

Author's Accepted Manuscript

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CEPHALOPATHY: NEW DIAGNOSTIC POSSIBI-
LITIES AND NEW THERAPEUTIC APPROACHES

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PII: S2307-5023(14)00074-5
DOI: <http://dx.doi.org/10.1016/j.nhtm.2014.11.057>
Reference: NHTM9

To appear in: *New Horizons in Translational Medicine*

Cite this article as: Marta Llansola, Carmina Montoliu, Ana Agusti, Vicente Hernandez-Rabaza, Andrea Cabrera-Pastor, Michele Malaguarnera, Belen Gomez-Gimenez, Alma Orts, Raquel Garcia-Garcia, Tiziano Balzano, Lucas Taoro, Vicente Felipo, TRANSLATIONAL RESEARCH IN HEPATIC ENCEPHALOPATHY: NEW DIAGNOSTIC POSSIBILITIES AND NEW THERAPEUTIC APPROACHES, *New Horizons in Translational Medicine*, <http://dx.doi.org/10.1016/j.nhtm.2014.11.057>

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TRANSLATIONAL RESEARCH IN HEPATIC ENCEPHALOPATHY: NEW DIAGNOSTIC POSSIBILITIES AND NEW THERAPEUTIC APPROACHES

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Focal points:

- **Benchside**

The mechanisms underlying cognitive and motor alterations in minimal hepatic encephalopathy (MHE) are beginning to be clarified in animal models. A number of therapeutic targets have been identified to improve cognitive and motor function in MHE. Also, serum level of 3-nitrotyrosine is the first peripheral biomarker identified for diagnosis of MHE in cirrhotic patients, with high diagnostic accuracy, high sensitivity and specificity.

- **Bedside:**

In the European Union more than 2 million patients with liver cirrhosis show MHE with mild cognitive impairment. MHE is an important, until now underestimated, health, social and economic problem. Early diagnosis and treatment of MHE will significantly improve quality of life and life span of the patients and reduce costs of hospitalization and treatment

- **Industry**

There are no specific treatments for the neurological alterations in MHE. A number of therapeutic targets have been identified in animal models to improve cognitive and motor function in MHE. This is a new market waiting for development of appropriate therapeutic treatments which would improve quality of life and survival of patients. Development of a kit for diagnosis of MHE in clinical practice is pending.

- **Governments - Regulatory Agencies**

Early diagnosis and treatment of MHE will significantly improve quality of life and life span of the patients and reduce costs of hospitalization and treatment. Screening of the presence of MHE in patients with liver diseases will reduce costs.

Executive Summary:

- In the European Union more than 2 million patients with liver cirrhosis show minimal hepatic encephalopathy (MHE) with mild cognitive impairment
- MHE is an important, until now underestimated, health, social and economic problem. Early diagnosis and treatment of MHE will significantly improve quality of life and life span of the patients and reduce costs of hospitalization and treatment
- Impaired function of the glutamate-nitric oxide (NO)-cGMP pathway in brain is responsible for some cognitive alterations in MHE
- Several factors affect the function of this pathway in MHE by related mechanisms: hyperammonemia, neuroinflammation, increased GABAergic tone and increased activation of NMDA receptors and of p38
- Treatments acting on these different brain targets restore the function of the glutamate-NO -cGMP pathway and cognitive function in rats with MHE
- Serum level of 3-nitrotyrosine is the first peripheral biomarker identified for diagnosis of MHE in cirrhotic patients, with high diagnostic accuracy, high sensitivity and specificity
- Development of a kit for diagnosis of MHE in clinical practice is pending

ABSTRACT

Chronic liver disease (e.g. cirrhosis) affects brain function. There is a high incidence of mild cognitive impairment and psychomotor slowing in patients with cirrhosis. This condition, known as minimal hepatic encephalopathy (MHE) affects more than 2 million people in the European Union and has serious health, social and economic consequences. There are no effective treatments for MHE.

Rat models of MHE reproduce cognitive and motor alterations seen in patients, showing reduced performance in different types of cognitive tests, including learning a conditional discrimination task in a Y maze.

Reduced ability to learn the Y maze task is due to reduced function of the glutamate-nitric oxide (NO)-cGMP pathway in cerebellum, assessed in vivo by microdialysis. This results in reduced formation of cGMP in response to activation of NMDA receptors and impairment of learning ability.

Both hyperammonemia and neuroinflammation contribute to impair this pathway. The effect is mediated by enhanced tonic activation of NMDA and GABA_A receptors and of MAP-kinase p38.

Based on these mechanistic studies new therapeutic strategies acting on specific targets in the brain have been designed and tested, which have successfully restored the function of the glutamate-NO-cGMP pathway in vivo and learning ability in rats with MHE. This can be achieved by therapeutic treatments using:

- a) phosphodiesterase 5 inhibitors (sildenafil, zaprinast), that increase cGMP levels by reducing its degradation
- b) extracellular cGMP
- c) antagonists of type A GABA receptors (bicuculline)
- d) neurosteroids that modulate GABAergic tone (pregnenolone sulfate)
- e) inhibitors of cyclooxygenase (ibuprofen) which reduce neuroinflammation
- f) inhibitors of MAP- kinase p38 (SB239063), that reduce microglial activation and neuroinflammation

Translation of some of these treatments to clinical practice would improve cognitive function, quality of life and life span of patients with cirrhosis and MHE and reduce health systems costs.

Key words: minimal hepatic encephalopathy, mild cognitive impairment, hyperammonemia, neuroinflammation, therapeutic treatment, NMDA receptors, cGMP, nitric oxide, GABAergic neurotransmission, phosphodiesterase inhibitors, MAP kinase p38, glutamatergic neurotransmission, neurosteroids, inflammation

Introduction

Chronic and acute liver diseases (cirrhosis, hepatitis, ..) affect cerebral function, leading to a wide range of neurologic and psychiatric alterations referred to as "hepatic encephalopathy". The term hepatic encephalopathy (HE) is used for all situations in which cerebral function is altered as a consequence of a previous failure in liver function. This covers a diverse array of situations from very mild cognitive and motor alterations in patients with liver cirrhosis to strong alterations for example in acute liver failure, leading to brain edema, increased intracranial hypertension which may lead to brain herniation and death (1).

The most frequent form is called "minimal hepatic encephalopathy" (MHE), is not clinically evident and is only detected by performing psychometric tests. Patients with MHE show attention deficits, psychomotor slowing, mild cognitive impairment and reduced visuo-motor and bimanual coordination. Although these alterations are mild, its combination reduces seriously the patients quality of life and ability to perform daily life activities (e.g driving cars). MHE increases their risk of suffering falls, fractures and more severe clinical HE and reduces their life span.

MHE affects around 40% of cirrhotic patients. More than 2 million people in USA and a similar number in the European Union suffer MHE, which is an important, until now underestimated, health, social and economic problem.

Advances in diagnosis of MHE

A relevant problem is the lack of simple diagnostic procedures for early detection of MHE. Currently the procedure to detect it is performing psychometric tests. However, this is not usual in most clinical settings. Cirrhotic patients attend hepatology-gastroenterology services which usually do not perform these tests, which are time consuming and require for corrections depending on age and education level. As a consequence, most patients with MHE remain undiagnosed and untreated worldwide.

Fortunately, this situation may change in the next future, as the first peripheral biomarker for early detection of MHE has been identified and patented. Determination of serum levels of 3-nitrotyrosine is useful to diagnose MHE with high sensitivity, specificity and positive and negative values (2). This was demonstrated by measuring 3-nitrotyrosine levels by high-pressure liquid chromatography. This procedure is not convenient in clinical practice.

Development of a diagnostic kit, based on antibodies against 3-nitrotyrosine, useful in clinical practice, would strongly improve early diagnosis. This would allow generalization of early diagnosis and treatment of MHE worldwide, thus preventing or delaying progression of neurological impairment. This would improve the quality of life of patients and reduce falls, accidents, hospitalizations and economical costs.

Synergistic effects of hyperammonemia and inflammation in the induction of neurological alterations in MHE

To develop effective treatments to improve cognitive and motor function in patients with MHE it is essential to understand the mechanisms leading to these neurological alterations.

It is well known that a main contributor to HE is hyperammonemia. In the last years there is increasing evidence that another important factor is inflammation, which seem to act synergistically with hyperammonemia to induce the neurological alterations (1). The presence and grade of MHE in cirrhotic patients correlates with serum levels of the pro-inflammatory cytokines IL-6 and IL-18 (3). In fact, it has been shown that the combination of hyperammonemia and inflammation is enough to induce mild cognitive impairment, even in the absence of liver failure, if the ammonia and inflammation levels exceed a threshold (4).

For example, some patients with non alcoholic steatohepatitis (NASH) or with keloids (a

dermatological disease) show cognitive impairment associated to hyperammonemia and inflammation in the absence of liver cirrhosis or any liver disease, respectively (4).

The current treatments for MHE are directed to reduce ammonia levels by different means: reducing protein intake, reduce ammonia transport from intestine to blood using non absorbable disaccharides such as lactulose, modulating the intestinal bacterial flora using antibiotics such as neomycin or rifaximin or, more recently, probiotics.

However, these treatments are not effective. It is very likely that treatments acting on brain targets involved in the mechanisms leading to specific cognitive or motor alterations would be more effective and successful to reverse, prevent or delay cognitive and motor alterations in patients with MHE. This has been already demonstrated in animal models of MHE.

Identification of mechanisms underlying cognitive and motor alterations in animal models of MHE.

Rat models of MHE reproduce many neurological alterations seen in patients, showing hypokinesia and reduced performance in different types of cognitive tests, including learning a conditional discrimination task in a Y maze.

The molecular mechanisms responsible for some cognitive and motor alterations have been recently identified in rat models of chronic hyperammonemia and MHE. Alterations in glutamatergic and GABAergic neurotransmission play a main role in these neurological alterations (5,6). On the basis of these mechanistic studies new therapeutic approaches have been designed and tested successfully in these rat models, allowing to restore motor activity and cognitive function

Rats with MHE due to portacaval shunts (PCS) show hypokinesia, similar to that present in cirrhotic patients. Cauli et al (7) showed that this is a consequence of a large increase in the extracellular concentration of glutamate in substantia nigra pars reticulata (SNr), which is about 10-fold higher in PCS than in sham-operated control rats. Glutamate activates metabotropic glutamate receptor 1 (mGluR1) in SNr and this increases the release and extracellular concentration of GABA in ventromedial thalamus. These increased GABA levels reduce the glutamate release and extracellular levels in cortex, resulting in reduced motor activity (7). Motor activity may be restored by stereotaxic injection of an antagonist of mGluR1 in SNr. This normalizes extracellular GABA in ventromedial thalamus and glutamate in cortex and restores motor activity (7).

Neuroinflammation plays a main role in hypokinesia in HE. Treatment with ibuprofen, an anti-inflammatory reduces extracellular glutamate in SNr and eliminates hypokinesia in PCS rats (8).

Rats with MHE reproduce the mild cognitive impairment present in patients and show reduced spatial learning and memory (9) and reduced ability to learn a conditional discrimination task in a Y maze (10). Different types of learning and memory are modulated by different mechanisms involving distinct neuronal circuits and brain areas. Impairment of spatial learning and memory would be due to impairment of NMDA receptor-dependent long-term potentiation in hippocampus (9,11).

Restoring the function of the glutamate-nitric oxide -cGMP pathway by treatments acting on different brain targets restores cognitive function in rats with MHE

The ability to learn the Y maze task is mainly modulated by the glutamate-nitric oxide (NO)-cGMP pathway in cerebellum. Activation of NMDA receptors increases calcium in the post-synaptic neuron, leading to activation of neuronal nitric oxide synthase and increased formation of NO. NO activates soluble guanylate cyclase and increases the formation of cGMP. This formation of cGMP induced by activation of NMDA receptors is necessary to learn the Y maze task.

The function of this pathway can be analyzed *in vivo* by microdialysis in freely moving rats by measuring extracellular cGMP before and after activation of NMDA receptors by NMDA added through the microdialysis probe (12).

The function of the glutamate-N-cGMP pathway (the increase in extracellular cGMP induced by NMDA) is reduced in cerebellum of rats with chronic hyperammonemia or MHE (12). This results in reduced formation of cGMP in response to activation of NMDA receptors.

It was then thought that this reduced formation of cGMP would be responsible for impairment of the ability to learn the Y maze task and that it would be possible to restore learning ability if cGMP levels are restored. It was assessed whether inhibiting cGMP degradation using inhibitors of phosphodiesterase 5 inhibitors restores cGMP levels and learning ability. This was the case, both zaprinast (13) and sildenafil (14) restored cGMP levels in cerebellum and the ability to learn the Y maze task in rats with chronic hyperammonemia or MHE. This confirms that reduced learning ability is due to reduced function of the glutamate-NO-cGMP pathway and cGMP formation.

The mechanisms by which hyperammonemia and MHE impair the function of the pathway were studied with the aim of designing therapeutic treatments to restore its function and, consequently, learning ability.

It was found that chronic hyperammonemia induces microglial activation and neuroinflammation (15), which contributes to impair this pathway. The function of the pathway and learning ability may be restored by treatment with anti-inflammatories such as

ibuprofen (16). However, the use of non-steroidal anti-inflammatory drugs such as ibuprofen is not recommendable in cirrhotic patients because it can exacerbate kidney damage in these patients.

A procedure to reduce microglial activation and neuroinflammation without affecting kidney function was assessed and it was found that inhibition of MAP kinase p38 reduces neuroinflammation and restores cognitive and motor function in rats with MHE without affecting kidney function (17). Inhibitors of p38 may be therefore, when available for clinical practice, useful to improve the neurological alterations in patients with MHE.

The steps of the pathway which are impaired in hyperammonemia and MHE were studied in more detail. A main contributor to the reduced function of the whole pathway is a reduction in the basal activity and in the activation in response to NMDA receptor activation of neuronal nitric oxide synthase (nNOS). This is due to: a) altered subcellular distribution of nNOS, with increased amount in cytosol and reduced amount in synaptic membranes and b) increased phosphorylation of nNOS in Ser847, which reduces its activity (18). The increased phosphorylation of Ser847 is due to increased activity of calcium-calmodulin kinase II (CaMKII), which in turn is a consequence of enhanced tonic activation of NMDA receptors in chronic hyperammonemia (19).

It has been reported that activation of GABA_A receptors reduces the function of the glutamate-NO-cGMP pathway in cerebellum (20). It was therefore assessed the possible contribution of enhanced "GABAergic tone" in cerebellum to the reduced function of the pathway in cerebellum of hyperammonemic rats. It was found that chronic hyperammonemia increases GABAergic tone (activation of GABA_A receptors) in cerebellum but reduces it in cerebral cortex (21). Reducing activation of GABA_A receptors by chronic treatment with the antagonist bicuculline restores the function of the pathway and the ability to learn the Y maze task in rats with chronic hyperammonemia (21). This confirms that enhanced GABAergic tone in cerebellum contributes to the impairment of the pathway and of learning and shows that modulation of GABAergic tone is also a useful therapeutic approach to improve cognitive function in MHE.

A family of compounds which may modulate both GABAergic tone and activation of NMDA or sigma receptors is that of neurosteroids. Different neurosteroids may modulate the glutamate-NO-cGMP pathway positively or negatively by acting on different receptors (22). One neurosteroid, pregnaonolone sulphate, enhances the function of the pathway and restores learning ability and motor coordination in hyperammonemic rats (23) likely by enhancing NMDA receptors and reducing GABA_A receptors activation.

This shows that neurosteroids or derivatives thereof could also have beneficial effects to improve cognitive and motor alterations in patients with MHE.

A summary of the treatments which improve cognitive and/or motor function in animal models of MHE is shown in Table 1.

Table 1. Treatments that improve cognitive and/or motor function in animal models of minimal hepatic encephalopathy

Treatment	Action	Reference
Sildenafil	Inhibitor of phosphodiesterase 5, increases cGMP	14. Erceg et al, 2005
Zaprinast	Inhibitor of phosphodiesterase 5, increases cGMP	13. Erceg et al, 2005
cGMP	Increases extracellular cGMP	13. Erceg et al, 2005
Ibuprofen	Anti-inflammatory	16. Cauli et al, 2007 8. Cauli et al, 2009 15. Rodrigo et al, 2010
Bicuculline	Antagonist of GABA _A receptors	21. Cauli et al, 2009
SB239063	Inhibitor of MAP kinase p38	17. Agusti et al, 2011
Pregnenolone sulfate	Neurosteroid that modulates activation of NMDA and GABA _A receptors	23. Gonzalez-Usano et al, 2014

Concluding remarks

In summary, based on mechanistic studies, new therapeutic strategies acting on specific targets in the brain have been designed and tested which have successfully restored the function of the glutamate-NO-cGMP pathway in vivo and learning ability in rats with MHE. This can be achieved by therapeutic treatments using:

- a) phosphodiesterase 5 inhibitors (sildenafil, zaprinast), that increase cGMP levels by reducing its degradation (13,14)
- b) extracellular cGMP (13)
- c) antagonists of type A GABA receptors (bicuculline) (21)
- d) neurosteroids that modulate GABAergic tone (pregnenolone sulfate) (23)
- e) inhibitors of cyclooxygenase (ibuprofen) which reduce neuroinflammation (15,16)
- f) inhibitors of MAP- kinase p38 (SB239063), that reduce microglial activation and neuroinflammation (17)

Translation of some of these treatments to clinical practice would improve cognitive function, quality of life and life span of patients with cirrhosis and MHE and reduce health systems costs.

ACKNOWLEDGEMENTS:

Supported in part by Ministerio de Ciencia e Innovación (SAF2011-23051, CSD2008-00005), and Consellería Educación Generalitat Valenciana (PROMETEO-2009-027, ACOMP/2012/066, ACOMP/2013/101, PROMETEOII/2014/033).

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