Scandinavian Journal of Pain 5 (2014) 121–128

Contents lists available at ScienceDirect

Scandinavian Journal of Pain

journal homepage: www.ScandinavianJournalPain.com

Topical review

GCH1 variants, tetrahydrobiopterin and their effects on pain sensitivity☆

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HIGHLIGHTS

- *GCH1* gene mutations decrease pain sensitivity in experimental models of pain in humans and mice.
- *GCH1* gene variants in the general population reduce pain sensitivity and chronic pain.
- Reduced *GCH1* function decreases pain only after pain sensitisation in experimental models or in injury-related pain conditions.
- Inhibition of GCH1 enzyme activity causes antinociception in rat models of neuropathic and inflammatory pain.
- Pharmacological inhibition of GCH1 causes less antinociception in mice than in rats.

ARTICLE INFO

Article history:
Received 6 October 2013
Received in revised form
23 December 2013
Accepted 26 December 2013

Keywords:
GTP cyclohydrolase 1
Tetrahydrobiopterin
DOPA-responsive dystonia
Acute nociceptive pain
Inflammatory pain
Neuropathic pain

ABSTRACT

Background: A great proportion of the variation in pain experience and chronicity is caused by heritable factors. Within the last decades several candidate genes have been discovered either increasing or decreasing pain sensitivity or the risk of chronic pain in humans. One of the most studied genes is the *GCH1* gene coding for the enzyme GTP cyclohydrolase 1 (GCH1). GCH1 catalyses the initial and rate-limiting step in the biosynthesis of tetrahydrobiopterin (BH4). The main function of BH4 is regulation of monoamine and nitric oxide biosynthesis, all involved in nociceptive signalling.
Methods: In this topical review we focus on the implication of the *GCH1* gene and BH4 in painful conditions. We discuss experimental evidence from our group in relation to relevant research publications evaluating the BH4 pathway in pain. Studies assessing the role of GCH1 and BH4 in pain consist of human and animal studies, including DOPA-responsive dystonia (DRD) patients and *hph-1* mice (a genetic mouse model of DRD) having mutations in the *GCH1* gene as well as preclinical studies with the GCH1 inhibitor 2,4-diamino-6-hydroxypyrimidine (DAHP). The hypothesis is that genetic and pharmacological reduction of GCH1 would result in lower pain sensitivity.
Results: Previous studies have demonstrated that a particular "pain protective" *GCH1* haplotype, found in 15% of the general human population, is linked to decreased pain sensitivity. We further support these findings in DRD patients, showing normal thresholds to mechanical and thermal stimuli, whereas a trend towards lower pain sensitivity is seen following chemical pain sensitisation. Consistent with these observations, non-injured *hph-1* mice displayed normal mechano- and thermosensation compared to wild-type mice. After peripheral inflammation with Complete Freund' Adjuvant or sensitisation with capsaicin the mutant mice exhibited lower sensitivity to mechanical and heat stimuli. Moreover, *hph-1* mice showed decreased nociception in the first phase of the formalin test.

Several studies report analgesic effects of GCH1 inhibition with 90–270 mg/kg DAHP in rat models of inflammatory and neuropathic pain. However, we could not completely replicate these findings in

DOI of refers to article: http://dx.doi.org/10.1016/j.sjpain.2014.02.001.

Abbreviations: CFA, Complete Freund' Adjuvant; DAHP, 2,4-diamino-6-hydroxypyrimidine; DHPR, dihydropteridine reductase; L-DOPA, L-3,4-dihydroxyphenylalanine; DRG, dorsal root ganglia; GCH1, GTP cyclohydrolase 1; GTP, guanosine triphosphate; NAS, N-acetylserotonin; NO, nitric oxide; NOS, nitric oxide synthase; PAH, phenylalanine hydroxylase; PP, pain-protective; PCD, pterin-4a-carbinolamine dehydratase; PTPS, 6-pyruvoyltetrahydrobiopterin synthase; BH4, tetrahydrobiopterin; SNI, spared nerve injury; SNP, single nucleotide polymorphism; SR, sepiapterin reductase; TH, tyrosine hydroxylase; TPH, trypthophan hydroxylase; TRPV1, transient receptor potential vanilloid 1.

☆ The topical review is based upon the PhD thesis of Dr. Arafat Nasser, titled Involvement of the GCH1 gene and the cofactor tetrahydrobiopterin in pain—a comparative study in mice and man.

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http://dx.doi.org/10.1016/j.sjpain.2013.12.001

mice. Fairly higher doses of DAHP (≥270 mg/kg) were needed to reduce inflammatory pain in mice, but the window between antinociception and toxic effects was small, since 400 mg/kg DAHP affected motor performance and general appearance. Also, the analgesic effects were marginal in mice compared to that observed in rats.

Conclusions: Variations in the *GCH1* gene in both humans and mice appear to regulate pain sensitivity and pain behaviours, particularly after pain sensitisation, whereas pain sensitivity to phasic mechanical and thermal stimuli is normal. Moreover, pharmacological inhibition of GCH1 shows antinociceptive effects in preclinical pain studies, though our studies imply that GCH1 inhibition may have a small therapeutic index.

Implications: The implication of the *GCH1* gene in pain may increase our understanding of the risk factors of chronic pain development and improve current pain therapy by personalised medicine. In addition, inhibition of GCH1 provides a potential target for analgesic drug development, though GCH1 inhibitors should possess local or partial effects to avoid serious side-effects to the central nervous system and cardiovascular system.

Contents

1. Introduction

There is a general consensus that there are individual differences in the development of chronic pain, experience of pain and response to analgesic compounds. The reason(s) for these variations can be the genetic variability, previous pain experience, psychosocial factors, age and sex differences [1,2]. Although many factors may contribute to the risk of chronic pain, studies in rodent models of inflammatory and neuropathic pain [3,4] and acute nociceptive pain [4,5] as well as twin studies [6] imply that a great proportion (30–60%) of the variation in chronic pain conditions is caused by heritable factors.

Within the last two decades, several candidate genes have been identified by single nucleotide polymorphism (SNP) association studies that either increase or decrease pain sensitivity or increasing the risk of pain chronicity in humans. They include genes encoding several receptors, enzymes and ion channels implicated in the transmission, processing and modulation of nociceptive messages (for review see [7,8]). One of the most studied genes is the *GCH1* gene coding for the enzyme GTP cyclohydrolase 1 (GCH1) involved in the biosynthetic pathway of tetrahydrobiopterin (BH4). This topical review focuses on the implication of the *GCH1* gene in painful conditions as well as down-stream effects of BH4. Discussion of findings from our group in relation to relevant research publications evaluating the BH4 pathway in pain is presented.

2. General aspects of BH4

BH4 is a low-molecular-weight (241.25 Da), non-protein organic compound that belongs to the group of pteridines composed of a pyrimidine and a pyrazine ring (Fig. 1). It participates in enzymatic reactions as a donor or acceptor of chemical groups or electrons [9]. Below is given a short introduction to the biosynthesis, regulation and main functions of BH4.

2.1. Biosynthesis of BH4

The biosynthesis of BH4 is highly controlled by three main pathways: (i) the *de novo* synthetic pathway, (ii) the salvage pathway and (iii) the recycling pathway (Fig. 2). Biosynthesis of BH4 proceeds from guanosine triphosphate (GTP) *via* three reactions. The first and rate-limiting step is catalysed by GCH1, which converts GTP to 7,8-dihydroneopterin triphosphate. This is subsequently converted to 6-pyruvoyl-tetrahydrobiopterin catalysed by 6-pyruvoyltetrahydrobiopterin synthase (PTPS). The final reaction generates BH4 through reactions catalysed by sepiapterin reductase (SR) (Fig. 2). SR may also contribute to the salvage pathway by catalyzing the conversion of sepiapterin into 7,8-dihydrobiopterin, which is then transformed into BH4 by dihydrofolate reductase. Sepiapterin may also bind to the enzymes aldose reductase and carbonyl reductase, alternatively synthesising BH4 through the salvage pathway (Fig. 2). When BH4 for instance catalyses the hydroxylation of aromatic acids (see Section 2.3), it is oxidised

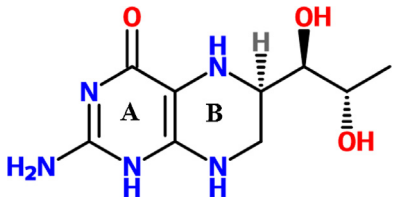

Fig. 1. Structure of tetrahydrobiopterin (BH4). BH4 is a small-molecule (241.25 Da) composed of a pyrimidine (A) and pyrazine (B) ring. Mainly, the heterocyclic ring system is involved in BH4 cofactor-dependent enzyme reactions [9].

to 4α-hydroxy-tetrahydrobiopterin. This in turn undergoes two conformations catalysed by pterin-4a-carbinolamine dehydratase (PCD) and dihydropteridine reductase (DHPR) regenerating BH4 (Fig. 2) [9].

2.2. Regulation of BH4 biosynthesis

The initial reaction carried out by GCH1 is presumed to be the key regulatory step of BH4 biosynthesis [9]. Though under basal conditions, sensory neuron activity of the synthetic pathway is low with the recycling and salvage pathways preserving BH4 homeostasis. New BH4 biosynthesis is therefore closely controlled [10]. The situation differs after injury or inflammation, as the *GCH1* gene, coding for GCH1, is markedly up-regulated in sensory neurons followed by increased GCH1 protein, GCH1 activity and BH4 biosynthesis [11]. This is further supported by *in vitro* studies showing substantial up-regulation of GCH1 in distinct cell cultures using pro-inflammatory cytokines [12], forskolin [13] and lipopolysaccharide [11].

2.3. Functions of BH4

BH4 is an essential cofactor for the three aromatic amino acid hydroxylases; phenylalanine hydroxylase (PAH), tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH). It is also required for the activity of the three isoforms of nitric oxide synthase (NOS), the neuronal NOS, inducible NOS and endothelial NOS. Consequently, BH4 is required for phenylalanine metabolism and for the synthesis of serotonin, dopamine, epinephrine, norepinephrine and nitric oxide (NO). Given the pleiotropic properties of BH4, induction or reduction of BH4 has been implicated in several pathological conditions such as pain [11,14], depression and anxiety (Nasser et al., unpublished work) as well as neurological diseases [9].

3. *GCH1* gene variants and pain

The association of the *GCH1* gene and pain was originally discovered in rats by Costigan et al. [15] using microarray based gene expression analysis. They demonstrated that the expression of the *GCH1* gene was considerably increased in dorsal root ganglia (DRG)

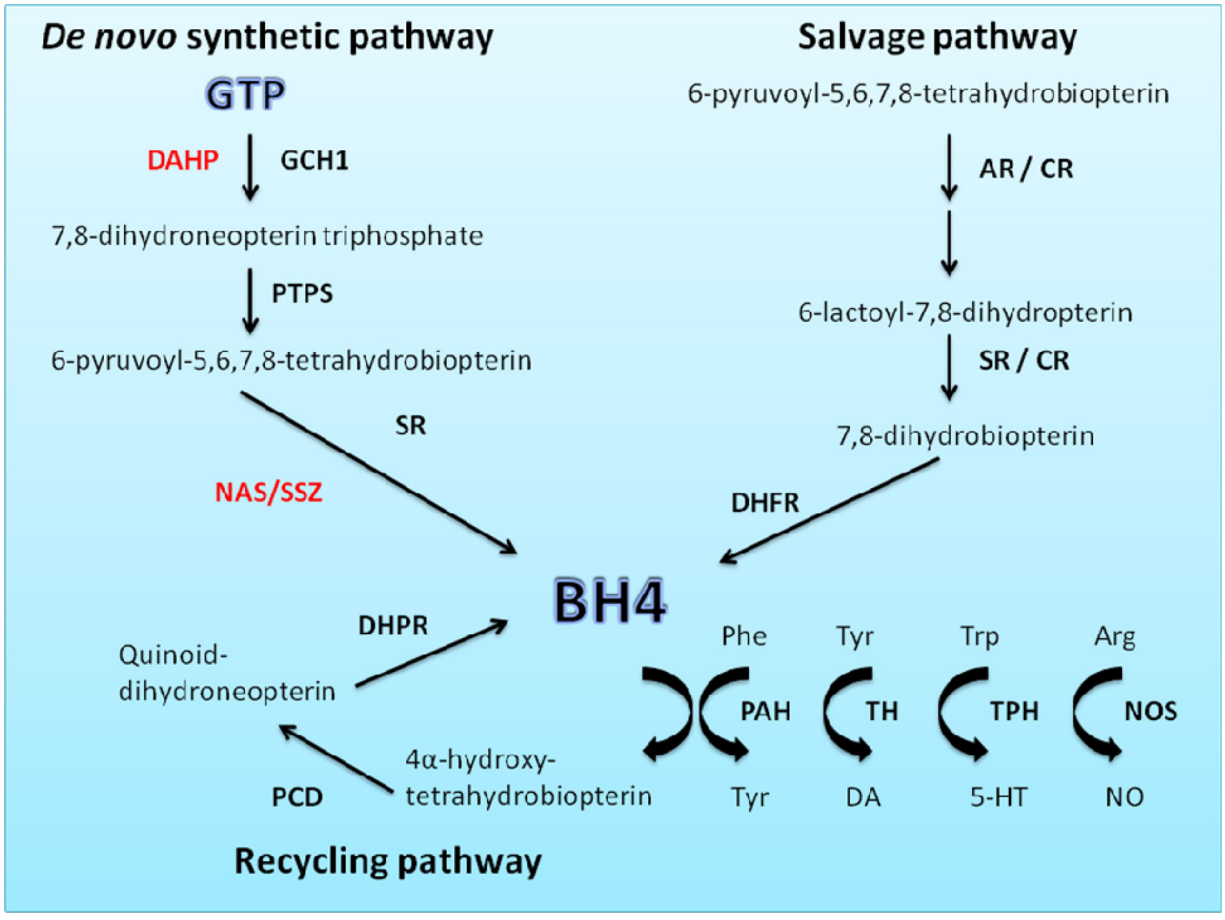

Fig. 2. The biosynthesis and main functions of BH4. Three main pathways control the biosynthesis of BH4: (i) the *de novo* synthetic pathway, (ii) the salvage pathway and (iii) the recycling pathway. BH4 is an essential cofactor for the aromatic amino acid hydroxylases (PAH, TH and TPH) as well as for all isoforms of NOS. Inhibitors of the synthetic pathway include DAHP, NAS and SSZ. *Abbreviations*: GTP, guanosine triphosphate; GCH1, GTP cyclohydrolase 1; PTPS, 6-pyruvoyltetrahydrobiopterin synthase; SR, sepiapterin reductase; AR, aldose reductase; CR, carbonyl reductase; DHFR, dihydrofolate reductase; Phe, phenylalanine; Tyr, tyrosine; Trp, tryptophan; Arg, arginine; PAH, phenylalanine hydroxylase; TH, tyrosine hydroxylase; TPH, trypthophan hydroxylase; NOS, nitric oxide synthase; DA, dopamine; 5-HT, serotonin; NO, nitric oxide; PCD, pterin-4-acarbinolamine dehydratase; DHPR, dihydropteridine reductase; DAHP, 2,4-diamino-6-hydroxypyrimidine; NAS, N-acetylserotonin; SSZ, sulfasalazine.

Table 1
Clinical studies investigating association of the "pain-protective" *GCH1* haplotype with experimental pain sensitivity, chronic pain and pain therapy.

References	Outcome measure/assessment	Phenotype/results
[11]	Mechanical, heat and ischaemic pain Chronic pain after lumbar discectomy	↓ Sensitivity to mechanical pain ↓ Post-surgical pain
[18]	Heat and cold pain Pain after removal of third molars	No association No association
[13]	Mechanical, heat, pressure and electrical pain Freeze lesion and capsaicin sensitisation	No association ↓ Pain hypersensitivity
[16]	Chronic and recurrent acute pancreatitis	No association
[29]	Capsaicin-evoked pain scores	↓ Pain sensitivity
[19]	Pain therapy in outpatients	↓ Duration of pain therapy
[17]	Chronic widespread pain	No association
[39]	Pain related outcomes during labour	No association
[20]	Cancer pain therapy	↓ Need for opioid therapy
[40]	ODI scores and NRS back pain scores after surgery for DDD	↓ Post-surgical pain ↓ ODI scores
[41]	Number of days until analgesia after third molar extraction	↑ Number of days until analgesia
[42]	Cancer pain therapy	No association
[43]	Persistent pain after mastectomy	No association
[44]	HIV-associated neuropathy	No association
[45]	Pain sensitivity in PVD	No association with pain sensitivity ↑ Benefit from HC therapy
[46]	Susceptibility and pain sensitivity in FM	↓ Susceptibility and pain sensitivity
[47]	HIV-associated neuropathy	No association

ODI: Preoperative Oswestry Disability; NRS, Numerical Rating Scale; DDD: degenerative disc disease; PVD, provoked vestibulodynia (genital); HC, Hormonal contraception; FM, fibromyalgia syndrome.

neurons after peripheral nerve axotomy. Later the same group reported that not only the *GCH1* gene but also the *SR* gene was up-regulated after nerve injury [11]. This was found to enhance the biosynthesis of BH4 in DRG neurons. Below, we review human and mice studies investigating associations between *GCH1* gene variants and pain.

3.1. Pain protective GCH1 haplotype in the general population

Tegeder et al. [11] were the first to identify a particular haplotype of the *GCH1* gene in humans, found to attenuate post-surgical pain after lumbar discectomy and pain sensitivity to mechanical pain in healthy controls (Table 1). This "pain protective" (PP) *GCH1* haplotype was found in 15% of the studied population, and consisted of 15 SNPs located in several non-coding regions [11]. The decreased pain sensitivity in carriers of the PP *GCH1* haplotype is thought to be a result of decreased transcription of *GCH1*, evaluated in *ex vivo* stimulated leukocytes, leading to reduced GCH1 function and BH4 biosynthesis [11,13]. Although several studies report positive association between *GCH1* and pain, several other groups failed to replicate these findings in both experimental and chronic pain conditions (Table 1). For instance, authors found no association with chronic pancreatitis [16], chronic wide spread pain [17] and pain following extraction of the impacted third molar [18]. It is difficult to define the cause of the negative results, but poor replication is a great caveat of association genetic studies [5]. It could also be that the effect of the PP *GCH1* haplotype depends on the type of pain measured, with the order of "pain protection" being neuropathic pain > inflammatory pain > acute nociceptive pain.

Variations in the *GCH1* gene do not only modulate pain sensitivity, but also pain therapy (Table 1). It has been demonstrated that carriers of the PP *GCH1* haplotype showed shorter pain therapy duration in outpatients with different chronic pain conditions as well as longer interval between cancer diagnosis and initiation of opioid treatment than heterozygous and non-carriers of the PP *GCH1* haplotype in cancer patients [19,20].

3.2. GCH1 mutations in humans

To further investigate the possible involvement of *GCH1* in pain, we utilised a different strategy evaluating if mutations in the *GCH1* gene influences pain sensitivity in humans (Møller A, Nasser A, Hellmund V, Bjerrum OJ, Jensen TS and Møller LB, unpublished work). Contrary to the PP *GCH1* haplotype leading to moderate reduction of BH4 biosynthesis and only after stimulation [11,13], autosomal dominant inherited "loss-of-function" mutations in the *GCH1* gene causes reduced baseline GCH1 activity and BH4 concentrations. This is associated with DOPA-responsive dystonia (DRD) [21,22], a rare movement disorder with the "classical" clinical characterisation of gait problem due to dystonia, mostly in the lower extremities with onset in childhood [23,24]. Treatment with L-3,4-dihydroxyphenylalanine (L-DOPA) either plain or with a decarboxylase inhibitor remains the most effective treatment of DRD [22,23].

The experimental human studies in patients suffering from DRD are still on-going; however preliminary work indicates that acute nociceptive thresholds to mechanical and thermal stimuli are similar in DRD patients and controls, while a trend for lower pain sensitivity was evident in patients following chemical pain sensitisation (Møller et al., unpublished work).

3.3. Gch1 mutations in mice

We also used the hyperphenylalaninemia 1 (*hph-1*) mouse model considered to be a model of DRD [9,25]. The mouse model was originally developed using the sperm mutagen, N-ethyl-N′-nitrosurea, which induces random point mutations [26]. Like DRD patients, the mutant mice exhibit marked basal reduction in *Gch1* expression accompanied by reduced GCH1 activity and BH4 biosynthesis. In addition, both humans and mice display reduced TH protein and TH activity in striatum associated with reduced production of dopamine [27,28]. In contrast to the DRD patients, *hph-1* mice do not appear to display any motor deficit or dystonia-like symptoms [14].

To elucidate whether the findings in DRD patients translates back to the *hph-1* mouse we set out to investigate if *hph-1* mice displayed altered phenotype in mouse behavioural models of acute and inflammatory pain. Comparable to the human studies, we demonstrated that non-injured mutant mice exhibited normal mechano- and thermosensation compared to non-injured wild-type mice. After peripheral inflammation with Complete Freund' Adjuvant (CFA) or sensitisation with capsaicin the mutant mice exhibited lower sensitivity to mechanical and heat stimuli [14]. These findings are not only consistent with findings in DRD patients, but also with previous studies showing that the pain protective effect of the *GCH1* haplotype appeared to be confined to pain hypersensitivity following capsaicin sensitisation, whereas the effect on acute nociceptive pain appeared subtle [13,29]. All these

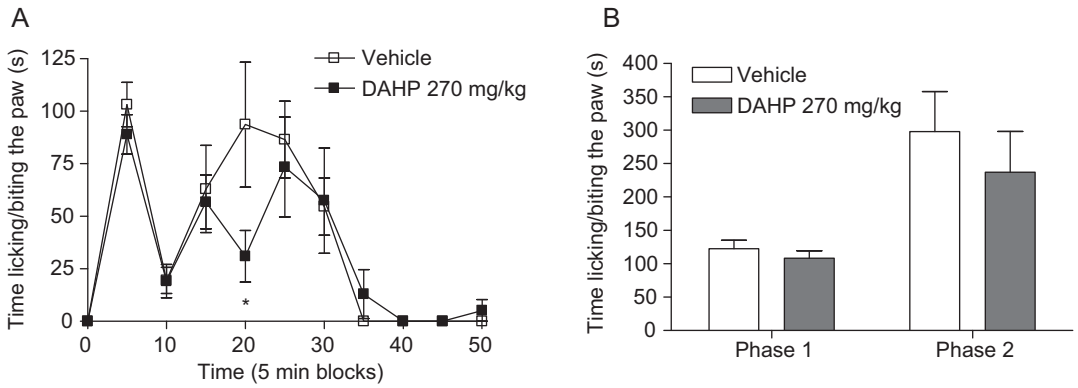

Fig. 3. Effect of 270 mg/kg DAHP on formalin-induced pain. (A) 20 μl of 2.5% (v/v) formalin was injected intraplantar into one hind-paw and the time spent licking or biting the paw was recorded during 50 min. For experimental details see [14]. Acute intraperitoneal administration of 270 mg/kg DAHP (Sigma–Aldrich) 60 min before formalin injection did not significantly change the pattern of the formalin test ($p > 0.05$). Pairwise comparisons revealed a statistical significant reduction in licking behaviour at 20 min after formalin injection (*$p = 0.035$). (B) The first phase and second phase were defined as pain behaviour during 0–10 min and 10–50 min, respectively. No statistical significant difference was observed between DAHP- and vehicle-treated mice in both phases ($p > 0.05$). Data are presented as mean ± SEM and + SEM, $n = 6$. Data were analysed with two-way RM-ANOVA with pair-wise comparisons using Fisher' LSD test or Student' *t*-test.

observations suggest that *GCH1* and hence BH4 modulates pain hypersensitivity after an injury or pain sensitisation. In addition to earlier findings, our studies imply that even though baseline *GCH1* expression and BH4 biosynthesis are substantially reduced, pain sensitivity to mechanical and heat stimuli are still not influenced. Nevertheless, an effect on acute nociceptive pain cannot be ruled out, since we demonstrated that *hph-1* mice had lower pain-like behaviours in the first phase of the formalin test. This is thought to correspond to acute nociceptive pain due to direct effects of formalin on the nociceptors [14].

4. Inhibition of BH4 biosynthesis and pain

A number of preclincal studies have demonstrated that inhibition of enzymes involved in the biosynthetic pathway of BH4 produces analgesic effects in different animal models of pain. These studies include acute and chronic administration of inhibitors either by systemic injections or intrathecal infusions. Inhibitors of BH4 have not yet been tested in clinical settings. Below, we review the different preclinical studies.

4.1. Inhibition of the GCH1 enzyme

Tegeder and co-workers were the first to discover BH4 as an endogenous regulator of inflammatory and neuropathic pain in rats [11]. As mentioned in Section 3, the authors demonstrated that peripheral inflammation and nerve injury up-regulated GCH1 activity with the result of enhanced biosynthesis of BH4 in DRG neurons. Inhibition of the enzyme GCH1 with 2,4-diamino-6-hydroxypyrimidine (DAHP), markedly decreased this excess BH4 production. This was found to reduce pain responses in the formalin test and the CFA model of inflammatory pain. Moreover, acute and chronic administration of DAHP attenuated mechanical and cold hypersensitivity in the spared nerve injury (SNI) model of neuropathic pain [11]. Unlike injury-evoked pain conditions, inhibition of GCH1 did not influence behavioural responses to phasic heat and mechanical stimuli. The authors also demonstrated that intrathecal injection of BH4 induced heat hypersensitivity in non-injured rats and increased pain-like hypersensitivity following nerve injury and inflammation [11]. These observations indicate a correlation between BH4 biosynthesis and chronic pain conditions. GCH1 inhibition has also been proposed to improve opioid treatment in cancer pain. Recently, a combination of GCH1 inhibition and morphine was found to increase and prolong the analgesic effects of morphine in a murine model of cancer pain, suggesting a possible co-therapeutic strategy of GCH1 inhibition and opioid treatment in cancer patients [30].

Although Tegeder et al. [11] reported significant effects of DAHP administration on inflammatory pain in rats, we were not able to fully replicate these findings in mice. Fig. 3 shows the behavioural responses in the formalin test using male C57BL/6 mice. Intraplantar (i.pl.) injection of 2.5% (v/v) formalin produced the classical biphasic response of licking and biting the paw with the first and second phase defined as responses during 0–10 min and 10–50 min, respectively. Statistical analysis showed no significant overall effect of 270 mg/kg DAHP treatment on formalin-induced pain behaviour ($p > 0.05$, Fig. 3A). Pairwise comparisons revealed a significant decrease in pain-like responses at 20 min after formalin injection ($p = 0.035$). The duration of nociceptive responses in the distinct phases were also not altered by injection of 270 mg/kg DAHP ($p > 0.05$, Fig. 3B), whereas a dose of 180 mg/kg inhibited nociception in this test in rats [11]. Increasing the dose to 400 mg/kg significantly decreased formalin-induced nociception in both phases compared with vehicle-treated mice. However, this was confounded by motor impairment, since 400 mg/kg markedly decreased performance in the rotarod test, a test used to evaluate balance and coordination (Nasser et al., unpublished observations).

Fig. 4A shows the effect of 180–350 mg/kg DAHP on mechanical pain hypersensitivity 24 h after CFA-induced paw inflammation. Statistical analysis showed an almost significant overall effect of DAHP treatment on mechanical hypersensitivity ($p = 0.091$). Subsequent pairwise comparisons revealed a significant reduction in mechanical hypersensitivity 180 min after injection of 270 mg/kg DAHP as well as 30 and 90 min after injection of 350 mg/kg DAHP ($p < 0.05$). Furthermore, we tested 270 mg/kg DAHP in the spinal nerve transection model and found a weak effect on neuropathic pain hypersensitivity (Nasser et al., unpublished observations). With these doses we observed no effects on motor performance in the rotarod test (Fig. 4B).

The reason (s) for the inconsistencies between our findings and those of Tegeder et al. [11] is not clear. It is possible that variability in pharmacokinetic properties, tissue distribution and metabolism of DAHP between rat and mice contribute to the observed discrepancy as well as species differences in both nociceptive and antinociceptive sensitivity. Additionally, C57BL/6 mice may be less sensitive to the effects of DAHP, and the outcome could be different in other mouse strains. In fact, the C57BL/6 mice have been shown to be less sensitive to a number of known analgesic drugs compared to other mouse strains [31]. Finally, methodological differences such as

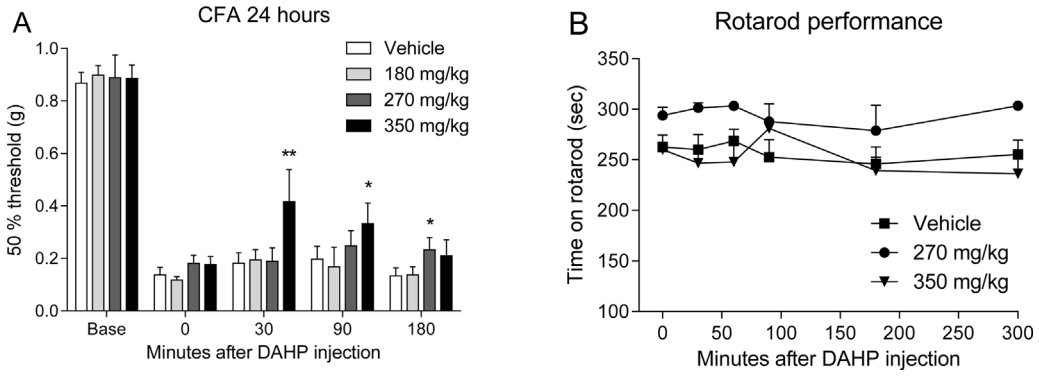

Fig. 4. Effect of DAHP on CFA-evoked mechanical hypersensitivity and motor function. (A) Mechanical pain hypersensitivity was measured using von Frey hairs and the up-and-down method. For experimental details see [14]. DAHP (Sigma–Aldrich) 180–350 mg/kg or vehicle were given intraperitoneal 24 h after intraplantar injection of CFA. The results showed that 270 mg/kg DAHP significantly changed mechanical pain hypersensitivity, but only 180 minutes after DAHP injection ($p > 0.05$). Administration of 350 mg/kg DAHP showed statistical significant reduction in mechanical hypersensitivity 30 and.90 min after injection. $^{*}p < 0.05$ *vs.* vehicle. $n = 8–16$. (B) 270 mg/kg and 350 mg/kg DAHP was administered intraperitoneal and the latency time (sec) to fall off the rotarod apparatus was tested 30, 60, 90, 180 and 300 min after injection. For experimental details see [14]. Data revealed that 270 and 350 mg/kg DAHP did not affect rotarod performance ($p > 0.05$). $n = 6$. Data are presented as mean + SEM, and were analysed by two-way RM-ANOVA with pair-wise comparisons using Fisher' LSD test.

variation in the scored behavioural response (licking *vs.* flinching) and stimuli modality (heat *vs.* mechanical) might also contribute to the observed discrepancy. Nevertheless, Tegeder et al. [11] did not report whether doses shown to have antinociceptive effects in rats produced motor impairment, and hence it cannot be ruled out that the observed effects could be secondarily to motoric confounds.

In summary, animal studies imply that molecules capable of inhibiting GCH1 may have antinociceptive properties. However, our findings that doses ≥270 mg/kg DAHP were required to observe antinociceptive effects, and that doses >350 mg/kg decreased motor function and affected general appearance of mice, suggest that the window between antinociception and toxic effects might be narrow. Compounds targeting GCH1 should therefore have local and/or partial effects in order to avoid side-effects related to both the central nervous system and the cardiovascular system. For instance, local disruption of GCH1 in DRG with small hairpin RNA introduced to the sciatic nerve have been demonstrated to show analgesic effects in the rat SNI model, suggesting GCH1 modulation as a potential gene therapy strategy for neuropathic pain treatment [32].

4.2. Inhibition of the SR enzyme

Competitive inhibition of the last step involving SR could also be a potential target. There are several SR inhibitors [33], though N-acetylserotonin (NAS) is the only compound that has been evaluated in animal pain models. Intrathecal infusion of NAS decreased the pathologically increased BH4 concentrations and resulted in attenuated inflammatory and neuropathic pain behaviours in rats [11]. An interesting hypothesis is that SR could be a more attractive drug target than GCH1, since basal BH4 levels may be conserved by the salvage pathway after SR inhibition (see Section 2.1 and Fig. 2), but not after GCH1 inhibition [10]. Therefore, further studies evaluating this hypothesis and testing the efficacy of SR inhibition in chronic pain is needed. A potential drug is sulfasalazine, currently used to treat rheumatoid arthritis and colitis ulcerosa. Sulfasalazine and its metabolites sulfapyridine and N-acetylsulfapyridine have recently been shown to be inhibitors of SR, and they were found to reduce intracellular BH4 concentrations in pheochromocytoma cells. These compounds also exhibited higher potency than that of NAS [34]. Sulfasalazine has been reported to show analgesic effects in streptozotocin-induced diabetic rats [35]. Since sulfasalazine is a well-known and relatively safe medicine, it is likely that it will also show clinical importance in the treatment of neuropathic pain in humans [10].

4.3. Inhibition of the PTPS enzyme

It is suggested that the PTPS enzyme is a less attractive target for drug development. The active site of the enzyme is more open than that for GCH1 rendering it more difficult to develop small molecules [33]. Additionally, the phosphate groups of dihydroneopterin triphosphate (Fig. 2) are central for effective substrate binding with substantial reduction in affinity with for instance dihydroneopterin monophosphate [33]. These observations may explain why no small-molecule inhibitors of PTPS exist. Consequently, PTPS inhibition has not yet been evaluated in preclinical models of pain, and the expression pattern of PTPS following injury is also not known.

5. Possible mechanisms of action of BH4

The exact mechanisms involved in the “pain producing” effect of BH4 has yet to be identified. Based on the physiological effects of BH4, it may act through mechanisms involving serotonin, catecholamines and/or NO. These molecules are known to play a complex role in pain signalling, exhibiting both facilatory and inhibitory effects on the nociceptive system [36,37]. So far data suggest that BH4 acts partly through NO related mechanisms [11]. After nerve injury neuronal NOS was up-regulated in DRG neurons followed by increased NO synthesis. This was completely reversed by inhibition of BH4 synthesis. Moreover, calcium imaging with cultured DRG neurons showed that BH4 concentration-dependently induced intracellular calcium concentrations. This enhanced calcium influx was partly reduced by the non-specific NOS inhibitor Nω-Nitro-L-arginine methyl ester [11]. The moderate reduction in BH4-induced calcium influx by NOS inhibition suggests the possibility of additional effects of BH4 unrelated to its cofactor function. The potential involvement of NO is also supported by experimental human studies showing that carriers of the PP *GCH1* haplotype exhibited lower up-regulation of inducible NOS transcript in lipopolysaccharide stimulated leukocytes compared to non-carriers [13]. It is therefore reasonable that reduced biosynthesis of BH4 results in lower NO release, which in turn results in decreased sensitisation of the nociceptive system during inflammatory and neuropathic pain conditions.

A direct effect of BH4 was noted by Tegeder et al. [13], whom suggested that BH4 may activate transient receptor potential vanilloid 1 (TRPV1) at primary afferent terminals. This was based on the fact that BH4 is a redox molecule and on studies demonstrating that TRPV1 can be activated by redox-active agents [38]. The

consequence of modulation of TRPV1 activity is facilitation of peripheral sensitisation leading to increased pain hypersensitivity. In our laboratory, unpublished work support the assumption of a direct effect of BH4 on the nociceptors. Work is on-going to elucidate whether this effect is mediated by TRPV1 receptors and/or other ion channels located at the primary afferent terminals.

6. Conclusions and future directions

Discovery of genetic variants that modulates pain sensitivity and chronicity may provide not only an understanding of the risk factors of chronic pain development, but also propose a more personalised pain therapeutic approach and potential new targets for analgesic drugs [5,8]. Our studies as well as other' indicate that the *GCH1* gene may be such a gene, though further studies are warranted to elucidate the exact roles of such genetic variability as well as their clinical implications.

Preclinical studies show that BH4 inhibition with GCH1 and SR inhibitors produce analgesic effects in rats. While this could indicate that these enzymes are potential new targets for development of analgesic drugs, we were not able to find significant analgesic effects of GCH1 inhibition in mice. Moreover, our studies imply that GCH1 inhibition may have a small therapeutic index. Therefore, further studies evaluating the effects of GCH1 and SR inhibition on chronic pain is highly needed.

Understanding the exact mechanisms of the BH4 pathway in pathological pain conditions is also important. Future studies should aim to explore its exact role in both the peripheral and central nervous system. On-going work is investigating the possibility of NO-related mechanisms and potential direct effects of BH4 on the nociceptors. However, the potential involvement of the biogenic amine neurotransmitters should not be neglected.

Funding sources

The original studies were supported by Lundbeck Foundation, Danish Medical Research Council, A.P. Møller Foundation, Brødrene Hartmann Foundation, Beckett Foundation and Aase & Ejnar Danielsens Foundation.

Conflict of interest

None to declare.

Acknowledgement

The PhD project was part of a translational study with collaboration between three organisations located in Denmark: Kennedy Center in Glostrup, Danish Pain Research Center in Aarhus and Faculty of Health and Medical Sciences in Copenhagen. Therefore, we wish to thank professor Troels S. Jensen, Dr.med. Anette Torvin Møller and Vibe Hellmund for organising and conducting the experimental human studies, and for the great scientific discussions. Arafat Nasser also owes his deepest gratitude to his co-supervisor professor Ole J. Bjerrum for his valuable support and supervision, and for proof-reading and commenting on this manuscript.

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