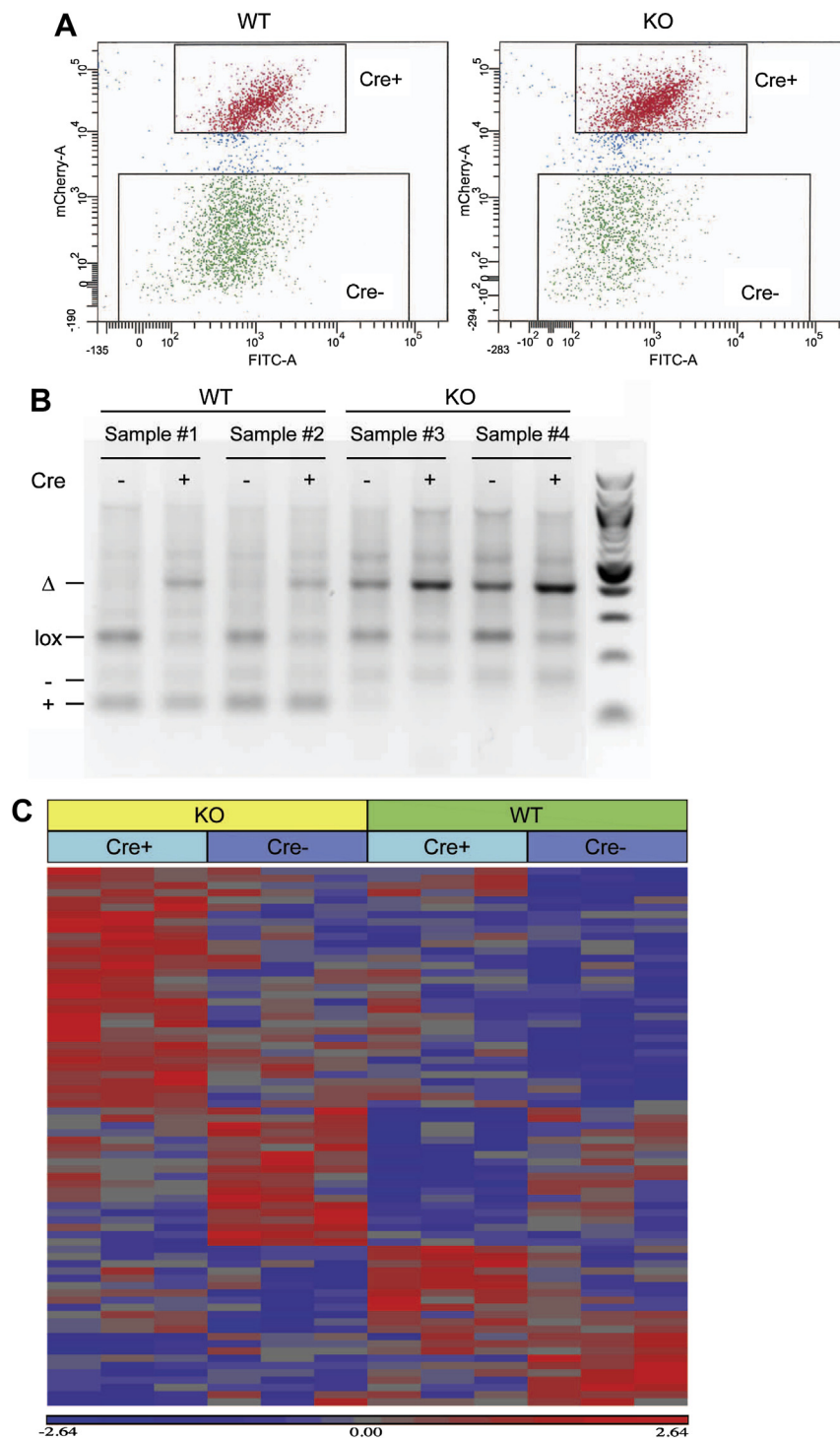
Figure 1: Glut4 neuron RNA profiling (A, J) Heatmap displaying the level of gene expression in different categories of neurotransmitter receptors (A) and ion channels (J) with control samples normalized to 1. The lighter color indicates higher gene expression, the darker color lower gene expression (*n* = 3). Individual gene expression for each subcategory of neurotransmitter receptors (B–I) and ion channels (K–N) was expressed as bar graphs (*n* = 3).

Figure 2: Sorting Glut4 neurons from WT and KO mice for gene expression analysis (A) Representative flow cytometry plots showing the different fluorescent cell populations collected in the hypothalami. (B) *Insr* genotyping in DNA extracted post-flow. We show two of the three independent datasets. (C) Heatmap displaying differentially expressed genes in non-Glut4 and Glut4 neurons sorted from WT and GIRKO hypothalami ($n = 3$, $p < 0.05$).

Does InsR ablation in Glut4 neurons affect their neurotransmitter profile? To answer this question, we queried the dataset for major categories of neurotransmitter receptors. Interestingly, GIRKO Glut4 neurons showed decreases in nearly all categories, as visualized by heatmaps (Figure 3A). We further analyzed the data by grouping major sub-classes of neurotransmitter receptors (Figure 3B—I). Even though most changes did not reach significance (Figure 3B—E, G—I), cholinergic receptors M4 and M5 were significantly reduced in InsR-deficient Glut4 neurons (Figure 3F). Of note, the predominant GABAergic receptor tended to be increased (Figure 3H). Expression of ion channels was reduced to different extents in InsR-deficient Glut4 neurons (Figure 3J). While most calcium, chloride, and sodium

Gene set description	# of markers	p-Value	Description
Cellular response to peptide hormone stimulus	91	0	KO down vs WT
Response to insulin stimulus	97	0	KO down vs WT
Anion homeostasis	36	0.0185	KO up vs WT
Rho guanyl-nucleotide exchange factor activity	52	0.020	KO down vs WT
Regulation of transcription initiation	15	0.020	KO down vs WT
Epidermal growth factor receptor binding	20	0.0208	KO down vs WT
Cellular protein complex assembly	152	0.0213	KO down vs WT
Regulation of cAMP metabolic process	95	0.0227	KO down vs WT

Table 2: Gene ontology analysis of RNA profiling data from WT and KO Glut4 neurons. Gene enrichment analysis showing the most significantly altered cellular pathways using RNA profiling data from flow-sorted WT and InsR-KO Glut4 neurons.

channels showed comparable or slightly reduced expression in InsR-deficient Glut4 neurons (Figure 3K—M), K-channel Kcnj10 was significantly reduced in InsR-deficient Glut4 neurons (Figure 3N).

Other top pathways with decreased expression in Glut4 neurons of GIRKO mice included guanyl-nucleotide exchange factor (GEF) activity, regulation of transcription initiation, cellular protein complex assembly, and regulation of cAMP metabolic processes. Conversely, anion homeostasis was the most significantly up-regulated gene set in GIRKO Glut4 neurons (Table 2).

3.3. Altered firing properties of InsR-deficient Glut4 neurons

The changes in anion homeostasis and neurotransmitter/ion channel expression would be expected to result in altered firing properties of Glut4 neurons. To test this hypothesis, we performed electrophysiological experiments (Figure 4A—D). We found that patch-clamped InsR-deficient Glut4 neurons had significantly reduced firing frequency (Figure 4C and D), despite similar resting membrane potential, and action potential amplitude compared with WT neurons (Figure 4A and B).

4. DISCUSSION

The key findings of our work are that hypothalamic Glut4 neurons are characterized by their enrichment in genes required for olfacto-sensory and metabolic processes, as well as the muscarinic cholinergic receptor M2. Moreover, ablation of InsR results in loss of insulin signal transduction properties, and decreased K_{ATP}-channel activity, as revealed also in patch-clamp experiments. The data are consistent with a primary metabolic coupling function of these neurons and their insulin responsiveness.

Although we found general enrichment of neurotransmitter receptors and ion channels in Glut4 neurons, we did not observe enrichment of particular sub-classes. This indicates that hypothalamic Glut4 neurons are a heterogeneous group of neurons and likely have multiple metabolic functions. Indeed, the distribution of Glut4 neurons to multiple neuroanatomical sites in the brain, including cortex, hippocampus, and hypothalamus [7] suggests that they have diverse biological functions.

The findings are largely consistent with the hypothesis that the presence of Glut4 in these neurons is a hallmark of metabolic functions. We should emphasize that in our studies we didn't probe the function of Glut4 itself in this process, let alone whether it responds to insulin in ways similar to muscle and fat Glut4. The present findings begin to explain why ablation of InsR in Glut4-expressing tissues has much more profound consequences on glucose homeostasis than ablation of InsR limited to "canonical" insulin-responsive tissues (i.e. muscle and fat) [7,11].

The identification of olfacto-sensory pathways in Glut4 neurons suggests that one potential function of this neuronal population is to connect sensorial and metabolic aspects of energy balance. These findings are reminiscent of findings using retrograde mapping of leptin receptors in the hypothalamus, demonstrating that they project to neurons in the premammillary tract that receive olfactory inputs [12]. Moreover, intranasal delivery of insulin is known to affect olfactory behavior via Kv1.3 channels [13]. In this regard, it's worth noting that six of the top ten genes downregulated in InsR-deficient Glut4 neurons encode olfactory receptors (Supplemental Table 1). The Quebec Family Study has found a genetic association between a cluster of olfactory receptors on human chromosome 19 and eating behavior [14]. Our data provide a potential lead on how olfacto-sensory cues might become integrated in energy balance and peripheral metabolism.

While Glut4 neurons don't appear to be enriched in a specific subset of neurotransmitters, they show significantly increased levels of the M2 muscarinic acetylcholine receptor. This receptor subtype in the hippocampus has been implicated in short- and long-term synaptic potentiation as well as neuronal plasticity that affect behavior [15], but considerably less is known about its role in the hypothalamus [16]. Our data provide a rationale to interrogate its metabolic contributions more specifically in this anatomical location.

Our gene enrichment analysis indicates the presence in Glut4 neurons of a gene cluster commonly associated with adipocyte differentiation that includes Ppar-γ, Tcf7l2, Klf4, C/ebp-α and -β, Lipin, and several others. Interestingly, Ppar-γ activation in the CNS has been linked to decreased insulin sensitivity [17]. Similarly Tcf7l2, a diabetes susceptibility gene [18] that had been originally linked to β-cell function, is expressed at high levels in the midbrain [19]— consistent with the present data — and has been found to regulate glucagon gene expression [20].

The transcriptome analysis of InsR-deficient hypothalamic Glut4 neurons supports key conclusions of our study and is consistent with an important role of these neurons in CNS insulin action. We have listed the top 100 most down- or up-regulated genes in InsR-deficient hypothalamic Glut4 neurons here (Supplemental Tables 1 and 2). First, gene ontology analyses uniformly indicate a loss of insulin and peptide hormone signaling pathways, as well as cAMP and transcriptional responses. The only class of genes whose expression increases following InsR ablation is involved in anion homeostasis. Interestingly, increased plasma anion concentrations are commonly seen in insulin-deficient states (e.g., diabetic ketoacidosis), and are thought to reflect increased generation of ketone bodies as an alternative energy source for neurons, possibly via glial cells — although this hypothesis remains controversial [21]. The present findings are consistent with the idea that, in conditions of impaired insulin signaling, there is an increase in the neuron's ability to utilize anions, possibly as a survival mechanism when glucose utilization is impaired.

As mentioned above, several olfactory receptors are sensitive to InsR ablation. Interestingly, one the top downregulated genes in InsR-deficient Glut4 neurons encodes a ribosomal protein (rsg24), whose decreased expression in the CNS has been linked to type 2 diabetes [22]. The Crh receptor also made the list of top downregulated genes, suggesting altered CNS stress response with insulin resistance.

5. CONCLUSION

In sum, our data define hypothalamic Glut4 neurons as a cell population with characteristics of hormone responsiveness, and with the potential to link insulin signaling to olfacto-sensory signaling. By systematic analyses of Glut4 neurons in additional CNS areas, it should be possible to gradually piece together an integrate anatomic and functional map of insulin action in the CNS.
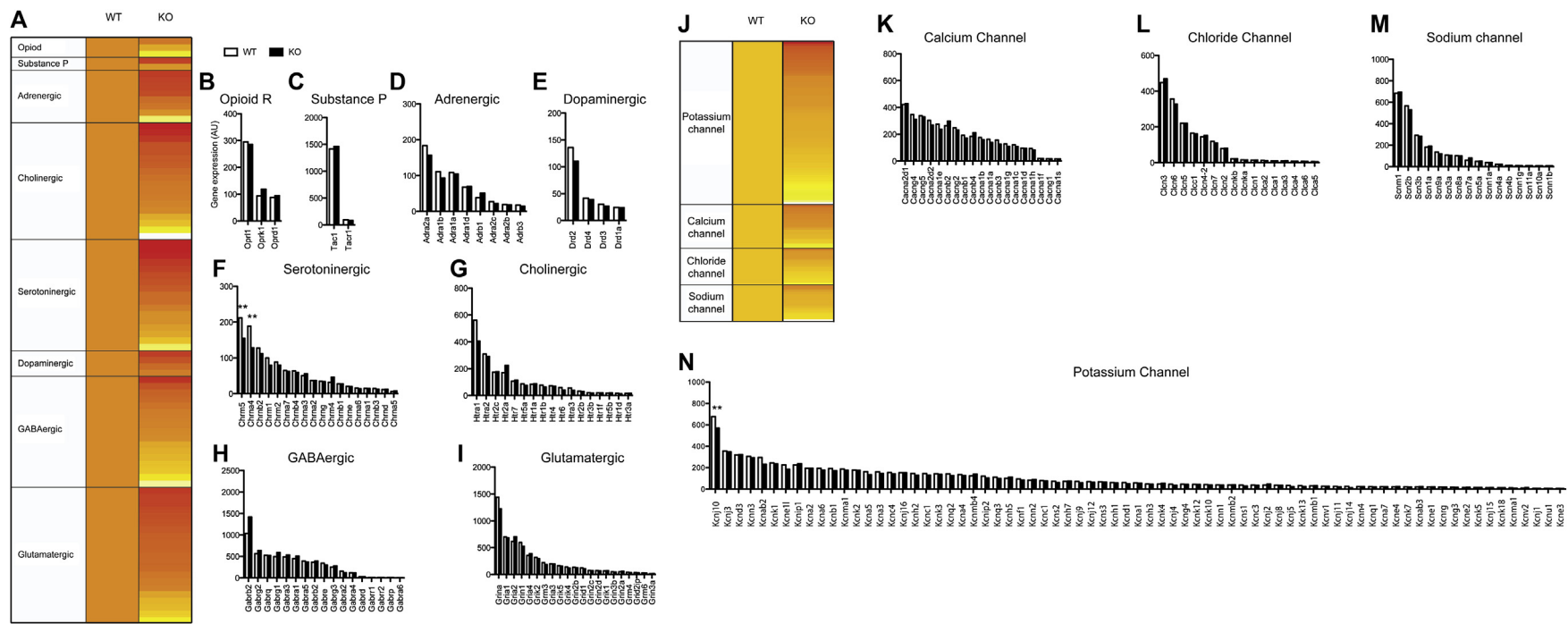
Figure 3: Glut4 neuron RNA profiling from WT and KO Glut4 neurons (A, J) Heatmap displaying the level of gene expression in different categories of neurotransmitter receptors (A) and ion channels (J) with control samples normalized to 1. The lighter color indicates higher gene expression, the darker color lower gene expression ($n = 3$). Individual gene expression for each subcategory of neurotransmitter receptors (B—I) and ion channels (K—N) was expressed as bar graphs ($n = 3$).
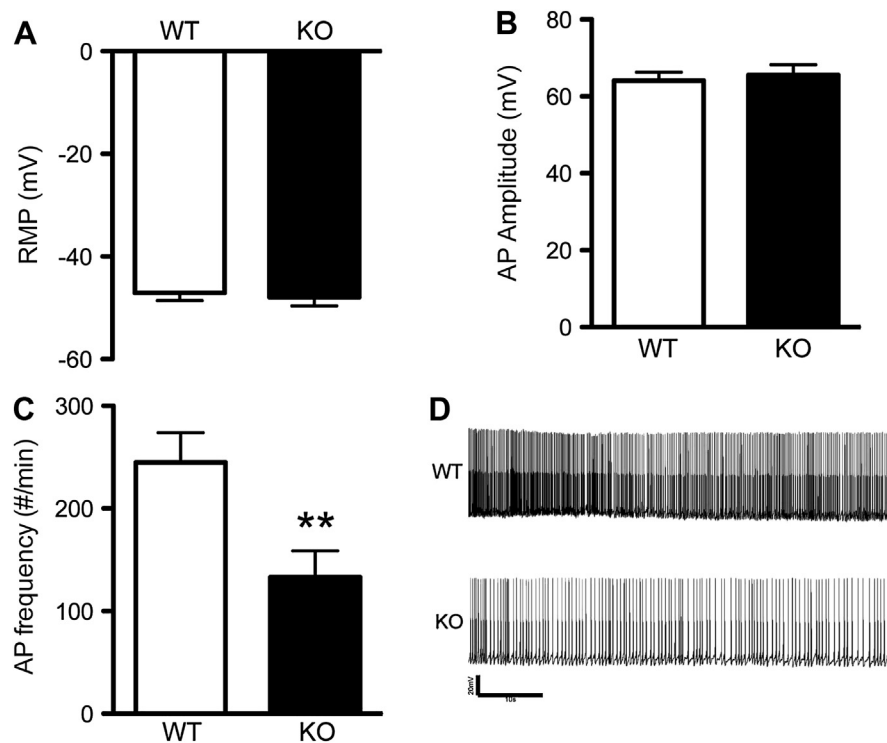
Figure 4: Electrophysiological characterization of Glut4 neurons from WT and KO mice (A—D) Electrophysiological properties of VMH Glut4 neurons. Patch-clamp recordings of the spontaneous firings of VMH Glut4 neurons in WT and GIRKO mice. (A) Resting membrane potential (RMP). (B) Amplitude of action potential. (C) Firing frequency. (D) Representative traces. Data show means ± SEM ($n = 19$—24) (** = $p < 0.01$).

AUTHORS' CONTRIBUTIONS

H.R. designed and conducted experiments, analyzed data, and wrote the article; B.Z. analyzed data; S.Y. and T.Y.L conducted experiments; O.A. analyzed data; D.A. analyzed data and wrote the article.

CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.molmet.2014.04.006.

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