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Chronic riluzole treatment increases glucose metabolism in rat prefrontal cortex and hippocampus

Golam M. I. Chowdhury[1,*], **Mounira Banasr**[2], **Douglas L. Rothman**[1], **Kevin L. Behar**[2], and **Gerard Sanacora**[2]

[1]Magnetic Resonance Research Center, Department of Diagnostic Radiology, Yale University School of Medicine, New Haven, Connecticut 06520, USA

[2]Department of Psychiatry, Yale University School of Medicine, New Haven, Connecticut 06520, USA

Abstract

Riluzole is believed to modulate glutamatergic function by reducing glutamate release and facilitating astroglial uptake. We measured the incorporation of ^{13}C during a 10 min infusion of [1-^{13}C]glucose into metabolites in prefrontal-cortex and hippocampus of urethane-anesthetized rats treated with riluzole (21d, 4mg/kg, i.p. daily) or saline. Total and ^{13}C-concentrations of metabolites were determined in extracts using ^{1}H-[^{13}C]NMR. In prefrontal-cortex (*P<0.05*) and hippocampus (*P<0.01*) riluzole increased ^{13}C-labeling over saline in **to** glutamate-C4 (112% and 130%), GABA-C2 (142% and 171%) and glutamine-C4 (118% and 233%) without affecting total metabolite levels (*P>0.2*). Our findings indicate that chronic riluzole enhances brain **glutamate labeling from [1-^{13}C]glucose**.

Keywords

glutamate; GABA; glutamine; TCA cycle; riluzole; nuclear magnetic resonance

Introduction

Glutamate (Glu) and GABA are the major excitatory and inhibitory neurotransmitters in the brain. These amino acid neurotransmitters play vital roles in the regulation of several important CNS processes, and have been linked to the pathogenesis and pathophysiology of several neurodegenerative disorders. Riluzole (2-amino-6-trifluoromethoxy benzothiazole), a neuroprotective agent with anti-glutamatergic actions, is effective in prolonging the median survival in patients with amyotrophic lateral sclerosis (ALS) (Miller *et al*, 2007). Although riluzole's mechanism of action is not completely understood, it's anti-glutamatergic pharmacological properties are believed to include inhibition of glutamate release through inactivation of voltage-dependent ion channels (Huang *et al*, 1997; Urbani and Belluzzi, 2000), effects on the expression and signal **transduction** through glutamate receptors (Du *et al*, 2007), and facilitation of astrocytic glutamate uptake (Frizzo *et al*, 2004). Recent clinical studies also suggest riluzole possesses antidepressant and anxiolytic-like effects in patients with depression and anxiety disorders (Pittenger *et al*, 2008), thereby suggesting that riluzole has been show to have effects on regions involved in emotion regulation. To better understand the potential mechanism of riluzole's action, we sought to determine the drug's effects on

*Correspondence: Dr. Golam M.I. Chowdhury (golam.chowdhury@yale.edu) TAC-MRRC, N150, P.O. Box 208043, New Haven, CT 06520, USA. Tel.: 1-203-785-6199; Fax: 1-203-785- 6643..

neuronal glutamate metabolism and the glutamate/glutamine cycle in the prefrontal cortex (PFC) and hippocampus (HC).

Nuclear magnetic resonance (NMR) spectroscopy used in conjunction with ^{13}C labeled substrates can provide dynamic information on neuronal and glial metabolism and neuron-glial trafficking of glutamate and GABA in the glutamate/GABA-glutamine cycle (Chowdhury *et al*, 2007a). [1-^{13}C]Glucose is oxidized to a quantitatively greater extent in neurons than glia, labeling the large and predominately neuronal glutamate pool. ^{13}C labeled glutamate released from active neurons (if gutamatergic neuron, but in GABAergic neuron glutamate further converted in to GABA) is taken up and converted to glutamine in nearby astroglia (figure 1A). The rates of ^{13}C incorporation into amino acid pools of neurons (Glu and GABA) and astroglia (Gln) using [1-^{13}C]glucose with NMR detection *in vivo* or *ex vivo* can provide unique information on neuronal and glial oxidative metabolism and substrate trafficking **(Rothman *et al*, 2003; Hertz *et al*, 2007)**.

In this study we examined the effects of chronic (21d) riluzole treatment using ^{13}C labeling from [1-^{13}C]glucose infused intravenously on the metabolism of the major brain amino acids in PFC and HC of anesthetized rats. Our findings suggest that riluzole enhances overall glutamate metabolism.

Materials and Methods

All experiments were conducted under protocols approved by the Yale IACUC. Two groups of male Sprague-Dawley rats (300-350g) were studied: (1) saline-injected controls, n=5; (2) Riluzole 4mg/kg/day, i.p, Sigma, n=5). Saline and riluzole were administered once daily for 21 days (21d). Lower (e.g., 2mg/kg) or higher (e.g., 8mg/kg) doses of riluzole were applied earlier reports showing neuroprotective effects in several models of neurodegeneration diseases (strock or parkinson's disease). We chose 4mg/kg dose on the basis of behavioral responses and our previous studies, e.g., Risterucci et al, 2006, suggesting this dose produced targeted effects of interest.

Sucrose preference test: The SPT was performed on day 15 and day 35 and consisted in 48 hours exposition to a palatable sucrose solution (1%, Sigma), followed by 4h of water deprivation and a 1-hour exposure to two identical bottles, one filled with sucrose solution and the other with water. Sucrose and water consumption was determined by measuring the change in volume of fluid consumed. Sucrose preference was defined as the ratio of the volume of sucrose vs. water consumed during the 1-hour test.

One day after the last exposure to riluzole or saline, rats were anesthetized with urethane (1.5 g/kg, i.p.) and a tail vein cannulated. The core body temperature was monitored and maintained near 37°C with a heating pad connected to a temperature-regulated recirculating water bath. Thirty minutes after injection of urethane, a solution of [1-^{13}C]glucose (99 atom%; Cambridge Isotopes, Andover, MA, USA) dissolved in water (0.75 mol per 200g body wt. per min) was infused for 10 min (Chowdhury *et al*, 2007a). Immediately after the infusions rats were euthanized using directed microwave irradiation (5kW, Model TMW 6402C, Muromachi Microwave Fixation System) to the head arresting brain metabolism in <1s, allowing brain tissue removal from prefrontal cortex (PFC) and hippocampus (HC). Blood was sampled from the heart following microwave euthanasia, centrifuged, and the plasma removed. Blood plasma was frozen in liquid N_2 and stored at -80°C for subsequent analysis. Cortical and hippocampus tissue extracts were prepared as described previously (Chowdhury *et al*, 2007b) using ethanol and the supernatants removed and lyophilized. A known quantity of [2-^{13}C]glycine was added during tissue extraction as an internal concentration reference. Lyophilized samples were resuspended in D_2O and H_2O (90:10 v/v) for NMR analysis at 11.7 T (^1H resonance frequency

of 500.13 MHz) using a Bruker AVANCE spectrometer (Bruker Instruments). Fully relaxed ^{1}H-[^{13}C] NMR spectra were acquired as two subspectra-one involving broadband ^{13}C inversion pulses applied in alternate scan blocks, while ^{13}C-decoupling was applied in both. Subtraction of the scans obtained with ^{13}C inversion (^{12}C-^{13}C) from those without inversion (^{12}C+^{13}C) gave the difference spectrum (2x^{13}C), containing only ^{13}C coupled ^{1}H resonances at twice the true intensity. The total carbon isotope composition was given by the ^{12}C+^{13}C subspectrum. The ^{13}C atom percentage enrichment was calculated as the ratio, ^{13}C/(^{12}C + ^{13}C) × 100, followed by subtraction of 1.1% to remove ^{13}C arising from natural abundance. Absolute concentrations of metabolites were determined relative to **the** [2-^{13}C]glycine, added during tissue extraction. The isotopic ^{13}C enrichments of Glu-C4, GABA-C2 and Gln-C4, were calculated from the ratio of the areas of these resonances in the ^{1}H-[^{13}C] NMR difference spectrum (2x^{13}C only) and the non-edited spectrum (^{12}C+^{13}C). The concentration and ^{13}C isotopic enrichment of plasma [1-^{13}C]glucose (H1α 5.2 ppm) and total acetate concentration were determined using ^{1}H NMR without ^{13}C decoupling using a repetition time of 20s. Formate was added to the sample as a concentration standard. The isotopic ^{13}C enrichment of glucose-C1 (5.2 ppm) was calculated by dividing the areas of the ^{13}C satellites with the total area (^{12}C+^{13}C) of their respective resonances.

Statistical Analysis

The statistical significance of differences in metabolite concentrations and ^{13}C enrichments between control and riluzole-treated rats were assessed by two-tailed, Student's t-test. Differences in mean values were considered significant for $P<0.05$. All values are presented as mean ± standard deviation (SD).

Results

Concentration and ^{13}C enrichment of plasma glucose

The plasma concentrations and percentage ^{13}C enrichments at the end of the 10 min infusions were similar in both groups of animals (Control: 21.1 ± 1.3 mmol/L and 36.1 ± 3.4% verses Riluzole: 19.7 ± 1.1 mmol/L and 37.5 ± 3.3%, $P>0.2$).

Effects of riluzole on levels of amino acids and metabolites in PFC and HC

The total concentrations of amino acids and metabolites were determined in the PFC and HC of [1-^{13}C]glucose-infused control and riluzole-treated rats (Table 1). Total concentration (μmol/g) of glutamate, glutamine and aspartate were higher ($P<0.01$) in PFC than HC in both groups, whereas GABA and alanine levels were lower ($P<0.05$). However, within same brain region no significant difference was found between control and riluzole-treated rats ($P=0.4$) (Table 1).

Effects of riluzole on amino acid ^{13}C labeling from [1-^{13}C]glucose in PFC and HC

Figure 1B depicts the percentage ^{13}C enrichments and concentrations of Glu-C4, GABA-C2 and Gln-C4 in the control and riluzole-treated rats infused with [1-^{13}C]glucose. Riluzole treated rats displayed greater ^{13}C labeling compared to controls when ^{13}C enrichment (%) and ^{13}C concentration (μ mol/g) for these amino acids in PFC ($P<0.05$, for each amino acids) and HC ($P<0.01$, for each amino acids) were analyzed (figure 1B). This enhance of ^{13}C labeling of Glu reflects label exchange (and not net synthesis) so that the increase seen with riluzole reflects increased TCA cycle flux.

Discussion

The results of this study demonstrate that chronic treatment with riluzole induces increases in ^{13}C-glucose metabolism in the PFC and HC. These effects were evidenced by increases in ^{13}C incorporation into glutamate-C4, glutamine-C4, and GABA-C2 following 21 days of riluzole treatment. The increased rate of incorporation of ^{13}C label into the major amino acids, indicative of an *enhancement* of oxidative metabolism by riluzole, was unexpected considering the presumed effect of riluzole in reducing glutamate release *in vitro*. The majority of brain glutamate is present in neurons and its turnover from [1-^{13}C]glucose reflects oxidative metabolism mainly in the neuronal TCA cycle. Riluzole-induced enhancement of Gln-C4 labeling is also consistent with increased glutamate/glutamine cycling between neurons and astroglia, because ~70-80% of Gln-C4 ^{13}C labeling from [1-^{13}C]glucose is derived directly through this pathway (Oz *et al*, 2004; Sibson *et al*, 2001). Thus, the increase in ^{13}C labeling is consistent with enhanced glutamatergic metabolism rather than with decreased glutamate release.

The plasma enrichment is ~37% and the concentration of glucose ~20 mM in both groups that indicate a resting glucose concentration of 0.63 × 20 or 13 mM. It is most probably due to the fact that all the animals were allowed free access to a sucrose solution for a period of 48 hours that ended 24 hours prior to the infusion of ^{13}C-glucose. The study was done also for behavioral assessment. Compared to saline-injected controls, no effect of riluzole was found in sucrose preference (data not shown). Based on studies by Sokoloff *et al*, 1988, hyperglycemia has no effect on glucose utilization over a range of 19-31 mM. In our previous studies we find that glucose oxidation is in good agreement with 2DG autoradiographic measurements in normoglycemic rats. In studies where metabsolic fluxes are measured using alternate substrates (e.g., ^{13}C-acetate), which do not raise blood glucose levels, the values found are in good agreement with rates determined using [1-^{13}C]glucose, indicating that any potential effects of hyperglycemia on the measured fluxes were not significant. As concluded from the study by Sokoloff *et al*, 1988, any effects of the hyperglycemia on blood flow, did not alter glucose consumption.

We did not measure the complete time course of ^{13}C labeling in the current study, but only a single early time point (10 min), which our previous studies (Chowdhury *et al*, 2007a) indicate is well within the linear portion of the turnover curves. The blood glucose infusion protocol raises plasma glucose to a near constant level within the first 1-2 min; thus, the ^{13}C label accumulated in brain Glu, GABA and Gln at 10 min, after normalization by the average plasma percentage ^{13}C enrichment over that interval for each animal, reflects the associated metabolic fluxes. Oxidation of [1-^{13}C]glucose in the glial TCA cycle will also result in labeling of Gln-C4, although the extent is less than for neurons comprising ~15-25% of total (Oz *et al*, 2004; Sibson *et al*, 2001). A noted limitation of this study is the fact that the studies were performed on anesthetized animals. While we can rule out the possibility that the effects of riluzole on metabolism seen in this study were indirectly related through an effect of riluzole on the depth of anesthesia, we saw no evidence that riluzole altered the level anesthesia in the animals.

Riluzole is currently the only medication indicated for the treatment of ALS by the US Food and Drug Administration. Although it is widely believed that decreased glutamatergic excitotoxicity is related to the drug's mode of action in delaying the progression of this disorder, there is no clear consensus regarding the exact pharmacological properties that are responsible for the effects on glutamatergic neurotransmission. More recent hypotheses support two pharmacological properties as playing critical roles in generating the drug's anti-glutamatergic effects **(Doble 1996)**. *In vitro*, riluzole reduces glutamate release by interaction with voltage-dependent ion channels (Wang *et al*, 2004). This has been posited as a likely mechanism through which the drug decreases glutamatergic excitotoxicity associated with neuronal

damage (Huang *et al*, 1997; Urbani and Belluzzi, 2000). However, several recent studies also demonstrate riluzole's ability to facilitate glutamate clearance from the extrasynaptic space (Fumagalli, *et al*, 2008; Frizzo *et al*, 2004), thus suggesting a second potentially important mechanism of decreasing the excitotoxic effects of glutamate.

Riluzole has been shown to restore levels of N-acetylaspartate (NAA), a marker of neuronal viability, in motor cortex of patients with **ALS**; increases in NAA within as little as 1d of treatment (Kalra *et al*, 1998) suggests relatively rapid metabolic effects. An increase in NAA was also observed in hippocampus of individuals with generalized anxiety disorder at 8 weeks of riluzole therapy (Mathew *et al*, 2007). Whether the increase in NAA reflects a direct effect of riluzole on neuronal metabolism or is a consequence of reduced excitotoxicity is not clear. Our finding that chronic riluzole treatment increased ^{13}C labeling from glucose in physiologically normal brain suggests that riluzole may have enhancing effects on mitochondrial function beyond those related to the suppression of excitotoxicity.

The idea that riluzole's neuroprotective effect is mediated, at least in part, by enhancing glutamate uptake from the extrasynaptic space is consistent with the model of neurotoxicity proposed by Hardingham (2006). This model proposes that Ca^{2+} in flux through excessive extrasynaptic NMDA receptor activity leads to increased release neuronal nitric oxide synthase (nNOS) and mitochondrial dysfunction. While stimulation of synaptic NMDA receptors promotes cellular signaling cascades that enhance neuronal survival. Thus, the ratio of extrasynaptic to synaptic NMDA receptor stimulation is a main determinant in processes of neurotoxicity and neuroprotection. Considering our present findings, riluzole led to increased Gln-C4 labeling from ^{13}C-glucose, suggesting increased glutamate/glutamine cycling with more glutamate being released and cycled through this pathway. As long as Glu removal keeps pace with release, extrasynaptic Glu levels would not be expected to rise. If, as found in several previous studies (Frizzo *et al*, 2004; Fumagalli, *et al*, 2008), riluzole is acting to enhance glutamate clearance from the extrasynaptic space, then it could be expected that chronic administration of this drug would result in decreased excitation of extrasynaptic NMDA receptors while allowing for an increase in synaptic stimulation. Synaptic NMDA receptor stimulation has been previously shown to have pro-survival effects on the brain (Hardingham, 2006).

A common model of glutamatergic disturbance has been purported for several neurological and psychiatric disorders. Interestingly, riluzole has recently been shown to have clinical efficacy across several neuropsychiatric disorders, perhaps reversing or preventing common pathophysiological effects of glutamate. The findings of this study suggest that chronic riluzole enhances pathway fluxes of glutamate metabolism from glucose in neurons and glia.

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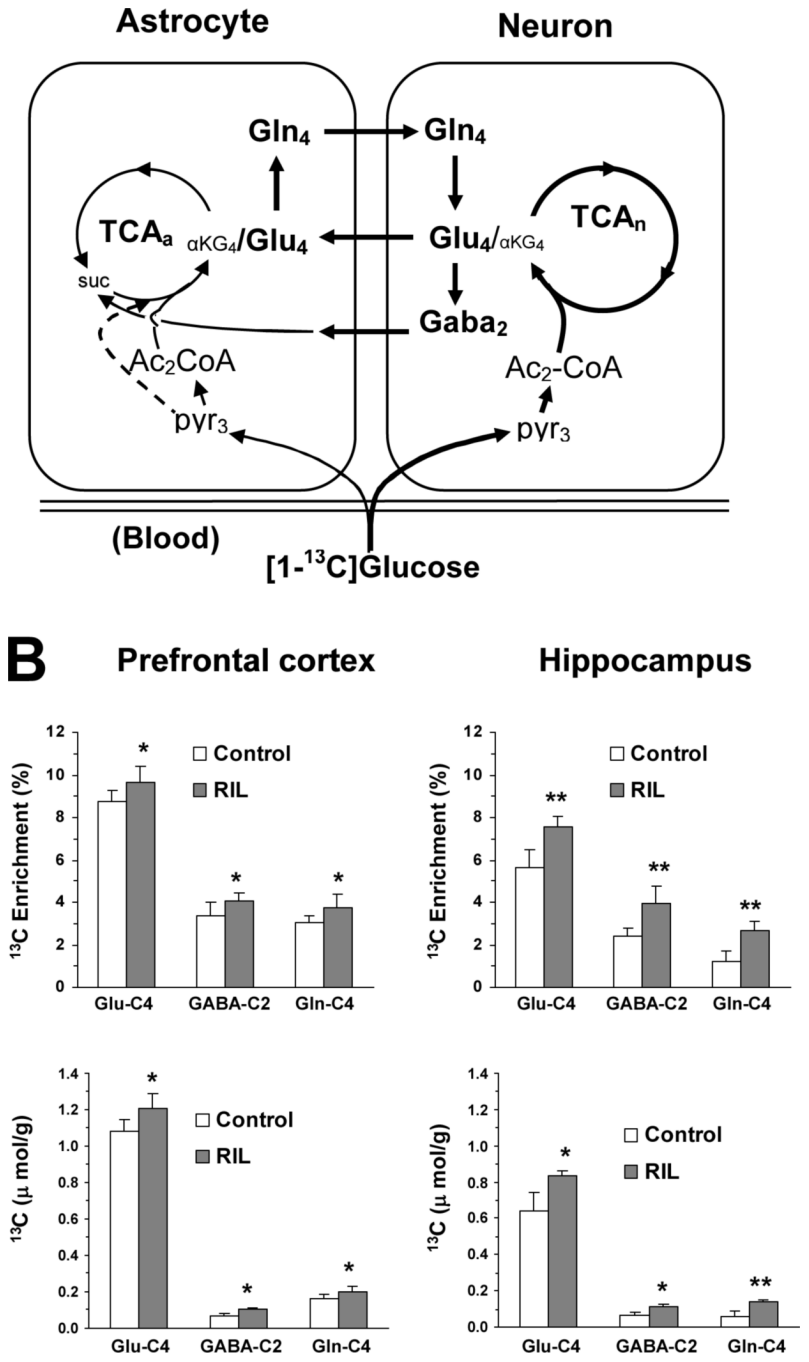

Figure 1.

A) Schematic depiction of Glu/Gln and GABA/Gln cycling between glutamatergic and GABAergic neurons and astroglia relevant to ^{13}C NMR experiments using [1-^{13}C]glucose. Metabolism of [1-^{13}C]glucose through glycolysis and the TCA cycle labels neuronal Glu-C4 (glutamatergic and GABAergic neurons) and Gaba-C2 (GABAergic neurons) with label transfer to astroglia and synthesis of Gln-C4 by neurotransmitter cycling. Abbreviations: (Subscripts: a, astroglia; n, neuron); $V_{TCA,}$ TCA cycle flux; α-KG, α-ketoglutarate; Suc, succinate; Ac_2-CoA, Acetyl-Coenzyme A; Pyr, pyruvate. B) ^{13}C labeling of the major amino acids of prefrontal cortex (PFC) and hippocampus (HC) in saline and riluzole (RIL, 4mg/kg/day, i.p) treated rats with [1-^{13}C]glucose for 10 min. ^{13}C concentrations were determined *ex*

vivo in the tissue extract using ^{1}H-[^{13}C] NMR spectroscopy. Concentrations were normalized to the plasma glucose-C1 enrichment of individual animals for each group. The value represent mean ± SD of 5 animals per group ($p<0.05$)

Table 1

Concentration (μmol/g) of amino acids and metabolites from [l-^{13}C]glucose (10 min) in prefrontal cortex (PFC) and hippocampus (HC)

Region	Subject	Glu	GABA	Gln	Asp	Suc	Ala	Lac	NAA	Cre_{tot}
PFC	Control	12.3±0.6**	2.3±0.1	57±0.4*	3.1±0.2**	0.9±0.1	0.6±0.1	2.3±0.3	8.9±0.5	7.8±0.4
	RIL	12.5±0.3**	2.5±0.2	5.4±0.3*	3.2±0.2**	1.0±0.2	0.5±0.1	3.1±0.9	9.2±0.7	8.7±0.5
HC	Control	11.3±0.4	2.7±0.5*	5.1±0.3	2.6±0.3	1.0±O.1	0.8±0.3*	2.3±0.6	8.4±0.3	7.9±0.9
	RIL	11.1±0.3	2.9±0.4*	5.2±0.3	2.5±0.1	0.9±0.1	0.7±0.1*	2.1±0.3	8.9±0.5	8.0±0.4

Concentration (μmol/g) major amino acids of prefrontal cortex (PFC) and hippocampus (HC) in control and riluzole (RIL, 4mg/kg/day, i.p.) treated rats with (l-^{13}C)glucose for 10 min. The value represent mean ± SD of 5 animals per group (PFC vs HC);

The significances indicated are between the two structures, not with and without riluzole.

** $p<0.01$

* $p<0.05$.