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.

Keywords Insulin signaling; CNS; Glut4 neurons; Neurotransmitter receptor; Ion channel

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

Gl(Rosa)26Sor-InsRlox/ytomRosa-Tomato mice were from the Jackson Laboratories. GIRK0 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 transgenics (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-thick coronal brain sections in cold sucrose-based cutting solution containing (in mM): 195 sucrose, 10 NaCl, 25 NaHCO₃, 25 glucose, 2.5 KCl, 1.25 Na₂HPO₄, 2 Na pyruvate, 0.5 CaCl₂, and 7 MgCl₂ (pH 7.3)
using a vibratome (Leica VT1000S). Slices were incubated in a submerged 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 NaH2PO4, 25 NaHCO3, 2 CaCl2, 1 MgCl2, 25 glucose (pH 7.3) and continuously bubbled with 95% O2 and 5% CO2. 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, 0.06 CaCl2, 0.6 EGTA, 2 MgATP, 0.2 Na2GTP, and 20 Phosphocreatine. We adjusted osmolality 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 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 Glut4 neurons).

Table 1: Gene ontology and pathway analysis of Glut4 neurons.

Table 1 shows the top categories in each group of gene ontology analyses performed using RNA profiling data from flow-sorted Glut4 neurons.
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).
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...
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<sub>STR</sub>-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 Chr 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 integrative anatomic and functional map of insulin action in the CNS.

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.

| Gene set description                                      | # of markers | p-Value Description               | Description                  |
|----------------------------------------------------------|--------------|----------------------------------|------------------------------|
| Cellular response to peptide hormone stimulus            | 91           | 0.0185                           | KO down vs WT                |
| Response to insulin stimulus                             | 97           | 0.0180                           | KO down vs WT                |
| Anion homeostasis                                        | 36           | 0.0180                           | KO up vs WT                  |
| Rho guanyl-nucleotide exchange factor activity           | 52           | 0.0200                           | KO down vs WT                |
| Regulation of transcription initiation                    | 15           | 0.0200                           | KO down vs WT                |
| Epidermal growth factor receptor binding                 | 20           | 0.0200                           | 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                |
Figure 3: Glut4 neuron RNA trafficking from WT and KO Glut4 neurons. (A) 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-L) and ion channels (n = 3) was expressed as bar graphs. The gray scale indicates the degree of gene expression.
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.

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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.

REFERENCES

[1] Woods, S.C., Lotter, E.C., McKay, L.D., Porte Jr., D., 1979. Chronic intracerebroventricular infusion of insulin reduces food intake and body weight of baboons. Nature 282:503–505.
[2] Sherwin, R.S., 2008. Bringing light to the dark side of insulin: a journey across the blood-brain barrier. Diabetes 57:2259–2268.
[3] Hill, J.W., Elias, C.F., Fukuda, M., Williams, K.W., Berglund, E.D., Holland, W.L., et al., 2010. Direct insulin and leptin action on pro-opiomelanocortin neurons is required for normal glucose homeostasis and fertility. Cell Metabolism 11:286–297.
[4] Gautron, L., Elmquist, J.K., 2011. Sixteen years and counting: an update on leptin in energy balance. The Journal of Clinical Investigation 121:2087–2093.
[5] Havrankova, J., Roth, J., Brownstein, M., 1978. Insulin receptors are widely distributed in the central nervous system of the rat. Nature 272:827–829.
[6] Koch, L., Wunderlich, F.T., Seibler, J., Konner, A.C., Hampel, B., Irlenbusch, S., et al., 2008. Central insulin action regulates peripheral glucose and fat metabolism in mice. The Journal of Clinical Investigation 118:2132–2147.
[7] Lin, H.V., Ren, H., Samuel, V.T., Lee, H.Y., Lu, T.Y., Shulman, G.L., et al., 2011. Diabetes in mice with selective impairment of insulin action in GLUT4-expressing tissues. Diabetes 60:700–709.
[8] Accili, D., 2004. Lilly lecture 2003: the struggle for mastery in insulin action: from triumvirate to republic. Diabetes 53:1633–1642.
[9] Grayson, B.E., Seeley, R.J., Sandoval, D.A., 2013. Wired on sugar: the role of the CNS in the regulation of glucose homeostasis. Nature Reviews. Neuroscience 14:24–37.
[10] Ren, H., Orozco, I.J., Su, Y., Suyama, S., Gutierrez-Juarez, R., Horvath, T.L., et al., 2012. Foxo1 target Gpr17 activates AgRP neurons to regulate food intake. Cell 149:1314–1326.
[11] Lauro, D., Kidd, Y., Castle, A.L., Zarnowski, M.J., Hayashi, H., Ebina, Y., et al., 1998. Impaired glucose tolerance in mice with a targeted impairment of insulin action in muscle and adipose tissue. Nature Genetics 20:294–298.
[12] Leshan, R.L., Louis, G.W., Jo, Y.H., Rhodes, C.J., Munzberg, H., Myers Jr., M.G., 2009. Direct innervation of GnRH neurons by metabolic- and sexual odorant-sensing leptin receptor neurons in the hypothalamic ventromedial nucleus. Journal of Neuroscience 29:3138–3147.
[13] Marks, D.R., Tucker, K., Cavallin, M.A., Maet, T.G., Fadool, D.A., 2009. Awake intranasal insulin delivery modifies protein complexes and alters memory, anxiety, and olfactory behaviors. Journal of Neuroscience 29:6734–6751.
[14] Choquette, A.C., Bouchard, L., Drapeau, V., Lemieux, S., Tremblay, A., Bouchard, C., et al., 2012. Association between olfactory receptor genes, eating behavior traits and adiposity: results from the Quebec Family Study. Physiology & Behavior 105:772–776.

[15] Seeger, T., Fedorova, I., Zheng, F., Miyakawa, T., Koustova, E., Gomez, J., et al., 2004. M2 muscarinic acetylcholine receptor knock-out mice show deficits in behavioral flexibility, working memory, and hippocampal plasticity. Journal of Neuroscience 24:10117–10127.

[16] Gautam, D., Duttaroy, A., Cui, Y., Han, S.J., Deng, C., Seeger, T., et al., 2006. M1-M3 muscarinic acetylcholine receptor-deficient mice: novel phenotypes. Journal of Molecular Neuroscience: MN 30:157–160.

[17] Lu, M., Sarraf, D.A., Talukdar, S., Sharma, S., Li, P., Bandypadhyay, G., et al., 2011. Brain PPAR-gamma promotes obesity and is required for the insulin-sensitizing effect of thiazolidinediones. Nature Medicine 17:618–622.

[18] Grant, S.F., Thorleifsson, G., Reynisdottir, I., Benediktsson, R., Mangelescu, A., Sainz, J., et al., 2006. Variant of transcription factor 7-like 2 (TCF7L2) gene confers risk of type 2 diabetes. Nature Genetics 38:320–323.

[19] Nagalski, A., Irimia, M., Szewczyk, L., Ferran, J.L., Misztal, K., Kuznicki, J., et al., 2013. Postnatal isoform switch and protein localization of LEF1 and TCF7L2 transcription factors in cortical, thalamic, and mesencephalic regions of the adult mouse brain. Brain Structure & Function 218:1531–1549.

[20] Shao, W., Wang, D., Chang, Y.T., Ip, W., Zhu, L., Xu, F., et al., 2013. The Wnt signaling pathway effector TCF7L2 controls gut and brain proglucagon gene expression and glucose homeostasis. Diabetes 62:789–800.

[21] Belanger, M., Allaman, I., Magistretti, P.J., 2011. Brain energy metabolism: focus on astrocyte-neuron metabolic cooperation. Cell Metabolism 14:724–738.

[22] Mirza, Z., Kamal, M.A., Abuzeinahad, A.M., Al-Qahtani, M.H., Karim, S., 2013. Establishing genomic/transcriptomic links between Alzheimer’s disease and type II diabetes mellitus by meta-analysis approach. CNS & Neurological Disorders Drug Targets 12:1–17.