様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

# 科学研究費助成事業　　研究成果報告書

平成　３０　年　　６　月　１１　日現在

機関番号：　１４２０２
研究種目：　基盤研究(C)（一般）
研究期間：　2015～2017
課題番号：　１５Ｋ１０５５６

研究課題名（和文）Therapeutic potential of the metabolic relationship between the product of two genetic markers for pain (gch1 and mthfr)

研究課題名（英文）Therapeutic potential of the metabolic relationship between the product of two genetic markers for pain (gch1 and mthfr)

研究代表者

Ｊ・Ｐ　Ｂｅｌｌｉｅｒ（Bellier, Jean-Pierre）

滋賀医科大学・神経難病研究センター・助教

研究者番号：８０３４６０２２

交付決定額（研究期間全体）：（直接経費）　　3,500,000円

研究成果の概要（和文）：BH4は、痛覚に関与する神経伝達物質の生合成のために必要な補因子である。GCH1はBH4de novo経路における生成経路の律速酵素である。また、BH4salvage経路は、メチルテトラレダクターゼ（MTHFR）を介して葉酸代謝経路に関連している。GCH1、MTHFRが慢性疼痛に関連しているBH4で疼痛マーカーおよび可用性として知られているので、痛みのそれらの代謝経路の可能性を評価した。代謝経路の関与は、内臓の痛み動物モデルで評価されました。これらの代謝経路の進化的保全を無脊髄動物モデルで評価されました。観察は、代謝経路は、種間で保全されていると、痛みのあるBH4の利用可能性に関与している。

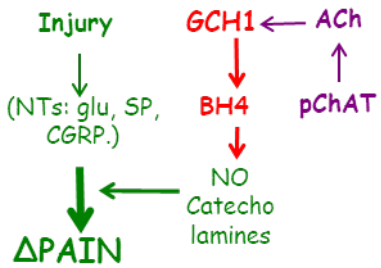
研究成果の概要（英文）：Tetrahydrobiopterin (BH4) is a cofactor required for the biosynthesis of neurotransmitters involved in the nociceptive neurotransmission. BH4 de novo synthesis is led by GTP cyclohydrolase 1 (GCH1) which is the first and rate-limiting enzyme in that synthetic pathway. In the other hand, BH4 salvage pathway is linked to the folate metabolic pathway via methyltetrahydrofolate reductase. Since GCH1, MTHFR are known as pain marker and availability in BH4 is linked to chronic pain, we evaluate the potential of those metabolic pathways in pain. Several approaches were used to leverage BH4 availability in vitro. The involvement of these metabolic pathways was assessed in animal model of visceral pain. Evolutional conservation of these metabolic pathways were assessed in higher invertebrate animal model. Our observation suggested that those pathways are conserved among species and involved in availability of BH4 in pain. However their modulation using drugs is facing specificity challenges.

研究分野：　麻酔・蘇生学

キーワード：　GTP cyclohydrolase 1　sensory neurons　tetrahydrobiopterin　pain　serotonin

## １．研究開始当初の背景

In vertebrate, sensory and nociceptive neurons carry information from the periphery to the central nervous system. They use excitatory neurotransmitters and neuropeptides to relay sensation (such as glutamate, substance P). However a variety of molecules including neurotransmitters (serotonin, nitric oxide) modulate that signal. I previously evidenced the synthesis of the neurotransmitter acetylcholine in the primary afferent neurons of the dorsal root ganglion by pChAT a peripheral form of choline acetyltransferase. Based on the known association existing between cholinergic nitrinergic, and catecholaminergic neurotransmissions, I formulated the hypothesis that acetylcholine synthesized by pChAT in the DRG may play a role in the modulation of sensory neurotransmission by regulating nitric oxide and serotonin. In this context, a possible link is GTP cyclohydrolase 1 that synthesizes tetrahydrobiopterin (BH4), a cofactor required for the biosynthesis of nitric oxide and catecholamines (such as serotonin)(shema 1).

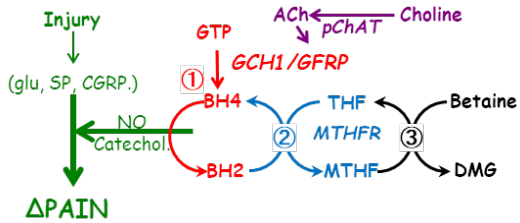



*Shema 1: Initial hypothesis showing the possible link between cholinergic system in the sensory neurons, BH4 production and pain.*

GTP cyclohydrolase 1 (GCH1) is the first and rate-limiting enzyme in the metabolic pathway of BH4. GCH1 has been shown to be a genetic pain marker, and a number of basic and clinical studies support the concept that BH4 is a key modulator in neuropathic pain. For instance the association between BH4 production and chronic pain has been confirmed in more than 12 independents cohorts, thought a common haplotype of GCH1.

Modulating BH4 metabolism through modulation of GCH1 activity has been proposed to control nociceptive mechanisms. However, GCH1 is an enzyme not only presents in the primary afferent neurons, but also, for instance, in the dopaminergic and serotoninergic neurons of the brain stem that have important cerebral function. Therefore controlling specifically the activity of GCH1 in primary afferent neurons without affecting activity of GCH1 in other regions of the nervous system (and other organs) represents a real challenge.

Current data indicate a relation between cholinergic system and BH4 production. In addition, I observed that some cholinergic drug may interact with betaine pathway, this suggested that the acetylcholine synthesis in the DRG by the peripheral form of choline acetyltransferase may regulate BH4 availability not only by acting on de novo BH4 synthesis but may he acting also on the BH4 salvage pathway that involve methylenetetrahydrofolate reductase (MTHFR) (schema 2). It should be noted that polymorphism in MTHFR gene is also associated with chronic pain.




*Shema 1: Working hypothesis showing possible de novo and salvage pathway for BH4 in the sensory neurons.*

## ２．研究の目的

The objective of this work is to explore pathway of BH4 synthesis between peripheral and central nervous system. In order to reveal difference that can be useful to block BH4 synthesis specifically in the peripheral sensory/nociceptive system. In that context, our specific objectives were:

i) To evaluate the anti-nociceptive potential of an antibody targeting BH4 synthesizing pathway in the periphery.

ii) To investigate the metabolic relations between BH4 salvage pathway and cholinergic system in sensory system.

iii) To evaluate the anti-nociceptive potential of drugs affecting previous

pathways in animal model of pain.

iv) To study conservation of those system in other species in order to isolate potential anti-nociceptive agent by biomimicry.

## ３．研究の方法

Previously, I developed sensitive and specific analytical tools to assess BH4 metabolism (HPLC method) and raised reliable antibodies against GCH1 and other protein involved in BH4 biosynthetic pathway. During the present project, we combined functional neuroanatomy and biochemical analysis.

i) Antibodies targeting BH4 synthesizing pathway were produced in animals. First, intracellular delivery was test in vitro in HuH7 cell line constitutively expressing GCH1. Several approaches were tested, such as liposome, nanoparticle and artificial mimic of viral particles. GCH1 activity and BH4 production were assessed using EDC-HPLC.

ii) To investigate the metabolic relation between BH4 salvage pathway and cholinergic system in sensory system, we investigated the expression of the enzymes involved in the synthesis of betaine and MTHFR, using RT-PCR, western-blot, and immunohistochemistry.

iii) To evaluate the potential anti-nociceptive effect of the previous pathway *in vivo*. We used an animal model of visceral pain, the TNBS-induced colitis rat model, which also develops visceral and somatic hypersensitivity, and neurochemical change in both primary afferent neurons of the enteric nervous system and of the DRG. Animal model was assessed using whole mount immunohistochemistry and imaging combined with 3D-reconstruction and 3D-modelization. A number of markers were followed including GCH1 and calcium associated protein such as calbindin 28.

iv) To study conservation of those system in other species and reveal potential anti-nociceptive agent. We conduct an immunohistochemical investigation in the nervous system of a superior invertebrate.

## ４．研究成果

i) A monoclonal neutralizing antibody against a particular form of BH4 synthesizing enzyme that is preferentially expressed in the peripheral nervous system was raised. Several clones appear suitable for use as blocking antibody. The study of the intracellular delivery of the antibody (where it should block the enzyme) shows very low efficiency for every used approach. We conclude that alternatives approaches of delivery, more efficient and reliable have to be applied. (**学会発表** 3〕

ii) We were able to put in evidence that choline drug alters betaine synthesis, however we do not obtain evidence of its involvement in nociception, may be because the drug currently available are lack of specificity. In addition, metabolic pathway which involves mthfr seems difficult to control. So far, BH4 salvage pathway is hard to evidence then control in the sensory neurons. In this context, we however succeeded to characterize better the system that may use the de novo synthesis pathway for BH, we have raised a novel polyclonal antiserum against GCH1 that allow high anatomical resolution of GCH1 in the enteric nervous system. It suggests that GCH1 involvement in pain might be also involved in visceral pain, and animal model of visceral pain might be a good model to study regulation of GCH1 in vivo. This newly raised polyclonal antiserum against GCH1 is also very potent for delineating the enteric nervous system (Figure 1). (**雑誌論文** 2, 5; **学会発表** 4)

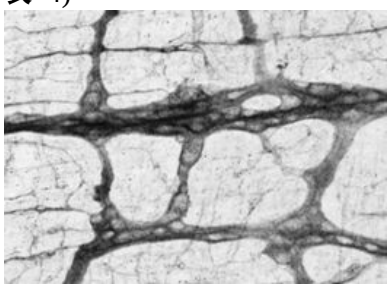


*Figure 1: IHC for the novel GCH1 antiserum in the rat enteric nervous system*

iii) We investigated neurochemical changes of BH4 metabolic pathway and

and neuchemical change associated with pain in the TBNS-induced colitis model in rat. Enteric nervous system marker was used and imaged using whole mount clarification method combined with 3D reconstruction. This approach allow an unprecedent visualization of the enteric nervous system connectivity in normal animal and animal model of visceral inflammation and pain. We in particular observed neurochemical change in the expression of calbidin 28 kda, asoociated with change in GCH1 expression, suggesting that both follow similar regulation, may be because bothe are calcium binding protein. In addtion severe change in the connectivity was observed, which might be involved in the deleterious effect of the inflammation in the animal model ( figure 2)(**学会発表** 2〕.

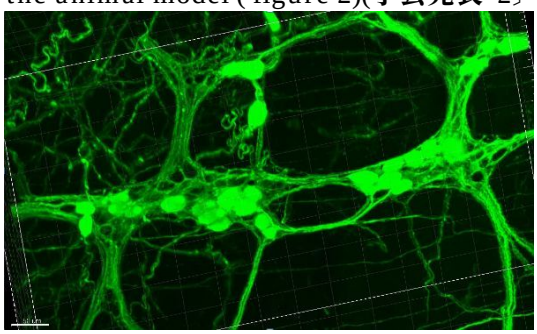
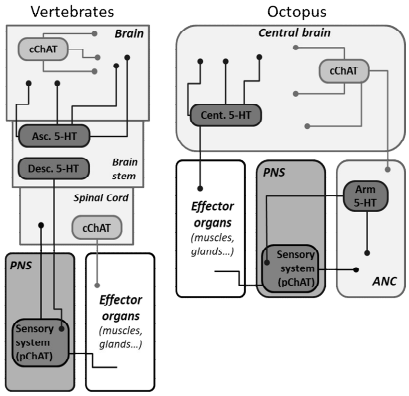


*Figure 2: whole mount/3d reconstruction for calbindin IHC in the rat enteric nervous system*

iv) Increase in pain feeling is a major trait of evolution. Pain in human seems to have evolved without limit and probably had a huge impact on human evolution. While in vertebrate ascending pain pathway (from nociceptor to brain) and anti-nociceptive pathway (from brain to second-order sensory neurons) are relatively well described, no similar system seems to exist in invertebrate. In a recent study, we observed in the cephalopod serotoninergic pathway, a striking similarity with mammal organization of the serotoninergic descending system that is involved in the control of the nociception (shema3). Considering the phylogenetic separation between mammal and cephalopod by over 500 million years, well before the appearance of the serotoninergic descending system, the presence of an equivalent system in cephalopod suggests a convergent evolution of the sensory/"nociceptive" processing mechanisms, and indicate that the mechanism (**雑誌論文** 1;**学会発表** 1,4,5〕.




*Shema 3: A comparison of neurotransmitter pathways between vertebrates and invertebrates*

## ５．主な発表論文等
**（研究代表者、研究分担者及び連携研究者には下線）**

**〔雑誌論文〕（計　5 件）**


1. Bellier JP, Xie Y, Farouk SM, Sakaue Y, Tooyama I, Kimura H. Immunohistochemical and biochemical evidence for the presence of serotonin-containing neurons and nerve fibers in the octopus arm. Brain Structure and Function. 222:3043-3061. 2017. **査読有**

2. Guan H, Yang H, Yang M, Yanagisawa D, Bellier JP, Nonaka T, Zhao S, Tooyama I. Mitochondrial ferritin protects SH-SY5Y cells against oxidative stress and modulates α-synuclein expression. Experimental Neurology. 291:51-61. 2017. **査読有**

3. Abdelalim EM, Bellier JP, Tooyama I. Localization of the Brain Natriuretic Peptide immunoreactivity in rat spinal cord. Frontiers in Neuroanatomy. 10:116. 2016. **査読有**

4. Wang X, Yang H, Yanagisawa D, Bellier JP, Morino K, Zhao S, Liu P, Vigers P, Tooyama I. Mitochondrial ferritin affects mitochondria by stabilizing HIF-1α in retinal pigment epithelium: implications for the pathophysiology of age-related macular degeneration. Neurobiology of Aging 47:168-17. 2016. **査読有**

5. Yang M, Yang H, Guan H, Bellier JP, Zhao S, Tooyama I. Mapping of mitochondrial ferritin in the brainstem of Macaca fascicularis. Neuroscience 328:92-106. 2016. **査読有**

**〔学会発表〕（計　6 件）**

1. Bellier JP, Xie Y, Sakaue Y, Tooyama I. The octopus arm neuroanatomy revealed by immunohistochemistry for 5-hydroxytryptamine and choline acetyltransferase isoforms. CONBIO 2017. 2017 **年度生命科学系学会合同年次大会**, Kobe;**神戸** 2017 **年** 12 **月** 6～9 **日**. Poster.

2. Bellier JP and Tooyama I. Visualization of calcium-binding proteins by whole tissue clarification methods. Japan Society for Histochemistry and Cytochemistry 2017. Ehime University, Ehime, Japan, 23 October 2017. Innovation for imaging metal ions. **第** 58 **回日本組織細胞化学会総会シンポジウム。金属イオンとイメージングの最前線。愛媛大学、愛媛県**（2017 **年** 9 **月** 23 **日**）。Oral presentation.

3. Bellier JP, Xie Y, Tooyama I, Kimura H. In vivo evaluation of interaction between GTP cyclohydrolase 1 and its regulatory protein GFRP in the rat brain stem. International Society of Neurochemistry, Paris, France (August 20-24, 2017); Poster

4. Bellier JP, Tooyama I. The peripheral type of choline acetyltransferase, a marker for peripheral cholinergic neurons in invertebrate and vertebrate. Japanese Association of Anatomists 2017, Nagasaki; **第** 122 **回日本解剖学会総会・全国学術集会**. **長崎、**2017 **年** 03 **月** 28～30 **日**. Oral presentation.

5. Bellier JP, Xie Y, Farouk SM, Sakaue Y, Tooyama I, Kimura H. Delineate the serotonin innervation in octopus arm by immunohistochemistry. Society for Neuroscience 2016, San Diego; Poster.

6. Bellier JP, Xie Y, Taché Y, Tooyama I, Kimura H. Antiserum as a pan-neuronal immuno-marker for enteric neurons in rodents. Japan Society of Histochemistry and cytochemistry 2016. Tokyo; Poster.

**〔図書〕（計　0 件）**

**〔産業財産権〕**

**○出願状況（計　0 件）**

**名称：**
**発明者：**
**権利者：**
**種類：**
**番号：**
**出願年月日：**
**国内外の別：**

**○取得状況（計　0 件）**

**名称：**
**発明者：**
**権利者：**
**種類：**
**番号：**
**取得年月日：**
**国内外の別：**

**〔その他〕**
**ホームページ等**

## ６．研究組織

**(1)研究代表者**
J・P Bellier（J・P **ベリエ** Bellier, Jean-Pierre）
**滋賀医科大学・神経難病研究センター・助教**
**研究者番号：**80346022

**(2)研究分担者**
**林 維光（ハヤシ イコウ**, Ding, Wei-Guang）
**滋賀医科大学・医学部・助教**
**研究者番号：** 80242973

**(3)連携研究者**
（　　）

**研究者番号：**

**(4)研究協力者**
（　　）