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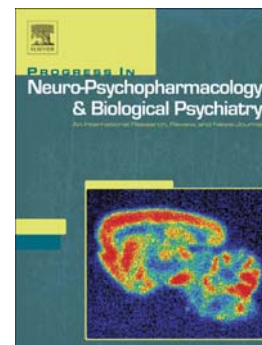
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Thiamine and benfotiamine improve cognition and ameliorate GSK-3 β -associated stress-induced behaviours in mice

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ABSTRACT

Thiamine (**vitamin B1**) deficiency in the brain has been implicated in the development of dementia and symptoms of depression. Indirect evidence suggests that thiamine may contribute to these pathologies by controlling the activities of glycogen synthase kinase (GSK)-3 β . While decreased GSK-3 β activity appears to impair memory, increased GSK-3 β activity is associated with the distressed/depressed state. However, **hitherto** direct evidence for the effects of thiamine on GSK-3 β function **has not been reported**. Here, we administered thiamine or, the more bioavailable precursor, benfotiamine at 200 mg/kg/day for 2 weeks to C57BL/6J mice, to determine whether treatment might affect behaviours that are known to be sensitive to GSK-3 β activity and whether such administration impacts on GSK-3 β expression within the brain. The mice were tested in models of contextual conditioning and extinction, a 5-day rat exposure stress test, and a modified swim test with repeated testing. **The tricyclic antidepressant** imipramine (7.5 mg/kg/day), was administered as a positive control for the effects of thiamine or benfotiamine. As for imipramine, both compounds inhibited the upregulation of GSK-3 β induced by predator stress or repeated swimming, and reduced floating scores and the predator stress-induced behavioural changes in anxiety and exploration. Coincident, thiamine and benfotiamine improved learning and extinction of contextual fear, and the acquisition of the step-down avoidance task. **Our data indicate that thiamine and benfotiamine have antidepressant/anti-stress effects in naïve animals that are associated with reduced GSK-3 β expression and conditioning of adverse memories. Thus thiamine and benfotiamine may modulate GSK-3 β functions in a manner that is dependent on whether the contextual conditioning is adaptive or maladaptive.**

Keywords: thiamine; benfotiamine; Glycogene-Synthase-Kinase-3-beta (GSK-3 β); depression; plasticity; animal models

1. INTRODUCTION

Thiamine (vitamin B1) is a pivotal regulator of mitochondrial function and metabolism. Thiamine is the precursor of thiamine diphosphate, and acts as a cofactor for several rate limiting enzymes in the Krebs cycle and the pentose phosphate pathway (Bettendorff et al., 2014). Compromised thiamine-dependent processes result in mitochondrial dysfunction and downstream oxidative stress, excitotoxicity, inflammatory changes, decreased neurogenesis and blood-brain barrier disruption (Abdou and Hazell, 2015). In addition, thiamine plays a role in the structural stabilization of neuronal membranes via non-enzymatic mechanisms (Mkrtchyan et al., 2015). Deficiencies in thiamine metabolism accompany chronic alcohol exposure and diabetes, and lead to neurodegenerative and depressive symptoms (Benton and Donohoe, 1999; Abdou and Hazell, 2015). Strikingly, the addition of a daily dose of 300mg thiamine **compared** to 6 weeks of standard fluoxetine treatment was shown to improve **Hamilton Depression Rating** Scores in patients with major depression in a recent randomized double-blind placebo-controlled clinical trial (Ghaleiha et al., 2016).

A number of thiamine precursors that exhibit improved bioavailability have been described, including benfotiamine, which has been investigated in animal models of Alzheimer's disease and other conditions to explore its efficacy (Balakumar et al., 2010; Portari et al., 2013). Both thiamine and benfotiamine suppress oxidative stress, inflammation and apoptosis (Abdou and Hazell, 2015). An 8-week treatment of benfotiamine reduced learning deficiencies (measured in the Morris Water maze), and decreased cortical amyloid plaque formation and phosphorylated tau levels in amyloid precursor protein/presenilin-1 overexpressing mice in a dose-dependent manner (Pan et al., 2010). Studies using supplementary thiamine in patients with Alzheimer's disease failed to reveal any clinical efficacy, but this was ascribed to limited intestinal absorption in these patients (Rodriguez-Martin et al., 2001).

The mode of action of thiamine in depressive illness and **neurodegenerative** disease is unclear. The anti-thiamine compound pyriethamine and diet-induced thiamine deficiency decrease the phosphorylation rates of Glycogen Synthase Kinase-3 β (GSK-3 β) and raise its enzymatic activity (Zhao et al., 2014). GSK-3 β activation has become a well-recognised marker of distress and depression (Beurel, 2015). Benfotiamine decreases GSK-3 β activity *in vitro* by increasing the phosphorylation of GSK-3 β at serine 9 (p9SGSK-3 β), which renders the enzyme inactive (Sun et al., 2012). Oral dosing of mice with thiamine or benfotiamine for 2 weeks increased brain thiamine levels and, for benfotiamine, an 8-week-dosing regimen reduced GSK-3 β activities via increasing the proportion of p9SGSK3 β in amyloid precursor protein/presenilin-1 overexpressing mice (Pan et al., 2010). Thus while downregulation of GSK-3 β has been implicated as the mediator of thiamine-dependent mechanisms in the CNS, to date, there has been no direct *in vivo* evidence for its role under normal conditions.

Inactivation of GSK-3 β results in learning and long-term potentiation deficiencies (Sintoni et al., 2013; Jurado-Arjona et al., 2016), while thiamine-mediated functions are important for brain plasticity (Bettendorff, 2014). Low GSK-3 β activity has been linked to anti-depressant changes (Kaidanovich-Beilin and Woodgett, 2011; Li et al., 2014), which, as mentioned above, were recently reported to result from chronic thiamine administration (Ghaleiha et al., 2016). Given these seemingly opposing effects of GSK-3 β inactivation on cognition and depression/stress-related mechanisms, we studied whether administration of thiamine or benfotiamine (200 mg/kg/day, p.o.) could alter GSK-3 β -dependent tasks such as the acquisition of memory, depressive-like behaviour and the stress response. Thiamine- or benfotiamine-treated C57BL/6J mice were studied in the modified swim test, where depressive-like behaviour has previously been shown to correlate with over-expression of brain GSK-3 β in the brain (Strekalova et al., 2016) and a 5-day rat exposure stress paradigm, which suppresses hippocampal neurogenesis and increases anxiety scores in mice (Strekalova et al., 2015b). Finally, contextual fear learning

and its extinction, in which GSK-3 β mechanisms are well known to have a pivotal role (Chew et al., 2015), are also investigated here (Vignisse et al., 2011, 2014).

2. METHODS

2.1. Animals

3.5-month-old male C57BL/6J mice used in the study were obtained from the Gulbenkian Institute of Science, Oeiras, Portugal. 14 days before the start of the behavioural tests, mice were housed individually **for acclimatization to a new facility**, under a reversed 12-h light–dark cycle (lights on: 21:00 h) with food and water ad libitum, under constant controlled laboratory conditions ($22 \pm 1^\circ\text{C}$, 55% humidity). All experiments were carried out in accordance with the European Committees Council Directives **with the European Union’s Directive 86/609/EEC and Council Directive 93/119/EC**, and had been approved by the **ethic committee for animal research of Maastricht University CPV and by General Directory of Ethical Committee of the New Lisbon University**.

2.2. Study design

Mice were administered with thiamine, benfotiamine or imipramine via drinking water for two weeks and subjected to the modified swim test, predation stress, or a set of memory tests (Fig.1A-C; group sizes are given in Figs.2-4) (see below for details). For the modified swim test, sessions of 6 minutes in duration were performed on Days 1 and 2 and 5, and animals were culled 10 minutes post-test, and simultaneously with naïve mice that were not exposed to the swim test or administration of substances. The hippocampus and prefrontal cortex were dissected and prepared for GSK-3 β RT-PCR analysis and an ELISA assay was used to determine total and proportion of 9-Ser-phosphorylation in the hippocampus (Fig.1A). **All procedures were carried out after at least 1-hour acclimatization time to experimental room.**

2.3 Behaviour

2.3.1 Swim test

In the modified swim test model, mice were exposed to a two-day swimming protocol (day 1 and day 2); they were then tested on again on day 5 (Markova et al., 2013, 2014; Strekalova et al., 2016). In each immersion, naïve mice or those dosed with imipramine, thiamine or benfotiamine were placed for 6 min in a transparent cylinder (Ø 17 cm) filled with water (+23°C, water height 13 cm, height of cylinder 20 cm, under subdued lighting). Floating behaviour, defined by the absence of any directed movement of the head or body, was manually scored using previously established criteria using Noldus EthoVision XT 8.5 (Noldus Information Technology, Wageningen, The Netherlands) and CleverSys (CleverSys, Reston, VA, USA) as described elsewhere (Malatynska et al., 2012). The total time spent floating was calculated for the entire duration of the test.

2.3.2 Elevated O-maze

The apparatus (Technosmart, Rome, Italy) consisted of a circular path (runway width 5.5 cm, diameter 46 cm) that was placed 45 cm above the floor. The two opposing arms were protected by walls (closed area, height 10 cm), and the illumination strength was 25 Lux. The apparatus was placed on a dark surface in order to maintain control over lighting conditions during testing. At the start, mice were placed in one of the closed-arm area of the apparatus, behaviour was videorecorded and assessed for a 5-minute observation period as described elsewhere (Vignisse et al., 2011, Strekalova et al., 2015a). The latency to the first exit into the open compartments of the maze and the number of exits to the open arms were recorded.

2.3.3 Novel cage test

The novel cage test was performed to assess vertical exploratory activity in a new environment (Strekalova and Steinbusch, 2010; Couch et al., 2013). Mice were introduced into a standard

plastic cage (21×21×15 cm) filled with fresh sawdust. The number of exploratory rears each minute was counted under red light for a 5-min period.

2.3.4 Step-down passive avoidance test

The step-down apparatus (Technosmart, Rome, Italy) consisted of a transparent plastic cubicle (25 cm × 25 cm × 50 cm) with a stainless-steel grid floor (33 rods 2 mm in diameter) onto which a square wooden platform (7 cm × 7 cm × 1.5 cm) was placed. A shocker was used to deliver an alternating electric current (AC, 50 Hz, Evolocus, Terrytown, NY, USA). In this paradigm, animals were trained not to step down from a platform onto a grid floor to avoid an electric shock. During the training session, mice were placed onto the platform inside a transparent cylinder for 30 s to prevent them from immediately stepping down. After removal of the cylinder, the time until the animal left the platform with all four paws was measured as baseline latency of step down. Immediately after step down, mice received a single electric foot-shock (0.5 mA, 2 s) and returned to their home cages. During the recall trial session, animals faced the same context as in the training session. The latency to the animal stepping-down was recorded 1h and 24h post-training, latency of step down with all four paws was measured until 180s elapsed. According to previously validated criteria for the acquisition of the step down avoidance task, an increase of latencies measured in animals during a recall session are taken as a sign of long-term learning (Stekalova et al., 2002, Vignisse et al., 2011, 2014). A percentage of “good learners”, i.e., mice in whom this measure was >30s at 24h post-training, was calculated (Vignisse et al., 2011) (Fig.1C).

2.3.5 Fear conditioning learning and extinction

In a separate study, mice were trained with a 2s foot-shock of “weak” (0.5mA, 50 Hz) or “strong” (0.7mA, 50 Hz) intensity in the fear conditioning paradigm, which was delivered after a 2-min acclimatization period using a shocker (Evolocus, Terrytown, NY, USA). The apparatus (Open Science, Russia and Technosmart, Rome, Italy) consisted of a transparent

plastic cubicle (25 × 25 × 50 cm) with a stainless-steel grid floor (33 rods/2 mm in diameter). After delivery of the current, the mouse was immediately placed back into the home cage. Twenty-four hours later, freezing behaviour was scored in a 180-s recall of extinction session. The occurrence of freezing behaviour was assessed every 10s, and each 10-s period was assigned to a freezing or non-freezing period, and the percentage of time spent freezing was calculated. Animals subjected to “strong” shocks were exposed to a memory extinguishing procedure immediately after a recall session (Strekalova et al., 2003, Vignisse et al., 2014). Therefore, mice were left for another seven minutes in the apparatus, so the total procedure of memory extinction was 10-min long. During this period, no foot shock was applied, and animals were free to explore the apparatus. Twenty-four hours later, freezing behaviour was scored again in a 180-s recall of extinction session as in the previous trial and percentage of time spent in freezing was calculated.

2.4 Predator stress

A previously established protocol was used (Strekalova et al., 2015a). Mice were introduced into cylindrical containers, which were placed into a rat home cage for 15h (over-night, from 18h00 to 9h00) for 5 nights. The containers were made from customized transparent plastic, size 15cm x Ø 8 cm, with holes in covers (Ø < 0.5cm), which ensured protection of the mouse from the rat, but allowed visual and odour contact. Afterwards, animals were scored for exploratory rears in the novel cage test, latency and number of exit(s) to the open arms of the O-maze (Vignisse et al., 2011; see also below for details); they were killed 5h post-stress and the hippocampus and prefrontal cortex were dissected for GSK-3β RT-PCR assay (*see below*), simultaneously with naïve controls that were not exposed to any manipulations (Fig.1B).

2.5 Brain dissection, RNA extraction and RT-PCR

Mice were killed by cervical dislocation as it was described elsewhere (Costa-Nunes et al., 2014). The brain of each mouse was dissected and the prefrontal cortex and the hippocampi

were isolated as previously reported (Couch et al., 2013; Strekalova et al., 2016) and stored at 80° C until later use. mRNA was extracted by using TRI Reagent (MRC, USA). First strand cDNA synthesis was performed using random primers and Superscript III transcriptase (Invitrogen, Darmstadt, Germany); 1 µg total RNA was converted into cDNA. Quantitative RT-PCR (qRT-PCR) for glycogen synthase kinase 3 beta (GSK-3β) gene and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed using the SYBR Green master mix (Bio-Rad Laboratories, Philadelphia, USA) and the CFX96 Real-time System (Bio-Rad Laboratories, Philadelphia, USA). The sequences of the primers used are indicated in *Supplementary Table 1, see below*). Data were normalized to GAPDH mRNA expression and calculated as relative-fold changes compared to control mice as described elsewhere (Couch et al., 2013; Strekalova et al., 2016). Results of qRT-PCR measurement were expressed as Ct values, where Ct is defined as the threshold cycle of PCR at which amplified product was 0.05% of a normalized maximal signal. We used the comparative Ct method and computed the difference between the expression of the gene of interest and GAPDH expression in each cDNA sample (2-ΔΔ Ct method). Data are given as expression-folds compared to the mean expression values in non-stressed control mice (Couch et al., 2013).

2.6 ELISA assays of total and phosphorylated forms of GSK-3beta

Total GSK-3β and p9SGSK-3β were measured by ELISA, and their ratio was calculated as described elsewhere (Strekalova et al., 2016). The hippocampi were homogenized in buffer containing 10 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na₄P₂O₇, 10% glycerol, 2 mM Na₃VO₄. A Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) was added immediately prior to homogenization. The [pS9]-GSK-3β ELISA kit and [total]-GSK-3β ELISA kit (Invitrogen Corporation, Carlsbad, CA, USA) were used for quantification of the level of GSK-3β protein phosphorylated at serine residue 9 and total level of GSK-3β. All procedures were performed according the manufacturer's instructions; the absorbance was measured at 450 nm using a plate reader (Wallac 1420 VICTOR, Waltham,

MA, USA). The results were normalized to total protein level in tissues homogenates. Protein concentrations were determined by the biuret assay using bovine serum albumin as a standard.

2.7 Drug delivery

Experimental solutions replaced normal drinking water. Thiamine, benfotiamine and imipramine were obtained from Sigma-Aldrich, St. Louis, MO, USA. All agents were dissolved in tap water and were changed every 4-5 days (Strekalova et al., 2006, 2015b). The solutions of benfotiamine and thiamine were adjusted to pH 7.0, drinking behaviour of mice was monitored by evaluating a 24-h liquid intake during the first three days of dosing. The dose of imipramine dose (7.5 mg/kg/day), was based on previous results showing that this dose had no effect on general locomotor behaviour (Strekalova et al., 2015b; Costa-Nunes et al., 2015).

2.8 Statistical analysis

Data were analysed with GraphPadPrism v.5.0 for Windows (San Diego, CA, USA) using one-way ANOVA and Tukey's post-hoc test unless otherwise stated. Repeated measures (RM) ANOVA or Fisher's exact test were also employed where appropriate. The level of confidence was set at 95% ($p < 0.05$). Data are given as mean \pm SEM.

3. RESULTS

3.1. Thiamine and benfotiamine inhibit floating behaviour and brain GSK-3 β in the modified swim test

Statistical values for all results are presented in the *Supplementary Tables 2-11*. There was no difference in floating duration on **day 1 and day 2** ($p > 0.05$, one-way ANOVA, *Supplementary Table 2A*). Vehicle-treated mice displayed a significant increase in floating time from day 2 to day 5 ($p < 0.05$, RM ANOVA). This increase was not observed in any of the pharmacologically treated groups ($p > 0.05$ for each, RM ANOVA; Fig.2A, *Supplementary Table 2B*). Thus the

administration of thiamine and benfotiamine, as did imipramine, prevented behavioural changes in the modified swim depression paradigm. The swim test generated differences in GSK-3 β mRNA expression in the hippocampus and prefrontal cortex (main effect: $p < 0.05$, one-way ANOVA, *Supplementary Table 3*). Compared to naïve mice, GSK-3 β mRNA was significantly elevated in vehicle-treated mice ($p < 0.05$, Tukey test; Fig. 2B, C), but not in imipramine-treated mice, nor in the thiamine and benfotiamine groups ($p < 0.05$). Hippocampal GSK-3 β , p9SGSK-3 β , as measured by ELISA, and their ratio was also found to be significantly affected by treatment (main effect: $p < 0.05$). Vehicle-treated mice showed an increase in total GSK-3 β , reduced p9SGSK-3 β and a reduced p9SGSK-3 β /totalGSK-3 β ratio compared to naïve animals ($p < 0.05$, Tukey test; *Supplementary Fig.1 and Table 4*). Imipramine-treated mice, as well as thiamine- or benfotiamine-treated animals showed no such change ($p > 0.05$) and gave rise to levels that were similar to those in animals treated with imipramine.

3.2. Thiamine and benfotiamine ameliorate stress-induced behaviour and GSK-3 β expression after a predation stress

There was a significant between-group difference in exploratory rears in the novel cage test for the experimental groups ($p < 0.05$, one-way ANOVA, *Supplementary Table 5*). In comparison with naïve mice, rearing behaviour was significantly decreased in stressed vehicle-treated mice ($p < 0.05$, Tukey test; Fig. 3A), but not in stressed imipramine-, thiamine- or benfotiamine-treated groups ($p > 0.05$) **that** displayed significantly higher exploration than stressed vehicle-treated animals ($p < 0.05$). In the elevated O-maze test, we found significant differences in the latency to exit the open arms and in the total number of exits ($p < 0.05$, one-way ANOVA; *Supplementary Table 6*). In comparison with naïve animals, those in the stressed vehicle-treated group made significantly fewer exits in to the open arms and had a tendency to prolonged latency to exit ($p < 0.05$ and $p = 0.07$, Tukey test; respectively; Fig.3 B&C). This stress-induced change was not observed in the imipramine-treated group, nor in the thiamine- or benfotiamine-treated mice stressed mice ($p > 0.05$).

ANOVA revealed significant differences between experimental groups in GSK-3 β mRNA in the prefrontal cortex, but not in the hippocampus ($p < 0.05$ and $p > 0.05$, respectively, *Supplementary Table 7*). Compared with naïve controls, stressed vehicle-treated mice displayed a non-significant change in GSK-3 β mRNA level in the hippocampus ($p > 0.05$, Tukey test; Fig. 3D) and a significant increase in levels in prefrontal cortex ($p < 0.05$, Fig. 3E). Stressed animals treated with imipramine, or thiamine, or benfotiamine showed no such changes ($p > 0.05$ vs. naïve mice). Thiamine and benfotiamine, as for imipramine, prevented the stress-induced changes in exploratory behaviour, anxiety-like behaviour and over-expression of GSK-3 β in the brain.

3.3. Effects of thiamine and benfotiamine on memory

In the step-down test (*Supplementary Tables 8,9*), there were no differences in the baseline step-down latencies, measured prior training, between all experimental groups ($p > 0.05$, one-way ANOVA; *data not shown*), suggesting that normal scores of anxiety and locomotion are preserved in the test groups. Step-down latencies measured 1 and 24 hours post-training were significantly different across experimental groups ($p < 0.05$, one-way ANOVA). In comparison with vehicle-treated controls, there was a significant increase of step-down latency in thiamine- and benfotiamine-treated mice 1h post-training, and in thiamine-treated groups 24h post-training ($p < 0.05$, Tukey test; Fig. 4A). **A higher percentage of animals were grouped as “good learners” among the thiamine- and benfotiamine-treated as compared to non-treated mice** ($p < 0.05$, Fisher test; Fig. 4B). The number of rears in the novel cage did not differ between experimental groups ($p > 0.05$, Tukey test; Fig. 4C, *Supplementary Table 10*), further suggesting a lack of effects of applied treatments on locomotor activity.

There was a significant difference in freezing behaviour observed during a recall session between the groups trained under “weak”, but not “strong” conditioning ($p < 0.05$ and $p > 0.05$,

one-way ANOVA, *Supplementary Table 11*). The thiamine- and benfotiamine-treated mice groups spent significantly longer time freezing after a “weak” training than vehicle-treated mice ($p < 0.05$, Tukey test; Fig.4D); no such difference was found in a “strong” training protocol ($p > 0.05$; Fig.4E). At the recall of extinction, there was a significant between-group difference in freezing behaviour ($p < 0.05$, one-way ANOVA). As compared with vehicle-treated controls, thiamine- and benfotiamine-treated mice spent significantly less time freezing, demonstrating effective fear extinction ($p < 0.05$, Tukey test; Fig.4F). Together, these data suggest improved short- and long-term contextual memory and its extinction in thiamine- and benfotiamine-treated animals compared with untreated animals, and a lack of non-specific locomotor changes.

4. DISCUSSION

Our study has demonstrated, for the first time that the two-week treatment of mice with thiamine or benfotiamine had beneficial effects in classical paradigms of learning and depression/stress-related behaviours. Our results also provide the first evidence that these treatments are associated with decreased GSK-3 β activity in normal mice. Previously reported *in vitro* and *in vivo* studies with benfotiamine have revealed its inhibitory effects on GSK-3 β activities in pathological conditions, such as models of Alzheimers pathology (Pan et al., 2010; Sun et al., 2012), but whether the beneficial effects might be induced in non-pathological states where hitherto unknown.

The treatment of mice with thiamine or benfotiamine, as for imipramine, inhibited changes induced by forced swimming or predation stress. Thiamine deficiency is known to induce floating behaviour in mice (Nakagawasai et al., 2007). A 30-day-long dosing regimen with thiamine in Wistar rats that were subjected to a chronic immobilization stress rescued normal learning in the T-maze, decreased freezing scores, increased locomotion in the open field, and reduced anxiety-like behaviours (Dief et al., 2015). The present study also supports the latest findings showing that thiamine supplementation has beneficial effects in depressed patients

(Ghaleiha et al., 2016), and improves mood in healthy adults (Schmidt et al., 1991; Benton et al., 1997) and is in accord with evidence for a link between lowered thiamine level and stronger symptoms of depression of various forms (Bell et al., 1991, 1992; Pepersack et al., 1999; Zhang et al., 2013). However, there is no prior literature available on the anti-stress/anti-depressant effects of benfotiamine in animal models.

Behavioural changes provoked by the swim-test and the exposure to a predation stress are both accompanied by inhibition of GSK-3 β mRNA expression in the hippocampus and the prefrontal cortex. Elevated GSK-3 β mRNA and lowered p9SGSK-3 β /totalGSK-3 β ratio in the brain are established markers for depression-like and distress syndromes (Kaidanovich-Beilin and Woodgett, 2011; Zhou et al., 2012; Li et al., 2014). As for imipramine-treated mice, thiamine-treated or benfotiamine-treated rescued levels of GSK-3 β mRNA, p9SGSK-3 β , totalGSK-3 β and p9SGSK-3 β /totalGSK-3 β ratio in the stressed animals and were no different to the values determined for naïve animals. In the modified swim test, dosing with either drug had no effect on behaviour on days 1 and 2, which was at a time when no changes in GSK-3 β mRNA were observed, but, on day 5, when increases of both floating and GSK-3 β on gene and protein levels were reported (Strekalova et al., 2016) the drugs were effective. Together, this association argue that that the effects of thiamine- and benfotiamine could be attributable to the downregulation of GSK-3 β activity.

As well as modifying stress and anxiety responses, the two-week treatment with thiamine or benfotiamine in naïve animals were shown to enhance contextual fear learning and its extinction, which, are known to require intact GSK-3 β function (Beurel, 2015; King, 2015). Our experiments showed that treatments with thiamine or benfotiamine are able to increase hippocampal-dependent memory in two classical tests for this **form** of learning that is generally in line with established views on the role of thiamine in neuronal plasticity and pro-cognitive effects of benfotiamine in an Alzheimer's disease model (Pan et al., 2010; Abbou and Hazell, 2015). To our knowledge, this is the first demonstration of such effects of these compounds in

naïve mice. The present data suggest that moderate pro-cognitive effects are associated with the administration of thiamine or benfotiamine, which improved fear conditioning after “weak” training. A lack of effect under “strong” training conditions is likely due to ceiling effect of the training, when physiologically adaptive learning cannot be enhanced any further. Similar difference in sensitivity of contextual learning during “weak” versus “strong” training to mnemotropic agents was shown in our previous studies to be correlated with outcome from “weak” and “strong” protocols for the induction of long-term potentiation paradigms, where the same factors were used (Strekalova et al., 2002).

A lack of group differences in baseline step down as well as in vertical activity **and measures of horizontal locomotion in the True Scan open field** (see *Supplementary Figure 2*) rule out the possibility that general changes in anxiety and locomotion in thiamine- or benfotiamine-treated mice might account for the differences and strongly argues for improved memory in these groups.

The outcomes in the learning and modified swim test are believed to share overlapping mechanisms, such as elements of contextual conditioning. In the modified forced swim model of depression, both behavioural outputs and GSK-3 β mRNA over-expression are triggered by contextual reminders of the testing procedure alone, such as a visual exposure of an animal to a swimming tank, without its experience of a swimming (Strekalova et al., 2016). As such, modified swimming test is considered as a model of inappropriately increased (maladaptive) contextual conditioning of adverse memories, which plays an important role in a pathophysiology of depression (Clark et al., 2009). It is generally viewed that contextual conditioning can play both adaptive and pathological roles. While learning of new environment is of adaptive value for an individual, enhanced retention of negative contextual associations can be an important element of a pathophysiology of depression, post-traumatic stress disorder and generalized anxiety (Diamond et al, 2004; Clark et al., 2009).

In the present study, thiamine and benfotiamine inhibited contextual conditioning dependent on depressive-like behavioural and molecular changes, while it improved acquisition and extinction of contextual memories of a new environment that can be considered as adaptive. Based on the current findings, it could be speculated that thiamine and benfotiamine modulate GSK-3 β functions and contextual conditioning in a manner that is dependent on whether the contextual conditioning is adaptive or maladaptive.

Other studies showed discordant changes in hippocampal plasticity and affective processes in mice with systemically altered GSK-3 β activity, either via genetic manipulations (Pardo et al., 2016), or chronic pharmacological treatments, such as valproate (Sintoni et al., 2013). For instance, GSK3 β knock-in mice with increased activity of this molecule displayed heightened vulnerability to the learned helplessness model of depression-like behaviour and showed no enhancement, but an impairment of some but not all forms of memory. Such forms of learning, such as novel object recognition and spatial processing, and not temporal order memory, were disrupted in GSK3 β knock-in mice. In rats, chronic dosing with valproate decreased baseline brain p9SGSK-3 β /totalGSK-3 β ratio, however, did not attenuate their response to novel stressors, while impaired contextual fear conditioning. It was suggested that these effects were due to altered dynamics of GSK-3 β phosphorylation at 9 Ser that was due to distinct mechanisms involved in two behavioural tests (Sintoni et al., 2013). Thus, an increase or a decrease of brain GSK-3 β activities induced by chronic systemic interventions do not ultimately lead to concordant changes in mood and cognition, which can be expected from general roles of GSK-3 β in these conditions. The functions and regulation of GSK-3 β functions are very complex and can be moderated via multiple mechanisms including post-translational modifications, substrate priming, cellular trafficking, protein complexes (Beurel, 2015). **Hence, a number of GSK-3 β regulated processes may contribute to the changes reported here and to those reported in earlier studies.**

Here, systemically administrated thiamine and benfotiamine were shown to increase brain thiamine levels to a similar extent (**Vignisse et al., 2016**). This could explain the similarity in the effects of the compounds in our study, despite the low reported bioavailability characteristic of thiamine. Indeed, in current experiments, thiamine was used at a high dose, exceeding by more than 10-fold the concentrations applied in the studies that reported its administration to be behaviourally ineffective (Rodriguez-Martin et al., 2001). Single or two-week administration of thiamine at the dose 100 mg/kg did not elevate thiamine brain levels (Volvert et al., 2008). In line with our results, chronic administration of thiamine produced antidepressant effect in a clinic. It is of note that the doses used in the clinical study (300 mg/day) was substantially lower than that used in the present work (Ghaleiha et al., 2016). Neither treatment affected thiamine diphosphate content (**Volvert et al., 2008, Vignisse et al., 2016**) suggesting that the effects reported here are mediated by coenzyme-independent mechanisms (Mkrtchyan et al., 2015). Hence, it can be suggested that both **anti-stress** / anti-depressant and pro-cognitive effects of employed here treatments are underpinned by non-coenzyme functions of thiamine, such as an increase of brain thiamine levels associated with subsequent modulation of GSK-3 β functions.

Apart from GSK-3 β -related mechanisms, other factors could **also underlie the effects reported here**, especially the pro-cognitive effects, such as the altered production and activity of reactive oxygen species, anti-inflammatory activities, or changes in BDNF levels affecting biosynthesis of monoamines, including serotonin (Nakagawasai et al., 2007; Abbou and Hazell, 2015; Bozic et al., 2015), resulting, **as for instance, in reductions in spine density and complexity, that have been associated with depression, stress and learning (Duman and Duman, 2015)**. These would be interesting to explore in future studies given the impact of the treatments on normal animals though many of these other effects can also be related back to GSK-3 β -mediated cascades (King, 2014; Beurel, 2015).

Conclusion

Our results demonstrate that administration of thiamine, or its precursor, decrease GSK-3 β activity in limbic structures under conditions of stress. The current study also provides the first evidence for antidepressant-like effects and pro-cognitive action of thiamine, or its precursor, in a pre-clinical assays. Remarkably, the precognitive actions were shown in naïve animals. Thus our data support a view that thiamine can be an important physiological regulator of stress response, learning and brain GSK-3 β and reveal the potential thiamine/thiamine precursors as antidepressants and cognitive enhancers.

Acknowledgements

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Figure legends

Figure 1. Schematic of the timelines for each study. (A) modified swim test, (B) 5-day predation stress, (C) step-down avoidance, (D) fear conditioning test.

Figure 2. Thiamine and benfotiamine normalise floating and brain GSK-3 β expression in the modified swim test. Significant group differences were observed on day 5 of the modified swim test in (A) floating behaviour (* $p < 0.05$ vs. Day2) and GSK-3 β mRNA levels as measured in (B) the hippocampus and (C) prefrontal cortex (* $p < 0.05$ vs. vehicle-treated, # $p < 0.05$ vs. naive mice).

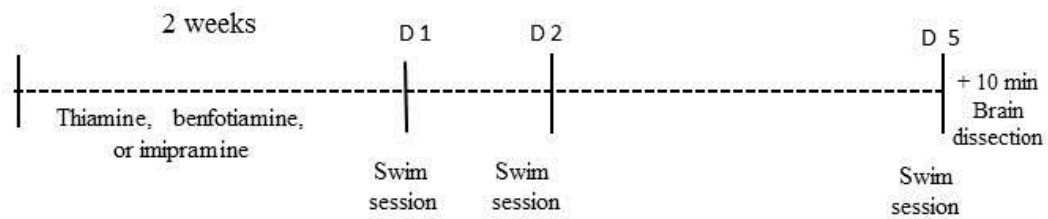
Figure 3. Thiamine and benfotiamine prevent stress-induced changes in novelty exploration, anxiety-like behavior, and brain GSK-3 β expression during predation stress. Significant group differences were found in the stressed animals in (A) novel cage rearing, (B) latency and (C) exit(s) to the open arms of the elevated O-maze. GSK-3 β mRNA levels in the (D) hippocampus and (E) pre-frontal cortex (* $p < 0.05$ vs. vehicle-treated, # $p < 0.05$ vs. naive mice).

Figure 4. Thiamine and benfotiamine enhance contextual conditioning and its extinction. The effect of thiamine and benfotiamine in the step-down avoidance study: (A) latency to step-down and (B) percentage of “good learners”, (C) novel cage rearing. Freezing behaviour in the contextual fear conditioning paradigm after (E) “weak” and (D) “strong”

training and (F) its extinction (#p<0.05 vs. vehicle-treated, 1h post-training; *p<0.05 vehicle-treated, 24h post-training).

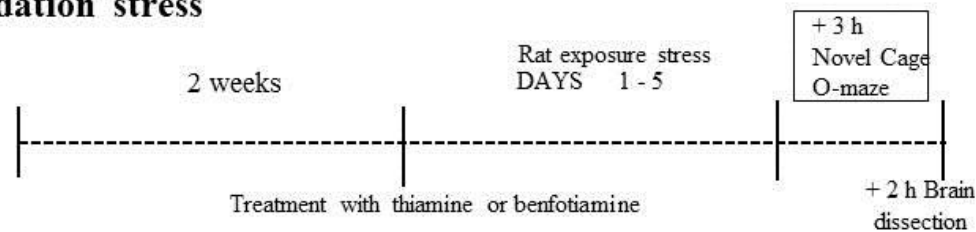
A

Modified swim test



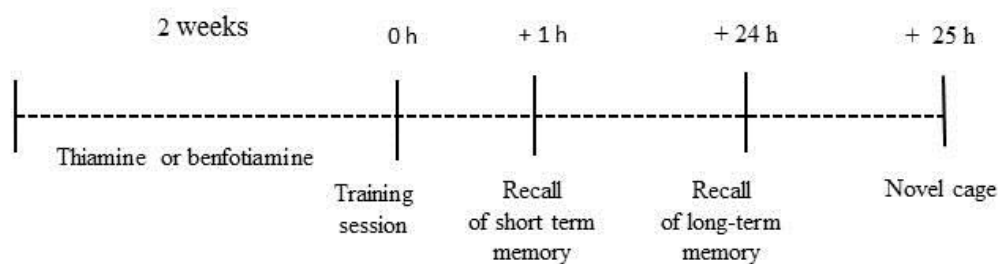
B

Predation stress



C

Step-down avoidance test



D

Fear conditioning learning and extinction

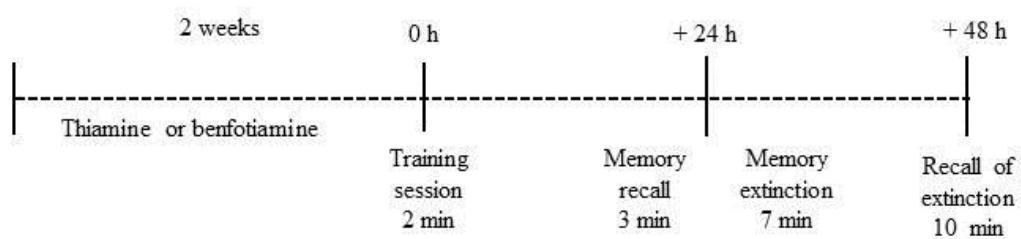
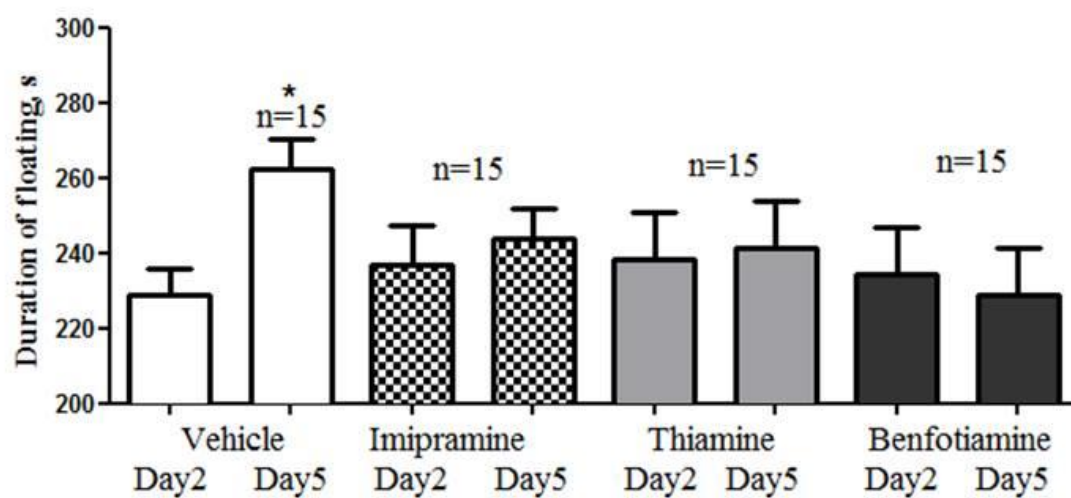
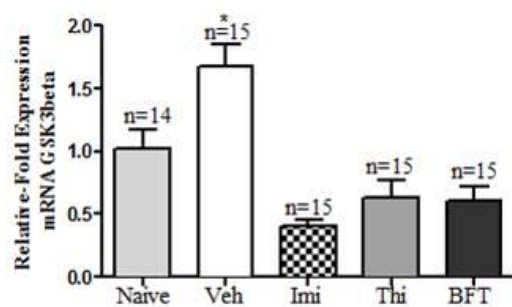


Figure 1

A Floating behaviour in Modified Swim Test



B GSK-3 β mRNA: Hippocampus



C GSK-3 β mRNA: Prefrontal cortex

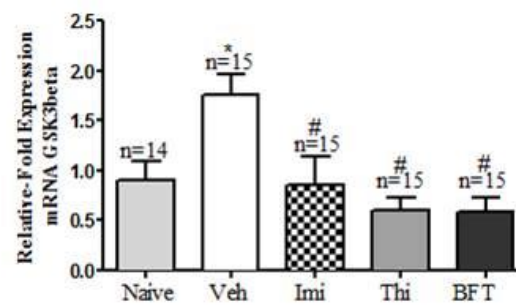


Figure 2

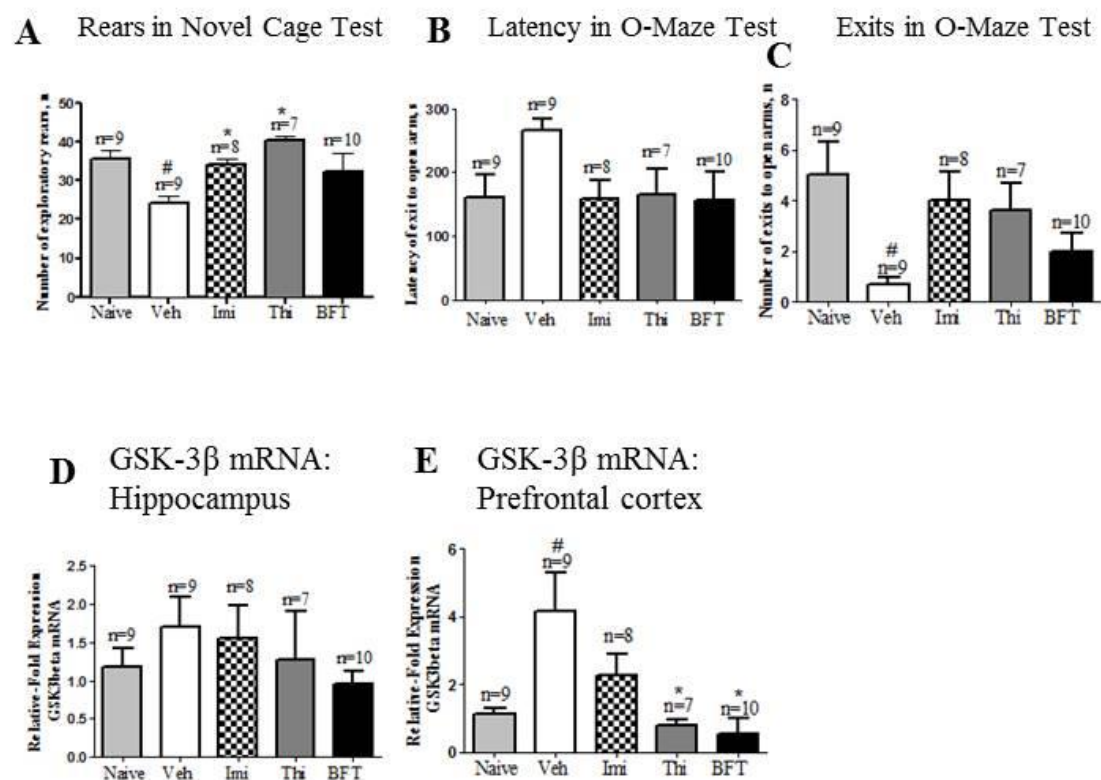


Figure 3

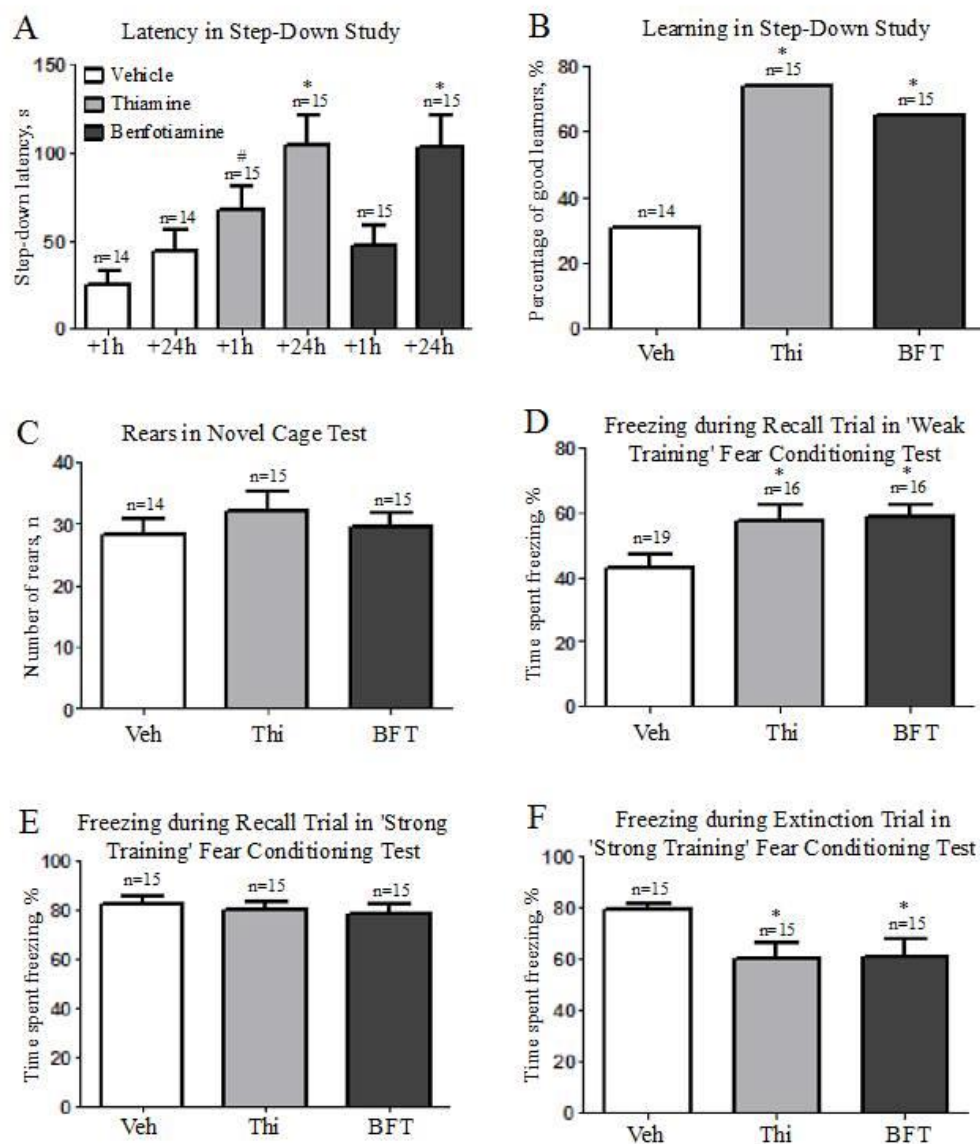


Figure 4

Highlights

- Thiamine and benfotiamine generate antistress and antidepressant-like effects in mice;
- Both molecules prevent brain activation of GSK3- β in a model of depression;
- Each also prevent stress-induced increases in anxiety and GSK3- β expression;
- Thiamine and benfotiamine enhance contextual memory and extinction in naïve mice;
- The effects on stress and learning are likely to be mediated via distinct mechanisms.