JOURNAL OF PROTEOMICS 80 (2013) 236–249

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Network of brain protein level changes in glutaminase deficient fetal mice

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ARTICLE INFO

Article history:
Received 15 October 2012
Accepted 5 January 2013
Available online 30 January 2013

Keywords:
Glutaminase
Gls
Schizophrenia
Protein network
LC–MS/MS

ABSTRACT

Glutaminase is a multifunctional enzyme encoded by gene Gls involved in energy metabolism, ammonia trafficking and regeneration of neurotransmitter glutamate. To address the proteomic basis for the neurophenotypes of glutaminase-deficient mice, brain proteins from late gestation wild type, Gls+/– and Gls–/– male mice were subjected to two-dimensional gel electrophoresis, with subsequent identification by mass spectrometry using nano-LC-ESI–MS/MS. Protein spots that showed differential genotypic variation were quantified by immunoblotting. Differentially expressed proteins unambiguously identified by MS/MS included neurocalcin delta, retinol binding protein-1, reticulocalbin-3, cytoskeleton proteins fascin and tropomyosin alpha-4-chain, dihydropyrimidinase-related protein-5, apolipoprotein IV and proteins from protein metabolism proteasome subunits alpha type 2, type 7, heterogeneous nuclear ribonucleoprotein C1/C2 and H, voltage-gated anion-selective channel proteins 1 and 2, ATP synthase subunit β and transitional endoplasmic reticulum ATPase. An interaction network determined by Ingenuity Pathway Analysis revealed a link between glutaminase and calcium, Akt and retinol signaling, cytoskeletal elements, ATPases, ion channels, protein synthesis and the proteasome system, intermediary, nucleic acid and lipid metabolism, huntingtin, guidance cues, transforming growth factor beta-1 and hepatocyte nuclear factor 4-alpha. The network identified involves (a) cellular assembly and organization and (b) cell signaling and cell cycle, suggesting that Gls is crucial for neuronal maturation.

1. Introduction

In the brain, the kidney-type glutaminase (KGA; EC 3.5.1.2) is a crucial neuronal enzyme that deamidates glutamine (Gln) to stoichiometric amounts of glutamate (Glu) and ammonia [1,2]. KGA is a heterotetrameric enzyme consisting of three 66 kDa subunits and one 68 kDa subunit in the inner membrane of mitochondria [3,4]. The catalytic rate of KGA is regulated by neuronal activity, dependent on phosphate for activation, and is strongly inhibited by its reaction products, Glu and ammonia. The predominant form of glutaminase in the brain is type 1 or brain-kidney type which is encoded by GLS on human

Abbreviations: ACN, Acetonitrile; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate; GABA, γ-aminobutyric acid; Gln, Glutamine; Glu, Glutamate; IPA, Ingenuity Pathway Analysis; KGA, Kidney-type glutaminase; LGA, Liver-type glutaminase; OGP, Octyl β-D-glucopyranoside; PMSF, phenylmethylsulphonyl fluoride; PVDF, polyvinylidene difluoride; SCZ, Schizophrenia; TBST, Tris-buffered saline and Tween 20.

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1874-3919/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.jprot.2013.01.013

chromosome 2q32–q34 [5,6] and mouse chromosome 1. Type 2 or liver-type glutaminase (LGA), which is encoded by GLS2 on human chromosome 12q13 and mouse chromosome 10, contributes only a small minority of glutaminase activity; it is found in nuclei suggesting a non-neurotransmitter role [7,8].

Glu is the predominant excitatory neurotransmitter in mammalian brain, a major source of cell energy, the precursor of γ-aminobutyric acid (GABA), and glutathione. After release into the synaptic cleft, Glu is taken up by adjacent astrocytes and converted to Gln by glutamine synthase. The majority of neurotransmitter Glu is recycled through this Gln–Glu shuttle between neurons and astrocytes [9–11]. While there has been a general acceptance of the Gln–Glu cycle as a major source of neurotransmitter glutamate [12], more recent findings have questioned this [13,14]. Indeed, three Gln-independent cycles for Glu trafficking involving tricarboxylic acid cycle intermediates have been identified [15].

Mice with heterozygous reductions in Gls show similar reductions in KGA [16] and elevations in Gln (the glutaminase substrate), and a global reduction in Glu/Gln ratios, showing that genetic compromise of Gls yields a neurochemical phenotype likely to impact Glu neurotransmission [17]. Both spontaneous and evoked synaptic inputs are reduced in the hippocampus, but not in the anterior cingulate cortex. This regional difference is consistent with the heterogeneity of systems that maintain Glu synaptic homeostasis, including neuronal Glu re-uptake [18] and anapleurosis from Gln via the tricarboxylic acid cycle [15].

Altered Gluergic neurotransmission is central to a wide range of neuropsychiatric conditions. High concentrations of extracellular Glu lead to excitotoxicity, neuronal damage by prolonged activation of Glu receptors in stroke and a range of other neurodegenerative disorders [19–21]. Release of KGA from dying neurons can extend the excitotoxic cascade, which has suggested that KGA inhibitors may reduce stroke size [22]. Alterations in the cortical Gluergic and GABAergic signal transduction are involved in depression [23] and increased KGA expression and activity has been found in postmortem brains of patients with schizophrenia (SCZ) [24,25], consistent with the dysregulation of Gluergic neurotransmission recognized in SCZ [26].

Gls null mice (Gls−/−) die shortly after birth [16]. Prior to their demise, Gls null mice show disorganized behavior, making it impossible for them to suckle, and they fail to ingest milk; however, dropper feeding them milk does not avert their demise. Thus, they appear to have a broader deficit in glutamatergic synaptic transmission accounting for their inability to coordinate their behavior. In Gls null neuronal cultures, baseline excitatory synaptic activity is unaffected while evoked excitatory synaptic responses are exhausted more rapidly. While other pathways produce sufficient Glu for baseline Gluergic transmission [15], Gls appears to be essential for maintaining the normal function of active synapses. Thus, Gluergic synapses lacking KGA show an activity-dependent deficit, presumably accounting for altered rhythmic neuronal activity affecting both breathing and coordinated motor behavior.

Consistent with the role of Gln as a major energy source [5], Gls−/− mice are born about 10% smaller than their wild type (WT) littermates [16], suggesting that they are at a metabolic disadvantage. In contrast, Gls heterozygous (het) mice (Gls+/−) are normal sized and display neither behavioral abnormalities nor SCZ-associated phenotypes; rather, the mice show a SCZ resilient phenotype [17]. Gls hets show diminished amphetamine-induced behavioral stimulation and striatal dopamine release, two animal correlates of positive symptoms in schizophrenia (SCZ). In contrast to patients with SCZ, Gls hets showed diminished ketamine-induced frontal cortex activation. They show enhanced latent inhibition, a behavioral measure typically diminished in SCZ and enhanced by antipsychotic drugs. Most strikingly, the mice show a focal hippocampal hypoactivity on brain imaging that is the inverse of the hyperactivity seen in patients with SCZ [27].

These results raise the question as to whether the SCZ resilient phenotype arises because of the constitutive reduction in KGA expression throughout life or reduced KGA activity in adulthood. More broadly, many transgenic studies assume that heterozygous mice do not differ from WT mice because of functional reserve. To address these issues, we examined the molecular consequences of Gls knockdown and knockout at the proteomic level during fetal development. This revealed a network of KGA-dependent proteins and their interaction partners that showed differential genotypic variation.

2. Material and methods

2.1. Animals

Procedures involving mice and their care were performed under protocols approved by the Institutional Animal Care and Use Committees of Columbia University and New York State Psychiatric Institute, following the guidelines of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Gls het (Gls+/−) mice with one copy of a floxed PGK*neo*-Stop cassette (stopGls allele) inserted ahead of the transcription initiation site in exon 1 of the *Gls* gene (Entrez Gene 14660) were kept on a 129SvEv/J background [16] and bred to yield WT (Gls+/+), heterozygous (Gls+/−) and null (Gls−/−) fetuses. At about 17–21 days gestation, dams were anesthetized with ketamine/xylazine and fetuses harvested to ice chips. Brains were rapidly extracted, flash frozen by immersion in isopentane on dry ice, and stored at −80 °C until analysis. Tail samples were sent to Transnetyx (Cordova, TN, USA) for automated genotyping for Gls WT and stopGls alleles and a y-chromosome maker for sex determination. A total of 10 dams, dissected on 7 separate dates spanning 8 months, provided the 30 male fetuses used.

2.2. Sample preparation for two-dimensional gel electrophoresis (2DE)

Whole brains were homogenized and suspended in 1.2 mL sample buffer (20 mM Tris, 7 M urea, 2 M thiourea, 4% w/v CHAPS, 10 mM 1,4-dithioerythritol, 1 mM EDTA, 1 mM PMSF, 1 tablet Complete™ from Roche Diagnostics (Graz, Austria), and 0.2% v/v phosphatase inhibitor cocktail from Calbiochem (Darmstadt, Germany)). The suspension was sonicated on ice for approximately 30 s and centrifuged at 15,000 ×*g* for 120 min at 4 °C. Desalting was carried out with an Ultrafree-4 centrifugal filter unit with a cut off molecular weight of 10 kDa (Millipore, Wien, Austria) at 3000 ×*g* at 4 °C until the eluted volume was