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ORIGINAL PAPER

Development of Mice with Brain-Specific Deletion of Floxed *Glud1* (Glutamate Dehydrogenase 1) Using Cre Recombinase Driven by the Nestin Promoter

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Abstract In the brain, *Glud1*-encoded glutamate dehydrogenase plays a major role in the recycling of the neurotransmitter glutamate. We recently reported a new model of brain-specific *Glud1* null mice (Cns-*Glud1*$^{-/-}$) lacking glutamate dehydrogenase in the central nervous system. Cns-*Glud1*$^{-/-}$ mice exhibit reduced astrocytic glutamate breakdown and redirection of glutamate pathways without altering synaptic transmission. Cns-*Glud1*$^{-/-}$ mice were generated using LoxP and Nestin-Cre technology. Nestin-Cre mice are widely used to investigate gene deletion in the central nervous system. However, the *Nes-Cre* transgene itself was reported to induce a phenotype related to body weight gain. Here, we review the potential side-effects of *Nes-Cre* and analysed Cns-*Glud1*$^{-/-}$ body weight gain. Overall, Nestin-Cre mice may exhibit transient and moderate growth retardation during the few weeks immediately following weaning. Pending appropriate controls and homogenization of the genetic background, Nestin-Cre technology is a valuable tool enabling disruption of genes of interest in the central nervous system.

Keywords Glutamate dehydrogenase · Glud1 · Brain · Nestin-Cre

Glucose is the major source of energy delivered to the central nervous system (CNS) by peripheral organs. However, within the brain, oxidative catabolism of the neurotransmitter glutamate might significantly contribute to ATP production and maintenance of energy homeostasis. Upon glutamate transmission, intersynaptic glutamate clearance is achieved mostly by astrocytes [1]. Active astrocytic internalization of glutamate also ensures detoxification of the extracellular space. However, altered glutamate handling is associated with various neurodegenerative diseases; such as Parkinson's disease, epilepsy, schizophrenia, and Alzheimer's disease [2]. Following its uptake by astrocytes glutamate may be amidated to glutamine and then recycled back to glutamate once transferred to neurons (Fig. 1a). Alternatively, astrocytic glutamate may be deaminated to α-ketoglutarate within mitochondria. This pathway is catalysed either by transaminases or by glutamate dehydrogenase (GDH) before oxidative catabolism in the TCA cycle, thereby promoting ATP generation [3]. In the CNS, GDH is predominantly expressed in astrocytes and to lower levels in neurons [4, 5]. GDH catalyses either the anabolic amination of α-ketoglutarate to glutamate or the catabolic oxidation of glutamate [3]. The enzyme forms homohexamers in mitochondria and is encoded by *Glud1* in all vertebrates [6]. Additionally, a second isoform is found in humans [7], being expressed mostly in astrocytes and testicular supporting cells [8].

We recently reported a new model of brain-specific *Glud1* null mice (Cns-*Glud1*$^{-/-}$) lacking GDH in the CNS [9]. In astrocytes lacking GDH, oxidative catabolism of glutamate to CO_2 is reduced, indicating lower astrocytic glutamate breakdown. However, brain-targeted NMR measurements have shown that Cns-*Glud1*$^{-/-}$ glutamate levels are maintained to control values, while glutamine concentrations are increased in Cns-*Glud1*$^{-/-}$ brains [9]. This modified pattern of glutamate/glutamine ratio does not induce over-excitability or hypo-excitability. These data

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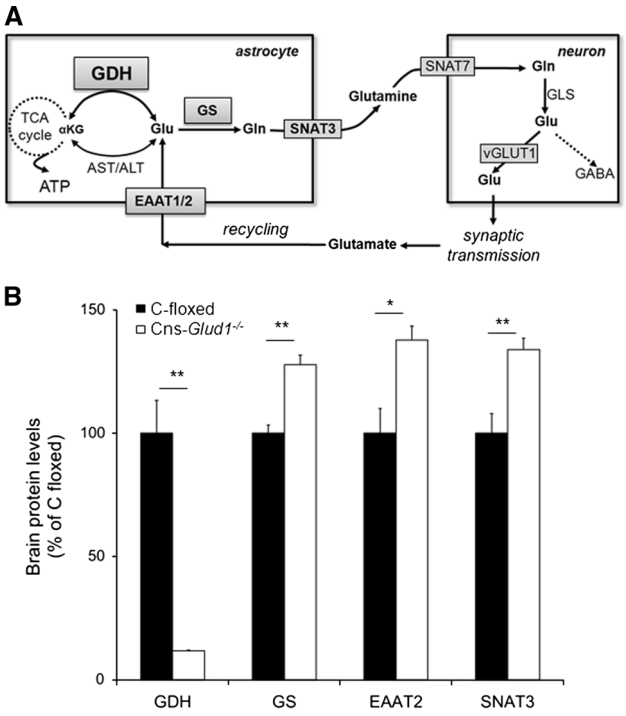

Fig. 1 Abrogation of GDH in brain affects glutamate handling. **a** Simplified representation of glutamate fates in astrocytes and neurons (αKG, α-ketoglutarate; ALT, alanine aminotransferase; AST, aspartate aminotransferase; EAAT 1/2, excitatory amino acid transporters 1/2; GDH, glutamate dehydrogenase; Gln, glutamine; GLS, glutaminase; Glu, glutamate; GS, glutamine synthetase; SNAT 3/7, sodium-coupled amino acid transporters 3/7; vGLUT1, vesicular glutamate transporter 1). **b** Protein levels analysed by immunoblotting [9] of enzymes/transporters related to glutamate pathway in brains from CNS-specific *Glud1* knockout (Cns-*Glud1*$^{-/-}$) and *Glud1*$^{lox/lox}$ floxed control (C-floxed) mice. Actin (for GDH) and ezerin (for GS, EAAT2 and SNAT3) served as loading control. Values are mean ± SE, $n = 2$–5, * $p < 0.05$ and ** $p < 0.01$ using the Student's *t* test

suggest preservation of glutamate homeostasis in GDH-null brains, at the expense of an increase in glutamine formation. Redirection of glutamate pathways in Cns-*Glud1*$^{-/-}$ brains is apparently favoured by up-regulation of astrocyte-type glutamate and glutamine transporters and of glutamine synthetase (Fig. 1b and [9]). The Cns-*Glud1*$^{-/-}$ mouse model represents a unique tool to explore the importance of GDH as a key enzyme connecting glucose and glutamate metabolism within the CNS. Furthermore, given the dys-regulation of glutamate in neurodegenerative diseases, GDH appears as a potent target for management of related neurological disorders.

We generated the Cns-*Glud1*$^{-/-}$ brain-specific GDH knockout mice by crossing *Glud1* floxed (*Glud1*$^{lox/lox}$) mice [10] with animals expressing the *Cre* recombinase under the control of Nestin *cis*-regulatory (*Nes-Cre*) sequence [11]. Heterozygous Cns-*Glud1*$^{+/-}$ were then crossed with *Glud1*$^{lox/lox}$ mice to obtain homozygous Cns-*Glud1*$^{-/-}$, while *Cre* was kept heterozygous [9]. In this specific study [9], animals were maintained on a mixed genetic background (C57BL/6 J × 129/Sv) to avoid inbred strain-specific phenotypes. Accordingly, floxed *Glud1*$^{lox/lox}$ littermates of Cns-*Glud1*$^{-/-}$ knockouts were used as control animals to optimize standardization of the genetic background between the two groups [9]. In other words, only Cns-*Glud1*$^{-/-}$ knockout mice carried the *Nes-Cre* transgene. No gender differences were noticed.

The Cre transgenic lines represent invaluable tools to explore the role of a given protein in a specific tissue (conditional knockout), that can additionally be time-specific (inducible Cre systems). Nevertheless, one should consider appropriate controls according to potential Cre-mediated effects. Some studies have reported side effects or phenotypes associated with Cre activity or transgene insertion [12–14]. For instance, the cause of haematological abnormalities after the systemic activation of CreER^{T2} described by Higashi et al. [15] was proposed to be the consequence of Cre-mediated genomic rearrangements. Such rearrangement within the mouse genome might happen at pseudo-*loxP* sites, which have been shown to serve as substrates for Cre recombinase [15–17]. Some of the unexpected phenotypes associated with the presence of the *Cre* transgene might be due to the disruption of the genome loci where the transgene was integrated.

Another potential confounding effect of Cre technology is the misplaced recombination of the floxed target-gene. This is illustrated by some mouse models used to study insulin-secreting cells. In order to target Cre-mediated recombination of floxed genes specifically in pancreatic beta-cells, Rip-Cre mice have been generated. These animals express the Cre recombinase under the control of the rat insulin (*Ins2*) promoter (*Rip-Cre*). However, infidelity of *Rip-Cre* transgene in the hypothalamus has been noticed in one transgenic line (*Tg(Ins2-cre)*^{25Mgn}) [18], although not particularly in another one (*Tg(Ins2-cre)*^{1Herr}) [19], potentially altering gene expression in nutrient-sensing neurons [20]. Such undesired hypothalamic effect might render the transgenic animals more likely to develop glucose intolerance under specific genetic backgrounds. Indeed, it was shown that perturbations in glucose homeostasis observed in a *Tg(Ins2-cre)*^{25Mgn} transgenic line [18] can be accounted for by differences in genetic background [21]. Therefore, misplaced recombination combined with C57BL/6 J background might influence metabolic homeostasis and potentially contribute to a phenotype not fully specific for the beta-cell.

Some studies may have attributed roles to a target-gene, which were in fact partly or fully due to Cre toxicity. Limitation of Cre technology should not hide other possible pitfalls, potentially more disturbing. Indeed, Cre-mediated effects do not rule out alternative major confounding factors, primarily the genetic background, as discussed

above. Whenever possible, the genetic background should be homogenized between the studied groups [22], similarly to human studies based on twins [23].

Nestin-Cre mice are widely used to investigate gene deletion and cell function in the CNS. However, the *Nes-Cre* transgene itself was reported to induce a phenotype related to body weight gain. Indeed, it was observed that Nestin-Cre mice have smaller body weights [24], an effect not observed by others [25]. These apparently conflicting results have not been particularly highlighted to the scientific community, as they appeared as Supplementary Materials [24] and Addendum [25] to their respective papers. Maintained on a pure C57BL/6J background, Nestin-Cre mice purchased from the Jackson Laboratory (Origin, R. Klein, EMBL) display mild hypopituitarism [26]. Discrepancies between laboratories regarding body growth suggest that *Nes-Cre* transgene may render animals more susceptible to factors such as the genetic background or subtle environment specificities. This might have physiological consequences, in particular when *Nes-Cre* transgene is associated with the deletion of genes implicated in metabolic control. The widespread use of Nestin-Cre mice has led to an increasing awareness of potential strain-dependent differences in body weight of these mice.

Given these strain differences, we further evaluated the Cns-*Glud1*$^{-/-}$ mice by comparing these conditional knockout animals with both Nestin-Cre controls carrying the *Nes-Cre* transgene (C-Cre) and *Glud1*-floxed controls lacking Nes-Cre but having *loxP* sites in the *Glud1* gene (C-floxed). Immunobloting analyses showed comparable GDH expression in C-Cre and C-floxed brains as well as in non-excitable peripheral tissues known to express GDH (Fig. 2a). Brain GDH enzymatic activity was not significantly different between the two transgenic control groups, *i.e.* C-Cre and C-floxed mice (Fig. 2b). On the contrary, in brain homogenates of Cns-*Glud1*$^{-/-}$ mice we observed abrogation of GDH activity, both in the oxidative deamination and in the reductive amination directions. Wild type control (C-wt) mice exhibited brain GDH activity

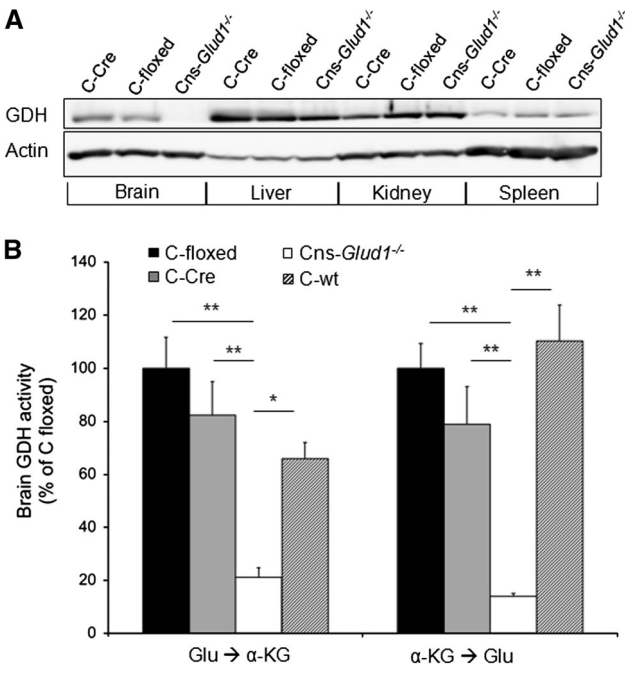

Fig. 2 Expression and activity of GDH in Cns-*Glud1*$^{-/-}$ mice. **a** Brain-specific GDH deletion in Cns-*Glud1*$^{-/-}$ mice was assessed by immunoblotting. GDH expression in brains was compared with non-excitable tissues collected from *Glud1*$^{lox/lox}$ floxed control (C-floxed), *Nes-Cre* control (C-Cre) and CNS-specific *Glud1* knockout (Cns-*Glud1*$^{-/-}$) mice. Actin served as loading control. Immunoblots are representative of 3 independent preparations. **b** Brain GDH enzymatic activity of C-floxed, C-Cre, Cns-*Glud1*$^{-/-}$, and wild type control (C-wt) mice measured in both oxidative and reductive directions [10], using glutamate (Glu) and α-ketoglutarate (α-KG) as substrates, respectively. Values are mean ± SE, $n = 5$–7, * $p < 0.05$ and ** $p < 0.01$ using one-way ANOVA

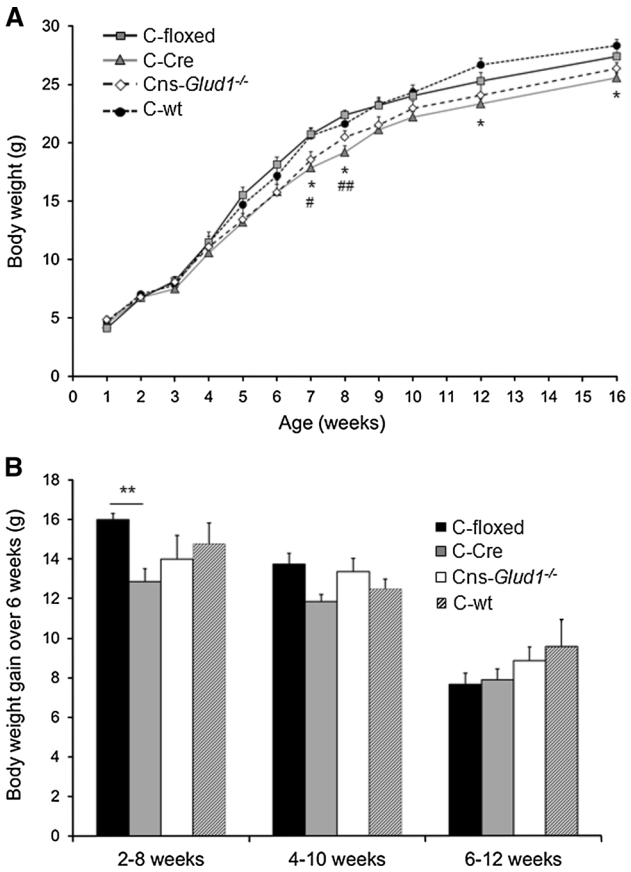

Fig. 3 Effects of *Nes-Cre* and Cns-*Glud1*$^{-/-}$ on body weight. **a** Body weights from *Glud1*$^{lox/lox}$ floxed control (C-floxed), *Nes-Cre* control (C-Cre), CNS-specific *Glud1* knockout (Cns-*Glud1*$^{-/-}$), and wild type control (C-wt) mice recorded over 16 weeks of life after birth. **b** Increases in body weight for periods of 6 weeks measured over 2–8, 4–10 and 6–12 weeks of age. Values are mean ± SE; $n = 7$–27; * $p < 0.05$, ** $p < 0.01$ C-Cre versus C-wt; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ C-Cre versus C-floxed, using one-way ANOVA

comparable to the transgenic controls C-Cre and C-floxed (Fig. 2b). Regarding body growth, we observed lower body weights associated with *Nes-Cre* transgene at the age of 7–8 weeks (Fig. 3a) contributed by reduced weight gain during the weeks immediately following weaning (Fig. 3b). The effect was transient and C-Cre animals could catch up body weight in comparison with the other transgenic groups by the age of 10 weeks when body growth stabilized. However, compared to wild type animals having more a C57BL/6J black 6 background, body weights remained slightly lower. Therefore, at the age of 10 weeks onwards, no significant differences in body weights were observed between transgenic mice maintained on a similar mixed genetic background, carrying or not *Nes-Cre* transgene; *i.e.* either C-Cre or Cns-*Glud1*$^{-/-}$ versus *Nes-Cre*-free C-floxed mice. According to these results, we favoured analyses of Cns-*Glud1*$^{-/-}$ mice at the age of 10 weeks onwards [9].

In conclusion, use of Nestin-Cre mice requires special attention regarding interpretation of body weight gain, in particular during the few weeks immediately following weaning. Pending appropriate controls and homogenization of the genetic background, Nestin-Cre technology is a valuable tool enabling disruption of genes of interest in the CNS. For instance, Cns-*Glud1*$^{-/-}$ mice lacking GDH in the CNS uncovered the fine tuning and compartmentalization of glutamate-glutamine handling in the brain.

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