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Increasing extracellular cGMP in cerebellum in vivo reduces neuroinflammation, GABAergic tone and motor in-coordination in hyperammonemic rats

Andrea Cabrera-Pastor, Tiziano Balzano, Vicente Hernández-Rabaza, Michele Malaguarnera, Marta Llansola, Vicente Felipo *

Laboratorio de Neurobiología, Centro Investigación Príncipe Felipe de Valencia, Spain

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ABSTRACT

Hyperammonemia is a main contributor to cognitive impairment and motor in-coordination in patients with hepatic encephalopathy. Hyperammonemia-induced neuroinflammation mediates the neurological alterations in hepatic encephalopathy. Intracerebral administration of extracellular cGMP restores some but not all types of cognitive impairment. Motor in-coordination, is mainly due to increased GABAergic tone in cerebellum. We hypothesized that extracellular cGMP would restore motor coordination in hyperammonemic rats by normalizing GABAergic tone in cerebellum and that this would be mediated by reduction of neuroinflammation.

The aims of this work were to assess whether chronic intracerebral administration of cGMP to hyperammonemic rats: 1) restores motor coordination; 2) reduces neuroinflammation in cerebellum; 3) reduces extracellular GABA levels and GABAergic tone in cerebellum; and also 4) to provide some advance in the understanding on the molecular mechanisms involved.

The results reported show that rats with chronic hyperammonemia show neuroinflammation in cerebellum, including microglia and astrocytes activation and increased levels of IL-1b and TNFa and increased membrane expression of the TNFa receptor. This is associated with increased glutaminase expression and extracellular glutamate, increased amount of the GABA transporter GAT-3 in activated astrocytes, increased extracellular GABA in cerebellum and motor in-coordination. Chronic intracerebral administration of extracellular cGMP to rats with chronic hyperammonemia reduces neuroinflammation, including microglia and astrocytes activation and membrane expression of the TNFa receptor. This is associated with reduced nuclear NF-κB, glutaminase expression and extracellular glutamate, reduced amount of the GABA transporter GAT-3 in activated astrocytes and reduced extracellular GABA in cerebellum and restoration of motor coordination.

The data support that extracellular cGMP restores motor coordination in hyperammonemic rats by reducing microglia activation and neuroinflammation, leading to normalization of extracellular glutamate and GABA levels in cerebellum and of motor coordination.

1. Introduction

Most patients with chronic liver diseases such as cirrhosis show minimal hepatic encephalopathy with attention deficits, mild cognitive impairment and motor in-coordination (Felipo, 2013). Hyperammonemia is a main contributor to the cognitive and motor alterations in these patients (Shawcross et al., 2004 and 2007; Felipo et al., 2012). Rats with chronic moderate hyperammonemia, similar to that present in cirrhotic patients also show cognitive impairment and motor in-coordination (Aguilar et al., 2000; Hernández-Rabaza et al., 2016a, b).

It has been also recently shown that chronic hyperammonemia induces neuroinflammation, which mediates cognitive and motor impairment (Rodrigo et al., 2010; Hernández-Rabaza et al., 2016a, b). Hyperammonemic rats show neuroinflammation in hippocampus that mediates the impairment in spatial learning and memory, which is restored by reducing neuroinflammation with sulforaphane (Hernández-Rabaza et al., 2016a). Hyperammonemic rats also show neuroinflammation in cerebellum that mediates the

* Corresponding author at: Centro Investigación Príncipe Felipe, Eduardo Primo Yufera, 3, 46012 Valencia, Spain.
E-mail address: vfelipo@cipf.es (V. Felipo).

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impairment in the ability to learn a conditional discrimination task in the Y maze and in motor coordination, which are also restored by treatment with sulforaphane (Hernández-Rabaza et al., 2016b).

Learning ability in the Y maze is also restored in hyperammonemic rats by increasing cGMP in cerebellum. This was achieved by chronic oral treatment with sildenafil, an inhibitor of phosphodiesterase 5, which reduces cGMP degradation and increases its levels in cerebellum (Erceg et al., 2005a). Sildenafil increases both intra- and extracellular levels of cGMP. Chronic intracerebral administration of extracellular cGMP also restores the ability to learn the Y maze task (Erceg et al., 2005b, Cabrera-Pastor et al., 2016a) as well as spatial reference memory but not working memory (Cabrera-Pastor et al., 2016b). This indicates that extracellular cGMP may restore some (but not all) of the neurological alterations induced by chronic hyperammonemia through induction of neuroinflammation.

A main neurological alteration in patients with hepatic encephalopathy and in hyperammonemic rats is motor in-coordination, which is mainly due to increased GABAergic tone (activation of $GABA_A$ receptors) in cerebellum (Hernández-Rabaza et al., 2016b). Reducing neuroinflammation restores motor coordination in hyperammonemic rats.

We have recently proposed that there is an interplay between neuroinflammation, cGMP, and GABAergic/glutamatergic neurotransmission in the induction of cognitive and motor alterations in hepatic encephalopathy (Agusti et al., 2017). We propose now that a similar interplay occurs in chronic hyperammonemia. Hyperammonemia reduces extracellular cGMP in cerebellum (Erceg et al., 2005a,b), induces neuroinflammation (Rodrigo et al., 2010), and alters GABAergic and glutamatergic neurotransmission in cerebellum (Hermenegildo et al., 1998; Cauli et al. 2009; Cabrera-Pastor et al. 2018). We propose that neuroinflammation, extracellular cGMP, and GABAergic/glutamatergic neurotransmission modulate each other and contribute to the induction of motor in-coordination in hyperammonemic rats (Fig. 1). As motor coordination is mainly modulated in cerebellum (Thach et al., 1992; Reeber et al., 2013; Koziol et al., 2014), we focused the present study in this area. It is not known if extracellular cGMP may reduce neuroinflammation in cerebellum or restore GABAergic tone or motor in-coordination in hyperammonemic rats.

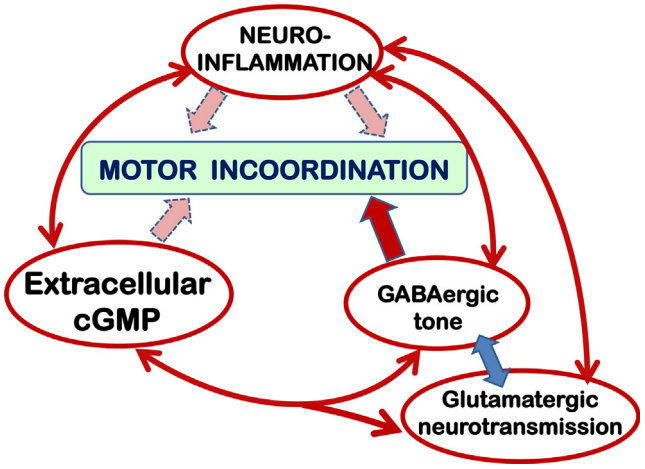

Fig. 1. Interplay between extracellular cGMP, neuroinflammation and glutamatergic and GABAergic neurotransmission. We propose that extracellular cGMP, neuroinflammation and glutamatergic and GABAergic neurotransmission modulate each other. Moreover, all of them may contribute to motor in-coordination indirectly by increasing GABAergic tone. This interplay would allow restoring motor coordination by acting on any of the above contributors, for example by increasing extracellular cGMP in cerebellum of hyperammonemic rats.

Taking into account the interplay proposed in Fig. 1, we hypothesized that increasing extracellular cGMP in cerebellum of hyperammonemic rats would restore motor coordination in hyperammonemic rats by normalizing GABAergic tone in cerebellum and that this would be mediated by reduction of neuroinflammation.

The aims of this work were to assess whether chronic intracerebral administration of cGMP: 1) restores motor coordination in hyperammonemic rats; 2) reduces neuroinflammation in cerebellum; 3) reduces extracellular GABA levels and GABAergic tone in cerebellum; and also 4) to provide some advance in the understanding on the molecular mechanisms involved.

To reach these aims extracellular cGMP was chronically administered intra-cerebrally to hyperammonemic rats using mini-osmotic pumps. Motor coordination was assessed using the beam walking test. Extracellular GABA and glutamate levels and GABAergic tone were measured in cerebellum in vivo by microdialysis in freely moving rats. Neuroinflammation was analyzed by immunohistochemistry by analyzing astrocytes and microglia activation and the pro-inflammatory markers IL-1b and TNF-a.

2. Methods

2.1. Model of chronic hyperammonemia in rats

Male Wistar rats (120–140 g, Charles River Laboratories, Barcelona, Spain) were made hyperammonemic by feeding them a diet containing standard diet supplemented with 20% ammonium acetate as in Felipo et al. (1988). The ammonia-containing diet was prepared as described in Azorín et al. (1989) and increased blood ammonia levels around 3-fold, an increase similar to that found in patients with liver cirrhosis. Rats remain hyperammonemic for long periods of time and reproduce many cognitive and motor alterations present in cirrhotic patients with hepatic encephalopathy (e.g. Hernández-Rabaza et al., 2016a,b). The experiments were approved by the Center and carried out in accordance with the European Communities Council Directive (86/609/EEC).

2.2. Continuous intracerebral administration of cGMP to rats using osmotic pumps

Rats were divided in four groups, two of control rats and two of hyperammonemic rats. For one group of control rats and one of hyperammonemic rats, the osmotic pumps were filled with 240 μM cGMP in sterile saline. For the other two groups with the vehicle solution, sterile saline. The pumps were implanted in the back of the rats two weeks after starting the ammonium-diet. These pumps released 0.25 μL per hour during 28 days and were connected to a cannula implanted in the cerebral ventricle as in Cabrera-Pastor et al. (2016b). cGMP is stable indefinitely inside the pump but may be degraded by phosphodiesterases once released. By this reason it is necessary to use the osmotic pumps to get a continuous controlled release of cGMP from osmotic pump to the brain. The dose used has been previously shown to be adequate to induce effects on brain of hyperammonemic rats. Under these conditions, the levels of extracellular cGMP in cerebellum in hyperammonemic rats are increased to levels similar to control rats (Erceg et al., 2005a; Cabrera-Pastor et al. (2016b). Osmotic pumps in the groups treated with extracellular cGMP contain cGMP in saline while in rats treated with vehicle they contain only saline. The effects induced must be therefore attributed to the release of this cyclic nucleotide.

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