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Evers, Roeland A F; van Vliet, Danique; van Spronsen, Francjan J

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# Tetrahydrobiopterin treatment in phenylketonuria: a repurposing approach

Roeland A.F. Evers[1], Danique van Vliet[1], Francjan J. van Spronsen[1].

[1]University of Groningen, University Medical Center Groningen, Beatrix Children's Hospital, Division of Metabolic Diseases, The Netherlands.

Address correspondence to:
Prof. dr. F.J. van Spronsen
University Medical Center Groningen
Hanzeplein 1
9700 RB Groningen
The Netherlands
Mail: f.j.van.spronsen@umcg.nl
Telephone: +31(0)50-3614147

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Abstract

In phenylketonuria (PKU) patients, early diagnosis by neonatal screening and immediate institution of a phenylalanine-restricted diet can prevent severe intellectual impairment. Nevertheless, outcome remains suboptimal in some patients asking for additional treatment strategies. Tetrahydrobiopterin ($BH_4$) could be one of those treatment options, as it may not only increase residual phenylalanine hydroxylase activity in $BH_4$-responsive PKU patients, but possibly also directly improves neurocognitive functioning in both $BH_4$-responsive and $BH_4$-unresponsive PKU patients. In the present review, we aim to further define the theoretical working mechanisms by which $BH_4$ might directly influence neurocognitive functioning in PKU having passed the blood-brain barrier. Further research should investigate which of these mechanisms are actually involved, and should contribute to the development of an optimal $BH_4$ treatment regimen to directly improve neurocognitive functioning in PKU. Such possible repurposing approach of $BH_4$ treatment in PKU may improve neuropsychological outcome and mental health in both $BH_4$-responsive and $BH_4$-unresponsive PKU patients.

Take-home message:

Tetrahydrobiopterin may directly improve neurocognitive functioning in phenylketonuria patients through several hypothesized mechanisms that all require further research.

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All authors were involved in the design and drafting of this review.

**Name of the corresponding author**
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## Introduction

Given its success, treatment of phenylketonuria (PKU; OMIM 261600) has classically focused on controlling blood phenylalanine (Phe) concentrations to prevent irreversible intellectual disability. This is mainly done by institution of a Phe-restricted diet following a positive neonatal screening test for PKU (Blau et al 2010). Additionally, 20-50% of PKU patients benefit from chaperone activity of tetrahydrobiopterin ($BH_4$) for the Phe hydroxylase enzyme (PAH; EC 1.14.16.1) (Keil et al 2013). In these so-called $BH_4$-responsive patients, pharmacological treatment with $BH_4$ results in increased PAH activity, leading to a decrease in blood Phe concentrations and/or an increase in natural protein tolerance. Notwithstanding the effects of the Phe-restricted diet and/or $BH_4$ treatment to prevent severe intellectual disability, some early-treated PKU patients still show mild impairments in executive and social-cognitive functioning and social skills, and are prone to develop anxiety and depressive symptoms (Smith and Knowles 2000, Jahja et al 2014, Jahja et al 2016). These findings have often been attributed to Phe neurotoxicity and cerebral neurotransmitter impairments (Dyer 1999, Christ et al 2010).

Interestingly, anecdotally, some $BH_4$-unresponsive PKU patients experience a better ability to concentrate and less behavioural problems on $BH_4$ supplementation during the short periods of time when being tested for $BH_4$-responsiveness, despite not showing a decrease in blood Phe concentrations (Cederbaum 2011). Although these reports may simply indicate placebo effects, it raises the question whether $BH_4$ could directly improve neurocognitive and psychosocial functioning beyond its effect of reducing blood Phe concentrations in $BH_4$-responsive PKU patients. This hypothesis is further substantiated by a pilot study in late-diagnosed PKU patients with maladaptive behaviour, of whom

most were $BH_4$-unresponsive, showing improved behaviour after 1 year of $BH_4$ treatment (20 mg/kg/day) (Moseley et al 2015). Thereby, if $BH_4$ will indeed be shown to directly improve neurocognitive functioning beyond its effect on the PAH enzyme, such a repurposing approach may extend the target population of $BH_4$ treatment.

Different working mechanisms, substantiated by different levels of evidence, may underlie such a possible direct beneficial effect of $BH_4$ on neurocognitive functioning in PKU patients as summarized in Figure 1 and Table 1. In the present review, we aim to further define these theoretical working mechanisms in order to stimulate further research on the possible neurocognitive effects of $BH_4$ to ultimately be able to use $BH_4$ in PKU patients to its full potential.

**$BH_4$ and the brain**

Before discussing several mechanisms through which $BH_4$ could directly improve neurocognitive functioning, this review will focus on the question to what degree $BH_4$ supplementation can increase cerebral $BH_4$ concentrations. It is known that orally administered $BH_4$ is largely rapidly excreted through feces and urine, the latter being facilitated by high-capacity organic anion transporters in the kidney (Ohashi et al 2012, Ohashi et al 2017). $BH_4$ furthermore shows slow transport across cell membranes, compared to related pterins such as sapropterin and dihydrobiopterin ($BH_2$) (Ohashi et al 2017). Next to this, $BH_4$ is an instable molecule that is easily oxidized to $BH_2$, although it appears $BH_2$ is then intracellularly reconverted into $BH_4$ (Ohashi et al 2016). Overall, $BH_4$ supplementation does increase plasma $BH_4$ concentrations (Zurflüh et al 2006), albeit very inefficiently, but it is unclear to what extent $BH_4$ then crosses the blood-brain

barrier (BBB). At least in part, this question is prompted by the experience of $BH_4$ treatment in patients with $BH_4$ deficiency, resulting in a disturbed function of PAH, as well as of tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH). Patients with $BH_4$ deficiency due to for example a defect in dihydropteridine reductase only benefit from $BH_4$ treatment with regard to liver Phe metabolism, and not with regard to cerebral TH and TPH activity (Ponzone et al 2004), suggesting $BH_4$ does not reach the brain. This similarly applies to patients with $BH_4$ deficiency as a result of 6-pyruvoyl tetrahydropterin synthase deficiency (Porta et al 2009). While this seems to contrast with the previously mentioned reports of direct neurocognitive effects of $BH_4$ in PKU patients, these discrepancies could be explained by a difference in $BH_4$ dosage. Namely, the dose of $BH_4$ in responsiveness testing and treatment in PKU can be much higher (up to 20 mg/kg body weight) compared to the dose of $BH_4$ in the treatment of $BH_4$ defects (usually below 10 mg/kg body weight). It has indeed been shown that $BH_4$ could pass the BBB and increase cerebral $BH_4$ in a dose-dependent manner in mice (Thony et al 2008). Moreover, some studies suggest that $BH_4$ could also reach the brain in humans. $BH_4$ administration (20 mg/kg/day) in children with autism spectrum disorder has also been shown to improve social awareness, autism mannerisms, hyperactivity, and inappropriate speech (Klaiman et al 2013). High doses of $BH_4$ are probably necessary to cross the BBB, as shown by increased biopterin concentrations in cerebrospinal fluid (CSF) in humans and rhesus monkeys following $BH_4$ administration at 20 mg/kg or higher (al Aqeel et al 1992, Ishida et al 1988, Kaufman et al 1982, Miller et al 1986), but not at lower doses (al Aqeel et al 1992). Overall, while some studies indicate that $BH_4$ supplementation may increase cerebral $BH_4$ concentrations, it is important to emphasize that the extent to which

this happens is not yet clear and this essential question requires further research. Higher dosages of $BH_4$ than currently used may be necessary to establish an increase in cerebral $BH_4$ that could result in relevant therapeutic effects. Alternatively, the use of sepiapterin, which is a precursor of $BH_4$, may be considered for this purpose. Although the exact mechanism has not yet been elucidated, it has been shown that administration of sepiapterin is more effective in increasing intracellular $BH_4$ concentrations compared to administration of $BH_4$ directly, with the latter resulting in a larger increase in intracellular $BH_2$ concentrations (Hasegawa et al 2005). Moreover, a very recent clinical trial showed that administration of a therapeutic formula of sepiapterin was well-tolerated in healthy subjects, and increased plasma $BH_4$ concentrations more efficiently compared to $BH_4$ administration (Smith et al 2019).

$BH_4$ toxicity

Some reports have warranted against the indiscriminate use of high-dose $BH_4$ treatment because of possible toxicity. In one study in $BH_4$-deficient mice, acute subcutaneous $BH_4$ administration at 300 mg/kg resulted in the death of 2 of 12 animals (Brand et al 1996), which has been suggested to be related to nonspecific stimulation of NO synthesis (Brand et al 1996, Klatt et al 1992). Such apparent toxicity, however, has not been observed in mice at subcutaneous administration of lower doses (30 or 90 mg/kg) (Brand et al 1996) or at subchronic oral administration up to 100 mg/kg (Thony et al 2008, Brand et al 1996) or acute oral administration up to 1300 mg/kg (Lewandowski, Combs and Folkers 1986). For subchronic intraperitoneal $BH_4$ administration in mice, the median lethal dose has been found to be 260 mg/kg (Lewandowski et al 1986). In PKU patients, $BH_4$ treatment (up to 20 mg/kg/day) is considered safe, with a low rate of adverse effects (Longo et al 2015).

**$BH_4$ and monoaminergic neurotransmitter synthesis**

As a first hypothetical working mechanism by which $BH_4$ may directly improve neurcognitive functioning in PKU patients, $BH_4$ is suggested to increase cerebral monoaminergic neurotransmitter synthesis. Cerebral monoaminergic neurotransmitter deficiencies are considered an important pathophysiological factor underlying brain dysfunction in PKU (Surtees and Blau 2000), resulting from insufficient brain uptake of their amino acid precursors (tyrosine (Tyr) and tryptophan (Trp)) and/or inhibition of TH and TPH – the enzymes being responsible for the rate limiting steps in dopaminergic and serotonergic neurotransmitter synthesis – by elevated brain Phe concentrations (de Groot et al 2010). In both living and deceased PKU patients, decreased Tyr and Trp levels in brain as well as reduced dopaminergic and serotonergic metabolites in CSF have been reported (Burlina et al 2000, McKean 1972). Moreover, a reduction in TH protein expression of 40% in medial prefrontal cortex of the BTBR *Pah-enu2* PKU mouse model has been observed (Pascucci et al 2012). No such reductions have been found for cerebral protein expression of TPH in this PKU mouse model, although enzyme activity levels were shown to be significantly reduced (Pascucci et al 2009).

$BH_4$ may directly stimulate cerebral monoaminergic synthesis in PKU patients, although the catecholaminergic and serotonergic system might respond differently to $BH_4$ administration. This hypothesis is substantiated by various findings. Firstly, with regard to the catecholaminergic system, TH activity and protein expression significantly increased in wild-type mice following oral $BH_4$ administration (20 and 100 mg/kg) (Thony et al 2008). Also, in $BH_4$ knock-out mice, chronic intraperitoneal $BH_4$ administration (50 mg/kg) improved TH activity, thereby increasing brain dopamine

concentrations (Sumi-Ichinose et al 2001), and the reduction of TH protein expression in striatum was partly reversed by $BH_4$ administration (50 mg/kg) (Sumi-Ichinose et al 2001, Homma et al 2013). Additionally, as observed by *in vivo* microdialysis, dopamine concentrations increased on $BH_4$ infusion in striatum of healthy rats, and this effect was further enhanced by continuous infusion of Tyr at a relatively low dose (Tsukada et al 1994). Secondly, with regard to the serotonergic system, acute $BH_4$ administration in $BH_4$ knock-out mice strongly increased brain serotonin concentrations without any effect on TPH activity (Sumi-Ichinose et al 2001). In line with that study, $BH_4$ treatment (50 mg/kg/day) in *Pah-enu1/2* mice led to a partial recovery of brain serotonin levels, but again without increased TPH activity (Scherer et al 2018).

Nevertheless, the effects of $BH_4$ administration in mice and rats are somewhat inconclusive, with $BH_4$ administration (20, 40 and 100 mg/kg) in one study not resulting in changed dopamine and serotonin concentrations in wild-type mice (Thony et al 2008), while other studies, in wild-type rats and $BH_4$ knock-out mice, respectively, showed increased biosynthesis of both monoaminergic neurotransmitters (Miwa, Watanabe and Hayaishi 1985, Brand et al 1996), although this effect was only achieved at toxic dose (300 mg/kg) in one of these studies (Brand et al 1996)

More recently, blood and urine melatonin and urine dopamine concentrations, that are thought to reflect brain serotonin and dopamine availability in the CNS, were found to be not changed by $BH_4$ administration in $BH_4$-responsive and $BH_4$-unresponsive patients (Yano et al 2016). On the other hand, observations in $BH_4$-responsive PKU patients suggested a direct positive effect of $BH_4$ on cerebral dopamine bio-availability beyond its effect through lowering blood Phe concentrations (van Vliet et al 2015). In this study, in

male $BH_4$-responsive PKU patients, blood prolactin concentrations – as a peripheral parameter to reflect cerebral dopamine concentrations – were found to be significantly lower on $BH_4$ treatment if compared to treatment without $BH_4$, even when correcting for blood Phe concentrations, and tended to be lower at increasing $BH_4$ dose. It is important to emphasize, however, that both studies assessed indirect measures of brain monoaminergic neurotransmitters.

Overall, while reports are still inconclusive, studies in PKU mice and patients suggest a benefit of $BH_4$ treatment to directly improve brain monoaminergic neurotransmitter synthesis in PKU (Table 1), which consequently might lead to improved neurotransmitter release and neurotransmission. The different responses of the catecholaminergic and serotonergic system, as well as the possible beneficial effects on neurocognitive functioning in PKU patients, certainly warrant further investigations.

**$BH_4$ and neuronal monoaminergic neurotransmitter release**

Besides stimulating monoaminergic neurotransmitter synthesis, $BH_4$ in PKU patients may also improve neurocognitive functioning by increasing neuronal dopamine and serotonin release either directly or through stimulation of NO production. Neuronal release of monoaminergic neurotransmitters has not been investigated in PKU patients, but research in the PKU mouse model has shown clear impairments in neuronal monoamine release (Pascucci et al 2009, Pascucci et al 2012). Although impaired neuronal monoaminergic neurotransmitter release in PKU could partly be explained by insufficient neurotransmitter synthesis, this may not be the only underlying mechanism. Firstly, disturbed NO metabolism in PKU might contribute to the observed impairments in

neuronal monoaminergic neurotransmitter release. Disturbed NO metabolism was reported in both PKU patients (Huemer et al 2012, Okano and Nagasaka 2013, Sanayama et al 2011) and in PKU mouse brain (Embury, Reep and Laipis 2005), and is hypothesized to result from increased oxidative stress. Cerebral NO is involved in both synaptic and nonsynaptic neurotransmission. As such, impaired cerebral NO metabolism in PKU can be suggested to contribute to dysfunctional monoaminergic neurotransmission. Secondly, alterations in neuronal $Ca^{2+}$ channels have been reported in cell studies, which may be related to impaired neuronal monoamine release in PKU (Dzhura et al 1998, Zhang and Gu 2005). These alterations in $Ca^{2+}$ channels are hypothesized to result from the disturbed amino acid balance in the PKU brain (Dzhura et al 1998, Kostyuk, Martynyuk and Pogorelaya 1991, Martynyuk, Savina and Skibo 1991).

Several studies, although mostly not related to PKU, suggest that $BH_4$ has an effect on monoaminergic neurotransmitter synthesis. *In vivo* microdialysis with intracerebral $BH_4$ infusion was found to increase neuronal dopamine and serotonin release in a dose-dependent manner in striatum from healthy rats (Koshimura et al 1990, Koshimura, Miwa and Watanabe 1994, Koshimura et al 1995, Mataga, Imamura and Watanabe 1991, Wolf et al 1991). For dopamine, a similar effect of $BH_4$ has been observed in rat frontal cortex (Mataga et al 1991). Furthermore, a more recent study showed increased metabolism of serotonin and dopamine in PKU mice despite unaltered concentrations of these neurotransmitters, which might be explained by an increase in synaptic monoaminergic neurotransmitter release (Winn et al 2016).

This effect of $BH_4$ may be exerted by two different mechanisms that are theoretically related to the causes of impaired neuronal monoaminergic neurotransmitter release in

PKU. Firstly, $BH_4$ is (together with flavin adenine dinucleotide and flavin mononucleotide) one of the essential cofactors for NOS, catalyzing the conversion of arginine into NO and citrulline, also in the brain. Research in both rats and mice indeed suggest an increase in cerebral NO production by NOS following $BH_4$ administration (Fabian, Perez-Polo and Kent 2008, Koshimura et al 2004). Extensive research, as reviewed by Kiss (2000), has shown that NO influences neuronal release of monoaminergic neurotransmitters (Kiss 2000). Although controversy exists concerning the exact role of NO in monoaminergic neurotransmission, the majority of data has indicated that NO stimulates dopaminergic, noradrenergic, and serotonergic neuronal release (Kiss 2000). Secondly, besides acting through NO, $BH_4$ has also been shown to directly enhance neuronal dopamine and serotonin release (Koshimura et al 1990, Koshimura et al 1994, Wolf et al 1991, Koshimura et al 2000, Koshimura et al 1992) independent of its cofactor activity. Such effect would be mediated by activation of $Ca^{2+}$ channels (Wolf et al 1991, Koshimura et al 2000, Shiraki et al 1996) via the cAMP protein kinase A pathway (Koshimura et al 2000).

To conclude, the possible effect of $BH_4$ treatment on neuronal monoaminergic neurotransmitter release and thereby neurotransmission has only been investigated in animal studies, mostly not related to PKU (Table 1). However, given the similarities in the possible mechanisms underlying impaired monoaminergic neurotransmission in PKU and the modes of action of $BH_4$, this might well offer a potentially useful therapeutic objective for $BH_4$ that deserves further research.

**$BH_4$ and glutamatergic synaptic transmission**

Although most research has focused on the possible effect of $BH_4$ on synthesis and neuronal release of monoaminergic neurotransmitters, $BH_4$ could also improve glutamatergic neurotransmission. Glutamate is the primary excitatory neurotransmitter in the brain, and has been shown to regulate neurogenesis, neurite outgrowth, synaptogenesis, and neuronal survival (Mattson 2008). As such, glutamatergic synaptic transmission is crucial for normal brain functioning, and impaired glutamatergic synaptic transmission has been associated with a diverse group of neurological disorders (Mattson 2008). Also in PKU, impaired glutamatergic synaptic transmission has been hypothesized to contribute to brain dysfunction (Martynyuk et al 2005). Both *in vitro* and *in vivo* studies in rats and PKU mice have shown that high Phe concentrations impair glutamatergic synaptic transmission through both presynaptic and postsynaptic effects (Glushakov et al 2002, Glushakov et al 2003, Glushakov et al 2005). This mechanism has been suggested to be responsible for impaired learning and memory in PKU mice (Glushakov et al 2005), and has been associated with susceptibility of BTBR *Pah-enu2* mice to audiogenic seizures (Martynyuk et al 2007).

As $BH_4$ has been found to induce neuronal glutamate release in striatum and frontal cortex from healthy rats (Mataga et al 1991), $BH_4$ might improve glutamatergic synaptic transmission in PKU and thereby improve neuropsychological functioning. The exact mechanism by which $BH_4$ would stimulate neuronal glutamate release has not been fully elucidated yet. The finding that increased glutamatergic neuronal release on $BH_4$ treatment was abolished by concomitant administration of 6-hydroxydopamine, destroying the dopaminergic nerve terminals, may suggest that the possible effect of $BH_4$

on striatal glutamate release is mediated by proper functioning dopaminergic neurons (Mataga et al 1991). Alternatively, it can be hypothesized that the effect of $BH_4$ is mediated by increased NO production, as NO has also been found to stimulate neuronal glutamate release (Neitz et al 2011). Overall, this hypothesis is mostly based on a single animal study not related to PKU (Table 1). Therefore, whether $BH_4$ treatment could stimulate glutamatergic synaptic transmission in PKU and thereby improve neurocognitive functioning still remains to be established.

**$BH_4$ and oxidative stress**

Theoretically, $BH_4$ may improve neurocognitive functioning in PKU patients by protecting against oxidative stress. Oxidative stress is defined as an imbalance between free radicals and antioxidant defense systems and is usually followed by oxidative cell injury and death. Research on this subject in PKU patients and mice, as reviewed by Ribas *et al.* (2011), suggests that oxidative stress could be an import mechanism leading to brain damage in PKU as a result of both increased reactive species production and decreased antioxidant defenses (Ribas et al 2011).

In oxidative stress, $BH_4$ plays a vital anti-oxidative role as a result of its interplay with nitric oxide synthase (NOS) (Thöny, Auerbach and Blau 2000, Schulz et al 2008). During oxidative stress, NOS generates superoxide, which is a reactive oxygen species, thereby further aggravating the oxidative situation. $BH_4$ is able to prevent the formation of superoxide by interacting with NOS, which is called 'NOS coupling'. However, especially during oxidative stress, $BH_4$ is oxidized to $BH_2$ leading to 'NOS uncoupling', thereby no longer protecting against free radicals. In case of oxidative stress, as observed

in PKU, a high intracellular $BH_4$ level might therefore be critical to maintain homeostasis, so that increased $BH_4$ availability in the brain might lead to a better protection against oxidative damage. However, it should be noted that the extent to which peripherally administered $BH_4$ could protect against oxidative stress largely depends on the increase of $BH_4$ concentrations relative to that of $BH_2$, e.g. the $BH_2/BH_4$ ratio, as studies in endothelial cells *in vitro* and in healthy rats indicate that an increase in this ratio would actually result in more oxidative stress (Crabtree et al 2008, Noguchi et al 2011). Thus, while $BH_4$ itself may decrease oxidative stress, $BH_4$ treatment may ultimately lead to too high $BH_2$ levels and by this may increase oxidative stress. Therefore, this possible negative effect of $BH_4$ treatment should be kept in mind and may be very relevant when considering the hypothesized benefits of $BH_4$ treatment in general.

The effect of $BH_4$ on oxidative stress has been investigated in research areas other than PKU. Some studies focusing on the vascular system, performed in both humans an mice, indeed suggest that $BH_4$ decreases oxidative stress (Cosentino et al 2008, Santhanam, d'Uscio and Katusic 2014), while other human studies show no effects of $BH_4$ on the cardiovascular system, possibly due to increased $BH_2$ levels (Cunnington et al 2012, Reverter et al 2015). Overall, $BH_4$ may in theory have positive effects on neurocognitive functioning of PKU patients by decreasing oxidative stress, but the effect of $BH_4$ on oxidative stress in the PKU brain has not yet been examined. Meanwhile, the possible negative effects of $BH_4$ treatment on oxidative stress deserve further attention as well.

**$BH_4$ and cerebral energy metabolism**

$BH_4$ might also have a positive effect on the brain by improving cerebral glucose metabolism through increased cerebral blood flow. Impaired cerebral energy status has been observed in both PKU patients (Hasselbalch et al 1996, Pietz et al 2003, Wasserstein et al 2006, Ficicioglu et al 2013) and PKU mice (Qin and Smith 2007). The role of such impaired cerebral energy metabolism on brain functioning in PKU is however largely unknown. Reduced cerebral energy metabolism has been suggested to relate to white matter abnormalities in PKU (Hasselbalch et al 1996), while increased cerebral glucose metabolism, which has been observed in particular brain areas in PKU patients (Ficicioglu et al 2013, Wasserstein et al 2006), has been suggested to reflect some compensatory mechanism (Ficicioglu et al 2013).

The effect of $BH_4$ (at 20 mg/kg/day) on brain glucose metabolism as measured by FDG-PET has been investigated in one study in $BH_4$-unresponsive PKU patients (Ficicioglu et al 2013). This study showed that, after 4 months of $BH_4$ treatment, glucose metabolism in left Broca's and right lateral temporal cortices was increased, which was accompanied by enhanced performance in a phonemic fluency test. Ficicioglu *et al.* hypothesized that this could have been the result of $BH_4$-induced vasodilation. As previously mentioned, $BH_4$ might lead to increased synthesis of NO, dopamine and serotonin. Whilst serotonin has a vasoconstrictive effect, dopamine and especially NO have strong vasodilatory qualities. Possibly, the increased blood flow enables certain brain regions to compensate for imbalances in glucose metabolism. Overall, this hypothesis is only supported by a single study in PKU patients (Table 1). Therefore, further research is necessary to establish the

effects of $BH_4$ on cerebral energy metabolism, and to investigate whether these effects could indeed be beneficial for neurocognitive functioning.

**$BH_4$ and white matter**

Finally, some evidence points towards a possible role for $BH_4$ in ameliorating neurocognitive functioning in PKU by influencing white matter. White matter abnormalities are one of the neuroradiological features characterizing PKU and have been observed in both untreated and early-treated (especially if not treated optimally) PKU patients (Anderson and Leuzzi 2010). While white matter pathology in untreated PKU is generally accepted to reflect hypomyelination, the observed white matter pathology in early-treated PKU patients is suggested to reflect intramyelinic edema rather than demyelination (Anderson and Leuzzi 2010). The clinical significance of the observed white matter abnormalities is still highly debated. However, PKU-related white matter abnormalities have been correlated to slowed information processing (Anderson et al 2004, Anderson et al 2007), which has been found to partly account for the executive function impairments seen in PKU (Janos et al 2012).

A possible relationship between $BH_4$ supplementation and improved myelination has been described in $BH_4$ deficiency patients (Wang et al 2006). When comparing their own results with a previous study on myelination in $BH_4$ deficiency patients (Chien et al 2002), Wang *et al.* (2006) showed more white matter abnormalities. In contrast to these differences in neuroradiological findings, age at which dietary treatment was initiated was comparable for both patient groups. $BH_4$ as well as neurotransmitter precursor treatment (levodopa and 5-HTP), however, was started at later age in the patient group

presenting with more white matter abnormalities, suggesting a role for $BH_4$ and neurotransmitter precursor treatment in the reversal of white matter pathology (Wang et al 2006). More recently, institution of $BH_4$ treatment in early-diagnosed and early-treated PKU patients has been shown to improve (and in some cases even fully correct certain aspects of) white matter abnormalities, which were significantly associated with reductions in blood Phe concentrations (White et al 2013). Whether these clear improvements were completely due to the blood Phe lowering effect of $BH_4$ remains to be established, as the study was only performed in $BH_4$-responsive PKU patients. In addition, neuroimaging findings by functional MRI (fMRI) in early-treated PKU patients have shown improved neural activation after 4 weeks of $BH_4$ treatment (20 mg/kg) even when blood Phe concentrations had not decreased (Christ et al 2013). Although additional research should further elucidate the possible relationship between these deficiencies of functional connectivity and white matter abnormalities in PKU, the results obtained by fMRI studies are in good agreement with the white matter abnormalities observed in PKU (Christ, Moffitt and Peck 2010, Christ et al 2012). Taken together, these results from studies in $BH_4$-treated PKU patients hold some promise for $BH_4$ treatment to improve neurocognitive functioning in PKU by influencing white matter. The underlying mechanism for such possible effect might be multifactorial and is not fully understood.

**Conclusion**

Besides lowering blood Phe concentrations in $BH_4$-responsive PKU patients, findings suggest that $BH_4$ treatment in PKU may also directly improve neurocognitive functioning. While the important question to what extent which peripherally administered

doses of $BH_4$ can increase cerebral $BH_4$ concentrations in humans necessitates additional investigation, the present review describes the working mechanisms that, theoretically, may underlie this possible direct neurocognitive effects of $BH_4$ in PKU. It should be emphasized that these hypothesized mechanisms are in large part based on studies in animal models and non-PKU-related research, but, taken together, they definitely justify further research on this topic. This research should at least focus on 1) further elucidating the possible beneficial effects of $BH_4$ treatment on neurocognitive functioning in PKU; 2) examining possible negative or toxic effects of $BH_4$ or its metabolites on brain function; and 3) the most effective way to increase $BH_4$ concentrations in the brain. Regarding the first aim, we suggest that the *Pah-enu2* mouse model for $BH_4$-unresponsive PKU could be used to identify if, and by which of the described mechanisms, $BH_4$ might improve neurocognitive functioning in PKU, beyond its effect through lowering blood Phe concentrations. Regarding the second aim, both this mouse model as well as *in vitro* studies could be used to investigate possible toxicity of $BH_4$ at different concentrations, especially focusing on the effect of $BH_4$, and the balance between $BH_4$ and $BH_2$, on oxidative stress. Thirdly, animal studies should also be used to investigate the optimal treatment regimen to increase cerebral $BH_4$ concentrations. This will possibly require higher $BH_4$ dosages than currently studied, or, alternatively, treatment with sepiapterin as this may be more effective in increasing intracellular $BH_4$ concentrations. Ultimately, the effect of $BH_4$ treatment on objective neurocognitive tasks in $BH_4$-unresponsive patients should be assessed in a placebo-controlled setting. If $BH_4$ indeed has beneficial neurocognitive effects, this may extend the target population of $BH_4$ treatment.

**Fig 1** A simplified schematic overview of the different hypothesized working mechanisms that may underlie a possible direct beneficial effect of $BH_4$ on neurocognitive functioning in PKU patients

**Table 1** An overview of human and non-human studies presenting results that are related to the possible neurocognitive effects of tetrahydrobiopterin treatment.

Abbreviations: PKU, phenylketonuria; $BH_4$, tetrahydrobiopterin; $BH_2$, dihydrobiopterin; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase; NO, nitric oxide.

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