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Thiamine and benfotiamine prevent stress-induced suppression of hippocampal neurogenesis in mice exposed to predation without affecting brain thiamine diphosphate levels

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Running title: **Anti-stress effects of thiamine and benfotiamine**

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Abbreviations

AD, antidepressant; AHN, adult hippocampal neurogenesis; BrdU, bromodeoxyuridine; S-BFT, stressed-treated with benfotiamine; S-NT, stressed-not treated; S-Thia, stressed-treated with thiamine; TD, thiamine deficiency; ThDP, thiamine diphosphate; ThMP, thiamine monophosphate.

Abstract

Thiamine is essential for normal brain function and its deficiency causes metabolic impairment, specific lesions, oxidative damage and reduced adult hippocampal neurogenesis (AHN). Thiamine precursors with increased bioavailability, especially benfotiamine, exert neuroprotective effects not only for thiamine deficiency (TD), but also in mouse models of neurodegeneration. As it is known that AHN is impaired by stress in rodents, we exposed C57BL6/J mice to predator stress for 5 consecutive nights and studied the proliferation (number of Ki67-positive cells) and survival (number of BrdU-positive cells) of newborn immature neurons in the subgranular zone of the dentate gyrus. In stressed mice, the number of Ki67- and BrdU-positive cells was reduced compared to non-stressed animals. This reduction was prevented when the mice were treated (200mg/kg/day in drinking water for 20 days) with thiamine or benfotiamine. Moreover, we show that thiamine and benfotiamine counteract stress-induced bodyweight loss and suppress stress-induced anxiety-like behavior, but do not effect plasma corticosterone concentrations. Both treatments elevated brain levels of thiamine, but not of thiamine diphosphate (ThDP), suggesting that the beneficial effects observed are not linked to the role of this coenzyme in energy metabolism. Our study demonstrates, for the first time, that thiamine and benfotiamine prevent stress-induced inhibition of hippocampal neurogenesis and accompanying physiological changes. This is not associated with normalization of hormonal stress response, but is likely to be mediated via the non-cofactor roles of thiamine. The present data suggest that thiamine precursors with high bioavailability might be useful as a complementary therapy in several neuropsychiatric disorders.

Keywords

Thiamine, benfotiamine, predator stress, proliferation, survival, immature neurons, hippocampus

1. Introduction

Increasing evidence suggests that thiamine (vitamin B1) and precursors with higher bioavailability can exert prominent neuroprotective effects in the mammalian brain (Gibson et al., 2016; Pan et al., 2016). It is well known that the principal phosphorylated derivative of thiamine, thiamine diphosphate (ThDP), is an essential cofactor for glucose metabolism, being required for the activity of transketolase and the mitochondrial pyruvate and oxoglutarate dehydrogenase complexes. It is, therefore, not surprising that thiamine deficiency has deleterious effects on brain activity, which heavily relies on oxidative glucose metabolism (Gibson and Blass, 2007). However, it has long been thought that a general impairment of brain energy metabolism does not adequately account for the selective vulnerability of diencephalic structures in thiamine deficiency (TD). This has led to the idea that thiamine may exert neuromodulatory or neuroprotective actions through mechanisms unrelated to its coenzyme role (Bettendorff, 1994; Bettendorff, 2013; Mkrtchyan et al., 2015).

The sensitivity of the brain to TD is thought to be owing to the slow absorption of thiamine through the intestinal epithelium and through the blood-brain barrier (Greenwood et al., 1982). Therefore, lipophilic precursors with higher bioavailability have been developed in order to increase the absorption of the vitamin. The widely used precursor benfotiamine (S-benzoylthiamine-O-monophosphate) is not lipophilic, but, after oral administration, it is dephosphorylated by intestinal ecto-alkaline phosphatases to the liposoluble S-benzoylthiamine, which in turn is converted to thiamine in liver and blood (Volvert et al., 2008). Benfotiamine had first been used as a possible treatment for microvascular complications of type-2 diabetes (Hammes et al., 2003; Marchetti et al., 2006; Beltramo et al., 2008). Further studies on various pathological conditions, including neurological disorders, have shown the beneficial effects of benfotiamine treatment in humans and in animal models (Balakumar et al., 2010; Sanchez-Ramirez et al., 2006; Pan et al., 2010).

As TD is associated with memory loss, even before the appearance of diencephalic lesions (Vetreno et al., 2011), and since cognitive impairment is generally associated with hippocampal dysfunction, such possible alterations were investigated in mice undergoing TD at a pre-pathological lesion stage (Zhao et al., 2008). In these deficient mice, learning abilities were markedly decreased and this was concomitant with an impairment of progenitor cell proliferation and neurogenesis in the dentate gyrus.

Many studies have shown that, in rodents and other mammals, exposure to stress causes a marked impairment of adult hippocampal neurogenesis (AHN) (Gould et al., 1998; Malberg and Duman, 2003). The discovery that concomitant treatment with antidepressants protected AHN against harmful effects of stress has raised much interest (Warner-Schmidt and Duman, 2006; David et al., 2009; Miller and Hen, 2015). Thus it appears that AHN is controlled by a number of different factors and it can be anticipated that several kinds of drugs (in addition to antidepressants) could be used to protect and boost neurogenesis when it is impaired by stressful events.

We considered the possibility that thiamine and/or benfotiamine might exert protective effects on AHN when mice are exposed to stressful events. Recently, the involvement of thiamine-dependent protective mechanisms in stress response was reported in forced swim and immobilization stress models (Dief et al., 2015). However, the effects of increased thiamine levels on neuroplasticity during stress response were not addressed and no data on the effects of thiamine or benfotiamine on brain neurogenesis have been available.

Here, we investigated possible protective effects of thiamine an benfotiamine treatment on mice subjected to predator stress. As stress, including predation stress, is known to inhibit hippocampal neurogenesis (Tanapat et al., 2001; Hanson et al., 2011a; Hanson et al., 2011b), we chose a previously validated 5-day stress paradigm (Strekalova et al., 2015) in which the stressed mice display reduced proliferation of progenitor cells and decreased survival of newborn neurons in the subgranular zone of the dentate gyrus. Stress protocol also results in a loss of body weight, and increased depression and anxiety-like behaviors. The results show that both thiamine and benfotiamine efficiently protect against the harmful effects of stress. They also protect against weight loss and anxiety-like behavior. Most importantly, these protective effects were not accompanied by any increase in brain content of the coenzyme ThDP, strongly suggesting that the beneficial effects of thiamine and benfotiamine are not due to boosting of brain energy metabolism and implicate non-cofactor roles of thiamine in the brain.

2. Methods

2.1. Animals

Three-month-old male C57BL/6J mice were supplied by Instituto Gulbenkian de Ciência, Oeiras, Portugal). All experiments were carried out in accordance with the European

Communities Council Directive for the care and use of laboratory animals 2010/63/EU and in compliance with ARRIVE guidelines.

2.2. Reagents

Thiamine and benfotiamine were from Sigma-Aldrich NV/SA (Diegem, Belgium). Thiamine (1.7 g/L or 5 mM) was dissolved in tap water and pH was adjusted to 7 with NaOH. Benfotiamine (1.7 g/L or 3.7 mM) was dissolved in alkalinized tap water and pH was adjusted to 7 with HCl. Bromodeoxyuridine (BrdU, Sigma-Aldrich) was dissolved in 0.9% NaCl and 0.007M NaOH. Primary antibody rat anti-BrdU (1:500, AbD Serotec, Raleigh, NC, USA), anti-rat and anti-mouse secondary antibodies (1:500, Jackson ImmunoResearch, Europe Ltd, Suffolk, U.K.) were used.

2.3. Experimental design

For stress studies, 3.5 month-old male C57Bl/6J mice were single housed under a reversed 12-h light–dark cycle (lights on: 21:00 h) with food and water *ad libitum*, under controllable laboratory conditions (22 ± 1°C, 55% humidity). The experimental design is summarized in Fig. 1. Mice (n=40) were randomly divided into 4 experimental groups (n=10 for each group): not stressed-not treated (NS-NT), stressed-not treated (S-NT), stressed-treated with thiamine (S-Thia) and stressed-treated with benfotiamine (S-BFT). In each group of 10 mice, 5 animals belonged to the first cohort (that would receive BrdU injections, see below) and the other 5 belonged to the second cohort. Mice were thus housed 5 by cage and received either vehicle (tap water), thiamine (200mg/kg/day) or benfotiamine (200mg/kg/day) in oral water *ad libitum* for the 20 days of experiment. Thiamine and benfotiamine solutions were replaced every 7 days. At day 14, before the first stress session, mice of the first cohort received four BrdU (50mg/kg) intraperitoneal injections, spaced one from the others by 2 hours. Between day 15 and 20, mice from 3 experimental groups (NS-NT, S-NT, S-Thia) underwent predator stress, *i.e.* rat exposure while in a small container (Strekalova et al., 2015): mice were introduced into transparent glass cylinder (15 cm high x 8 cm diameter) and placed into the rat cage. 15-h exposure was performed between 18.00 PM and 9.00 AM for 5 consecutive nights. Mice did not have access to food and water during stress sessions; treatment with thiamine or benfotiamine continued throughout stress exposure.

To perform Ki67 and BrdU detection (Fig. 1A), mice of the first cohort (4 x 5 animals) were sacrificed 24h after the last stress session. They were deeply anaesthetized

with Nembutal (CEVA, Santé Animale, Brussels, Belgium, 0.01ml/g body weight) and transcardially perfused with 0.9% NaCl followed by 4% paraformaldehyde (PFA, Fluka Chemika, Breches, Switzerland). Brains were removed and post-fixed in 4% PFA for 12h at 4°C, followed by immersion for cryoprotection in 30% sucrose in 0.1M phosphate-buffered saline (PBS), pH 7.4, for 12h at 4°C. Brains were then frozen in 2-methylbutane.

In the second experiment (Fig.1B), a cohort of 4 x 5 animals was used and treatment as in A but without BrdU injection. The mice were weighted at days 1 and 20 and were submitted to step-down anxiety tests, first prior to rat exposure, then 3 h post-stress. They were subsequently sacrificed 6 h post-stress for plasma cortisone evaluation. In the third experiment (Fig. 1C), a cohort of 4 x 5 animals were treated as in B until day 20 and used for biochemical analysis. Animals were sacrificed by cervical dislocation 24h after replacement of treatment by normal water and 12h after the last stress session. Trunk blood was collected in EDTA blood collection tubes. Blood, liver, cortex and hippocampus were gathered and snap frozen (for determination of thiamine derivatives by HPLC (Bettendorff et al., 1991; Gangolf et al., 2010) or placed in *RNA later* (for detection of BDNF expression). Plasma cortisone levels were also determined.

2.4. Step-down anxiety test

The step-down apparatus (Evolocus LLC Tarrytown, NY, USA and Open Science, Moscow, Russia) consisted of a transparent plastic cubicle (25 cm x 25 cm x 50 cm) with a stainless-steel grid floor (33 rods 2 mm in diameter), onto which a square wooden platform (7cm x 7cm x 1.5cm) was placed. Mice were placed onto the platform inside a transparent cylinder and after removal of the cylinder, the time until the animal left the platform with all four paws was taken as a measure of anxiety (Strekalova and Steinbusch, 2010).

2.5. Study of cell proliferation and survival in the hippocampus

All steps were performed as described previously (Beukelaers et al., 2011; Strekalova et al., 2015). 40-µm-thick free-floating sagittal sections were obtained using a MSE (UK) microtome with a freezing table in sagittal plane from lateral 3.5 to lateral 0.3 along the medial-lateral axis (approx. Paxinos Franklin 2001 mouse brain). Every 4th section was taken from each hemisphere of each brain. Sections were collected in PBS, transferred to the cryoprotective anti-freeze solution and stored at -20°C.

To perform BrdU and Ki67 detection, sections underwent antigen retrieval for 30 minutes at 90°C in Target Retrieval Solution (Dako, Glostrup, Denmark). Sections were washed 3 x 10 min in Tris-buffered saline (TBS), pH 7.6, and DNA was denatured with 2N HCl for 30 min at 37 °C, followed by washing with 0.1M borate buffer pH 8.5 for 10 min. The sections were then incubated overnight at 4°C with primary antibody rat anti-BrdU (1:500, AbD Serotec, Raleigh, NC, USA) and primary antibody mouse anti-Ki67 (1:500, BD Biosciences, San Jose, CA, USA) diluted in TBS containing 0.1% Triton, 0.1% Tween 20 and 5% normal donkey serum (blocking solution). After 3 washings in TBS, sections were incubated for 1 hour at room temperature in TBS containing 0.1% Triton, 0.1% Tween 20 and the corresponding secondary antibodies (1:500, Jackson ImmunoResearch, Europe Ltd, Suffolk, U.K.), either coupled to fluorescein isothiocyanate (FITC) or Rhodamine Red-X. Finally, sections were rinsed in TBS and mounted between slide and cover slide using VectaShield Hard Set mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). The slides were stored in the dark at 4 °C.

Quantifications were realized by an experimenter blind to the experimental conditions. To evaluate cell proliferation and 5 days cell survival in the dentate gyrus, the granule cell layer and the subgranular zone BrdU-positive and Ki67-positive cells were exhaustively counted under a 40 × objective in a sampling of every fourth 40 µm thick sagittal section (560 µm) from the beginning of the dentate gyrus to the end of the dentate gyrus (*i.e.*, 0.36 mm to 2.52 mm from lateral bregma; approximately 14 sections of 40 µM). Cell numbers were expressed as the number of cells per mm^3 of the granule cell layer as described previously (Beukelaers & al. 2011).

Fluorescence images were acquired using the NIS-Element confocal system equipped with the Nikon A1R hybrid resonant confocal inverted microscope (Nikon). Fields were acquired using Z-scan with a step of 1.5 µm between each confocal plane. All sections prepared for comparison were analyzed at the same time, using the same acquisition parameters.

2.6. Biochemical analyses

For gene expression analysis, cortex and hippocampi were dissected directly at the end of the behavioral test and were stored in RNA*later* (Sigma-Aldrich, MO, USA) at -80°C until use. mRNA was extracted by using RNA isolation Nucleospin®RNA XS kit (Macherey-Nagel). First strand cDNA synthesis was performed on 1 µg of total RNA using random primers and *ProtoScript®II First Stand cDNA Synthesis* Kit (*New ENGLAND BioLabs®Inc.*).

Quantitative RT-PCR was performed using the *FastStart SYBR Green Master (*Roche) and the Light Cycler 480 II System (Roche). Sequences of primers used were the following: for actin, 5'-GACGGCCAGGTCATCACTAT-3' (forward) and 5'-ATGCCACAGGATTCCATACC-3' (reverse); for HPRT, 5'-GGTGGATTACATTAAAGCACTGAAT-3' (forward) and 5'-AAAGTTTGCATTGTTTTACCAGTGT-3' (reverse); and for BDNF 5'-CGGCGCCCATGAAAGAAGTA-3' (forward) and 5'-AGACCTCTCGAACCTGCCCT-3' (reverse). All primers were purchased from Integrated DNA technology (Leuven, Belgium). Relative quantification was performed with the 2-ΔΔ Ct method. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was the reference gene for the hippocampus and actin was the one for cortex. Data are given as expression-folds compared to the mean expression values in non-stressed control mice (Guide to performing quantitation of gene expression using qRT-PCR, Applied Biosystem).

For the estimation of thiamine derivatives, mice were sacrificed by cervical dislocation and hippocampus, sensor-motor cortex, liver and blood were isolated for determination of thiamine derivatives by HPLC as previously described (Bettendorff et al., 1991; Gangolf et al., 2010).

For plasma corticosterone assay, trunk blood was collected in EDTA at sacrifice, stored at 4°C overnight and centrifuged at 10 x g for 10 minutes. Plasma was collected and stored at -80°C until use. All samples were run in duplicate. For plasma corticosterone levels, a commercially available ELISA kit (Sigma-Aldrich, MO, USA) was used according to manufacturer's instructions. The average intra- and inter-assay coefficients of variation for all corticosterone assays fell below 10%. The assay had a sensitivity of 3.7 ng/mL.

2.7. Cell culture and cell survival

Neuroblastoma cells (Neuro2a) were grown for 7 days in DMEM medium devoid of thiamine but supplemented with 10% fetal calf serum as previously described (Bettendorff et al., 1995; Volvert et al., 2008). The only source of thiamine is the fetal calf serum and the final thiamine concentration is approximately 14 nM, a value close to circulating thiamine concentrations in animals. Under these conditions, the cells grow normally. After 7 days, the cells were subcultured in triplicate at a density of $5x10^4$ cells / 200 µl in 96-well culture plates and grown overnight. Thiamine (50 µM) or benfotiamine (50 µM) were added 1 h prior to the addition of 0.25 mM paraquat (Sigma-Aldrich). No additional thiamine was added in control cells.

After 24 h, the medium was changed and the cells were incubated in DMEM medium

for 3 h with 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (0.15 mg/ml, MTT, Sigma-Aldrich) at 37 °C in 5% CO_2. The medium with MTT was removed and replaced by 0.07 N HCl in isopropanol. Absorbance was measured at 580 nm using the ELISA plate reader (Microplate Reader, Thermo Labsystems). Viability was expressed as a percentage of control cells (100%).

In a parallel experiment, the intracellular content of thiamine derivatives was determined. The cells were grown as described above in DMEM medium devoid of thiamine and transferred in triplicate in 6-well culture plates for 3 days at a seeding density of $1x10^5$/ 2 ml. They were then incubated with thiamine (50 µM) or benfotiamine (50 µM) for 1 or 25 h. The cells were then washed twice with cold PBS (Lonza) and detached by scrapping and placed in an Eppendorf vial. After addition of trichloroacetic acid (12 %, Sigma-Aldrich), they were centrifuged at 5000 g (4 °C) for 15 min. and extracted with diethyl ether (3 x1.5 mL). They were stored frozen at -20 °C until determination of thiamine by HPLC (Bettendorff et al., 1991; Gangolf et al., 2010).

The pellets were dissolved in NaOH 0.8 N and used for protein determination (Peterson, 1977).

2.8. Statistical analysis

Analysis was performed using GraphPad Prism software version 5.03 for Windows (San Diego CA, USA). One-way ANOVA followed by Tukey's post-hoc test was applied to compare three or more groups, except CORT data, which were treated by Kruskal Wallis test due to non-normal distribution. The level of confidence was set at 95% ($p < 0.05$) and all data are expressed as mean ± SD.

3. Results

3.1. Thiamine and benfotiamine prevent stress-induced suppression of hippocampal neurogenesis

In agreement with recently reported data (Strekalova et al., 2015) exposure of the mice to predator stress (5-day rat exposure) reduced the number of progenitor cells and short-term survival of new immature neurons in the subgranular gone of the dentate gyrus. As shown in Fig. 2, both thiamine or benfotiamine treatment (concomitant with stress exposure) were effective to prevent the stress-induced impairment of hippocampal neurogenesis. Benfotiamine was particularly powerful, increasing the density of Kir7-positive progenitor

cells by a factor of four to five (Fig. 2A). The density of BrdU-positive cells was significantly reduced in the stressed non-treated group, as compared to the non-stressed control group. This reduction was counteracted by thiamine and benfotiamine treatment. The seemingly stronger effect of benfotiamine compared to thiamine may be related to the fact that benfotiamine administration is much more efficient to raise blood thiamine concentrations than thiamine administration (Volvert et al., 2008).

3.2. Thiamine and benfotiamine protect against stress-induced weight loss and anxiety-like behavior

After 20 days of treatment (immediately before sacrifice), mice subjected to predator stress exhibited a 15% reduction in bodyweight, but it was significantly reversed when the mice were simultaneously treated by either thiamine or benfotiamine (Fig. 3A). This suggests that the two compounds have an anti-stress action. In line with this idea, the latency of step-down from a platform, a well-established measure of anxiety-like behavior, was increased in stressed animals and this was counteracted by thiamine treatment (Fig. 3B). In naive non-stressed mice, no effect of thiamine or benfotiamine treatment on latency of step-down could be found (not shown). Taken together, these results suggest that thiamine and benfotiamine protect against the harmful effects of high-intensity stress.

A possible explanation for these anti-stress effects would be that thiamine and benfotiamine could relieve a harmful hormonal stress response, e.g. by decreasing plasma corticosterone levels. As shown in Fig. 3, there is indeed some increase of plasma corticosterone 6h or 24h after stress exposure, although this does not reach statistical significance. In both cases, however, the treatment by thiamine or benfotiamine failed to decrease corticosterone levels. Thus, the anti-stress effects of the applied compounds are unlikely to be mediated by an effect on the hypothalamic-pituitary axis.

3.3. Brain levels of ThDP are not increased by thiamine and benfotiamine treatment

Ever since thiamine and its precursors were found to have neuroprotective actions, this was considered to be due to an increase in blood levels of thiamine, that caused a secondary increase in brain content of the cofactor ThDP. This was supposed to boost glucose utilization and brain energy metabolism, which are essential for neuronal activity (Gibson and Blass, 2007). We therefore compared the contents of thiamine and its phosphorylated derivatives in the blood, liver, hippocampus and sensorimotor cortex of mice subjected to different

treatments (non-stressed, stressed-not treated and stressed treated with thiamine or benfotiamine). In whole blood (Fig. 4A), there were no significant differences between the four groups (except for one small significant increase in thiamine monophosphate (ThMP) level after benfotiamine treatment). Likewise, no significant differences were found in liver content of thiamine or ThDP (Fig. 4B). With regard to benfotiamine effects, those results might appear to be at variance with previously published results (Volvert et al., 2008) showing strong increases in blood and liver thiamine after benfotiamine treatment. These increases were transient, however, and most of the excess thiamine was eliminated after a few hours. In the present study, we estimated blood, liver and brain thiamine contents one day after the replacement of treatment solutions with water. This delay appears to be sufficient to eliminate most excess thiamine from the blood and liver but not from the brain, as it is known that thiamine transport through the blood-brain barrier is very slow (Greenwood et al., 1982).

In the brain, data displayed in Fig. 4C and D show that thiamine and benfotiamine treatment of stressed mice causes a small but significant increase (1.5-fold) in free thiamine content, both in hippocampus and cortex. Concerning ThDP, no differences between the 4 groups were found. Small differences in ThMP content were observed in cortex (as in blood) but significance of these effects (if any) is unknown. It should also be noted that stress exposure by itself has no effect on the levels of thiamine compounds in blood, liver or brain. We conclude from these data that, in mice subjected to predator stress, the only observed effect of thiamine or benfotiamine in the brain is a modest increase in free thiamine content, while the level of cofactor ThDP is unaffected. This strongly suggests that the anti-stress effects of thiamine and benfotiamine are unrelated to stimulation of brain energy metabolism.

We also tested the effects of predator stress and treatments with thiamine and benfotiamine on a few other biochemical parameters in the brain. As was reported in a mouse model of Alzheimer's disease (Pan et al., 2010), benfotiamine treatment increased the phosphorylation (and hence reduced the activity) of glycogen synthase kinase beta (GSK-3β), we tested the effect of the different treatments on the phosphorylation level of GSK-3β and Akt (the main kinase responsible for GSK-3β phosphorylation). In both cases, neither thiamine nor benfotiamine treatments were able to increase the phosphorylation level of those kinases (data not shown).

Finally, we measured the expression of brain-derived neurotrophic factor (BDNF) in mice subjected to predator stress and thiamine or benfotiamine treatment. Previous studies

have shown that chronic unpredictable stress decreases the expression of BDNF in the rodent hippocampus (Autry et al., 2009; Bath et al., 2013). In the cortex of our mice, predator stress indeed decreased the expression of BDNF and this was reversed by benfotiamine (but not thiamine) treatment (Fig. 5A). In the hippocampus, however, no significant effects could be found (Fig. 5B). Thus, it appears that, in mice subjected to predator stress, the observed boosting of AHN in the dentate gyrus (Fig. 2) after thiamine or benfotiamine treatment is not related to an increase in expression of BDNF.

3.4. Benfotiamine protects against oxidative stress in cultured neuroblastoma cells

As our results suggest that benfotiamine treatment does not act by boosting energy metabolism in the hippocampus, other possible mechanisms must be considered. Previous studies have shown that stressful events, such as acute restraint stress in rats, have harmful effects on the hippocampus and these effects are related, at least partially, to oxidative damage and inflammation (Liu et al., 1996; Spiers et al., 2013; Chen et al., 2016). It is thus plausible that the inhibition of cell proliferation and survival that were observed after predator exposure may be linked to oxidative stress and inflammation. In order to check this hypothesis, we induced oxidative stress in cultured Neuro2a cells using paraquat, which is well-known to induce oxidative stress (Nunes et al., 2016; Singh et al., 2016). Paraquat significantly reduced cell viability and its effect was efficiently counteracted by benfotiamine, but not by thiamine (Figure 6A). In a parallel experiment, we determined intracellular thiamine derivatives at the end of the experiment after 25 h. It is clear from the present data that both thiamine and ThDP levels are strongly increased, but while benfotiamine was more efficient than thiamine in raising intracellular thiamine concentrations, both compounds were equally efficient concerning ThDP. Hence it is unlikely that the protective effects of benfotiamine are linked to increased cofactor levels. It is more likely that it acts through thiamine or some unknown compound, confirming the conclusion obtained on intact animals (Fig. 4).

4. Discussion

In the present work, we show for the first time, that treatment with thiamine and its precursor benfotiamine efficiently prevents stress-induced suppression of adult hippocampal neurogenesis (AHN) in mice exposed to predator stress. The stress protocol resulted in a significant decrease in the proliferation of progenitor cells in the subgranular zone of the dentate gyrus. Short survival of newborn neurons was also decreased. These effects on AHN

were concomitant with physiological changes such as lowering of body weight and increased signs of anxiety-like behavior. Therefore, this protocol appears to be an effective, relatively easy and rapid way to impair AHN and simultaneously induce physiological and behavioral symptoms that are well known to be associated with high-intensity unpredictable stress.

Perhaps the most important finding reported here is that treatment of the stressed mice with thiamine or benfotiamine is remarkably efficient in being able to boost both progenitor cell proliferation and short-term survival of newborn neurons in the subgranular zone of dentate gyrus. This is reminiscent of the results obtained in a mouse model of anxiety/depression-like state induced by corticosterone treatment (David et al., 2009). The authors found that the antidepressant fluoxetine induced a large increase of cell proliferation in the SGZ of corticosterone-treated animals. Many other studies (for review see Warner-Schmidt and Duman, 2006; Miller and Hen, 2015) have shown that treatment with various antidepressant drugs protected AHN against harmful effects of stress, especially chronic unpredictable and inescapable stress. However, none of the drugs used in these studies were as potent as benfotiamine proved to be for increasing cell proliferation and survival of newborn neurons. The use of thiamine and precursors with high bioavailability could therefore be considered as a complementary therapy in states of anxiety and depression. Of note, a recent clinical study showed antidepressive effects of thiamine administration to depressed patients (Ghaleiha et al., 2016).

A straightforward interpretation of our results would be that the observed protective effects on AHN are linked to elevated levels of blood thiamine, resulting in increased contents of thiamine compounds in the brain parenchyma after prolonged treatment with thiamine or its precursor. The higher potency of benfotiamine compared to thiamine does not seem unexpected as it was previously shown in normal wild-type mice that oral treatment with benfotiamine induces a strong increase in plasma thiamine levels (Volvert et al., 2008). This effect was transient and excess thiamine was nearly completely eliminated in less than 12 hours. Nonetheless, it remains possible that brain thiamine levels could increase significantly after daily administration of high doses of benfotiamine, as was the case in this study. However, we found that the 20-day treatment with thiamine or benfotiamine only caused a modest (1.5-fold) increase in brain thiamine (but not ThDP) content. It is also notable that benfotiamine was not more effective than free thiamine to increase brain thiamine content. Indeed, we found that the only effect of thiamine or benfotiamine treatment on brain content of thiamine compounds is a 1-5-fold increase in free thiamine content. Moreover, in cultured

neuroblastoma cells, incubation in the presence of benfotiamine protected against paraquat-induced stress while incubation with thiamine did not, while both compounds equally raised intracellular ThDP content. These results suggest that the effects observed in this study are not due to coenzyme function but either to thiamine or to some unknown metabolite.

In order to elucidate the mechanisms of AHN protection by thiamine and benfotiamine, it is desirable to have a clear understanding of how predator stress, and for that matter any kind of unpredictable high-intensity stress, results in impairment of AHN. This impairment is generally linked to forms of stress that are known to precipitate and worsen depressive symptoms (Duman, 2004). Interestingly, treatments known to improve anxiety/depression states (antidepressant drugs, environmental enrichment, physical exercise, electroconvulsive therapy) tend to reverse the effects of stress and boost AHN (Warner-Schmidt and Duman, 2006; Stranahan et al., 2006; Ming and Song, 2011; Miller and Hen, 2015). The mechanisms underlying AHN impairment by high-intensity stress are complex and involve many factors. The most important seem to be an overproduction of stress hormones (adrenal steroids such as corticosterone and, in the brain, oxidative stress (Liu et al., 1996; Spiers et al., 2013; Chen et al., 2016) and abnormal production of inflammatory factors (Schoenfeld and Gould, 2012). Our results do not favour the hypothesis that thiamine and benfotiamine could act by reducing blood levels of stress hormones, as we observed no decrease of blood corticosterone after treatment of the stressed mice with thiamine or benfotiamine. We also considered the possibility that those compounds might act by increasing the expression of the growth factor BDNF, which is known to favor AHN (Duman and Monteggia, 2006; Castrén et al., 2007). We found no decrease in BDNF expression in the hippocampus when the mice had been subjected to predator stress, and treatment with thiamine and benfotiamine had no significant effect either. This is in line with the suggestion b that the main action of BDNF in neurogenesis is to favor the maturation of new neurons rather than boosting the proliferation and survival of progenitor cells (Chan et al., 2008).

In view of these data, we rather favour the hypothesis that the beneficial effects of thiamine and benfotiamine are linked to antioxidative and/or anti-inflammatory actions. Indeed, it is known that inflammation reduces cell proliferation and survival of new neurons in the dentate gyrus (Ekdahl et al., 2003) and increased levels of cytokines such as interleukin-1 reduce progenitor cell proliferation in the subgranular zone of dentate gyrus (Koo and Duman, 2008). In contrast, inflammatory blockade by indomethacin was shown to restore AHN (Monje et al., 2003).

A number of previous studies support the view that thiamine and benfotiamine can act as antoxidative and anti-inflammatory agents. Antioxidative effects of benfotiamine were demonstrated *in vivo* (Schmid et al., 2008; Bozic et al., 2015b) and *in vivo* (Wu and Ren, 2006; Marouf et al., 2011). Here we show that benfotiamine has a protective action against the harmful effects of the prooxidant compound paraquat in neuroblastoma cells. Other studies have shown that benfotiamine can exert anti-inflammatory effects as well (Sanchez-Ramirez et al., 2006; Shoeb and Ramana, 2012; Bozic et al., 2015a). It was recently reported that, in rats subjected to chronic isolation stress, there was a decrease in the level of nuclear factor erythroid 2-related factor 2 (Nrf2) in the hippocampus; there was a concomitant increase in the level of NFκB (Djordjevic et al., 2015). This is consistent with the idea that Nrf2 plays an important role in brain inflammation, as it can antagonize the NFKβ pathway, a hallmark of inflammation. An attractive working hypothesis would be that the neuroprotective effects of benfotiamine implicate Nrf2 as a key target in the hippocampal formation.

Finally, it is important to emphasize that we found no significant effect of thiamine or benfotiamine treatment on brain levels of the coenzyme ThDP, an indispensable cofactor for brain energy metabolism. This strongly suggests that the protective effects of thiamine and benfotiamine cannot be explained primarily by an enhancement of glucose consumption and oxidation. The present results are in agreement with previous data (Volvert et al., 2008) showing that, in normal WT mice, oral treatment with high doses of benfotiamine had no effect on brain ThDP levels. Likewise, in a mouse model of Alzheimer's disease, benfotiamine also failed to increase brain ThDP (Pan et al., 2010). Actually, increases in brain ThDP by thiamine treatment were found only in animals previously submitted to treatments causing a strong deficiency of thiamine (Gibson and Blass, 2007).

From these data, we conclude that, in mice subjected to predator stress, the beneficial effects of thiamine and benfotiamine treatment do not primarily seem to involve the regulation of brain energy metabolism. They are more likely to be related to effects of free thiamine or unidentified metabolites on functions such as neurotransmission, cell-signaling and neurogenesis, possibly through antioxidative and anti-inflammatory actions. Hopefully, the elucidation of these mechanisms could lead to the development of new compounds that would be more active than benfotiamine. It should be recalled that the beneficial effects of this precursor require prolonged treatment with high doses. Nonetheless, it should be stressed that, even at high doses, benfotiamine has no known toxicity or side effects (Stracke et al., 2008). We believe that the development of new thiamine precursors acting at lower concentrations represent a promising strategy to protect the hippocampal formation from

harmful effects of free radicals and inflammatory factors. This might be helpful not only in stress-induced states of anxiety and depression but also in neurodegenerative disorders such as Alzheimer's disease.

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Legends to figures

Fig. 1. Schematic timeline of the experimental protocol. (A) Impact of predator exposure on proliferation of progenitors (Ki67 labeling) and survival of newborn immature neurons (BrdU labeling) in the subgranular zone of the dentate gyrus. (B) Impact of predator exposure on body weight and anxiety-like behavior. (C) Biochemical analyses: impact of predator stress on blood corticosterone concentrations and levels of thiamine derivatives in different tissues.

Fig. 2. Effect of thiamine and benfotiamine on the proliferation of progenitor cells and short-term survival of new immature neurons in the subgranular zone of the dentate gyrus after predator stress. (A) Number of Ki67-positive cells per mm^3 of cell granular layer in non stressed-not treated (NS-NT, n=5), stressed-not treated (S-NT, n=3), stressed-treated with thiamine (S-Thia, n=5) and stressed-treated with benfotiamine (S-BFT, n=5) mice. Data (expressed as mean ± SD) were analyzed by one-Way ANOVA followed by Tukey's post-hoc Multiple Comparison Tests (*$p<0.05$, **$p<0.01$ as compared to NS-NT; #$p<0.05$, ###$p<0.001$ as compared to S-NT). (B) Representative photomicrographs of Ki67-labeling in NS-NT, S-NT, S-Thia and S-BFT groups (C) Number of BrdU-positive cells per mm^3 of cell granular layer in the same groups of animals as in A. Data analysis as in A. (D) Representative photomicrographs of BrdU-labeling in the same groups as in B.

Fig. 3. Effect of thiamine and benfotiamine on predator stress-induced bodyweight loss, anxiety-like behavior and corticosterone levels (A) Body weight was measured in non stressed not treated (NS-NT, n=5), stressed-not treated (S-NT, n=5), stressed-treated with thiamine (S-Thia, n=5) and stressed-treated with benfotiamine (S-BFT, n=5) groups at the beginning (day 1) and end of treatment (day 20). Body weights of day 20 are presented as percentage from body weight of day 1. (#$p<0.05$, ###$p<0.001$ vs S-NT). (B) Latency of step-down from a platform, a measure of anxiety-like behavior. (C, D) Corticosterone levels in the plasma of non stressed-not treated (NS-NT), stressed-not treated (S-NT), stressed-treated with

thiamine (S-Thia) and stressed-treated with benfotiamine (S-BFT) groups sacrificed 6 h **(C)** or 24 h **(D)** after the last stress session. (*p<0.05 vs NS-NT).

Fig. 4. Effect of predator stress and treatment with thiamine or benfotiamine on the contents of thiamine derivatives in the blood (A), liver (B) and brain (C, D) of C57BL mice. The animals were sacrificed 24 hours after the last treatment with thiamine, benfotiamine or vehicle. Data (expressed as mean ± SD) were analyzed by one-Way ANOVA followed by Tukey's post-hoc Multiple Comparison Tests (* p<0.05 vs NSNT, #p<0.05 vs SNT on day 20) and by repeated two-way ANOVA followed by a Bonferroni post hoc test (day 20, $$$ p<0.001 vs day 1).

Fig. 5. Effects of by stress and thiamine or benfotiamine treatment on BDNF in the cerebral cortex and hippocampus of C57BL mice. BDNF mRNA levels obtained by qRT-PCR in cortex and hippocampus are expressed as fold of mRNA levels in non stressed – not treated mice (NS-NT). Represented groups are non stressed - not treated (NS-NT, n=5), stressed - not treated (S-NT, n=5), stressed - treated with thiamine (S-Thia, n=5) and stressed - treated with benfotiamine (S-BFT, n=5). Data (expressed as mean ± SD) were analyzed by one-Way ANOVA followed by Tukey's post-hoc multiple comparison test (*p<0.05 vs NS-NT).

Fig. 6. Effect of thiamine and benfotiamine on cell viability (A) and intracellular thiamine and ThDP levels (B) after 25 h in cultured mouse neuroblastoma cells after paraquat-induced oxidative stress. The cells were incubated in low thiamine medium (14 nM) in the presence of paraquat (0.25 mM) for one hour prior to addition of 50 µM thiamine or benfotiamine After 25 hours, cell survival and intracellular thiamine derivatives were determined. A one-way ANOVA was performed ($p < 0.0001$, $n = 21$) followed by Tukey's multiple comparisons test. Paraquat significantly reduced cell survival ($p < 0.0001$). A significant rescue was observed only with benfotiamine ($p < 0.0001$). For analysis of intracellular thiamine derivatives a two-way ANOVA was performed ($p < 0.0001$).

Figures

Figure 1

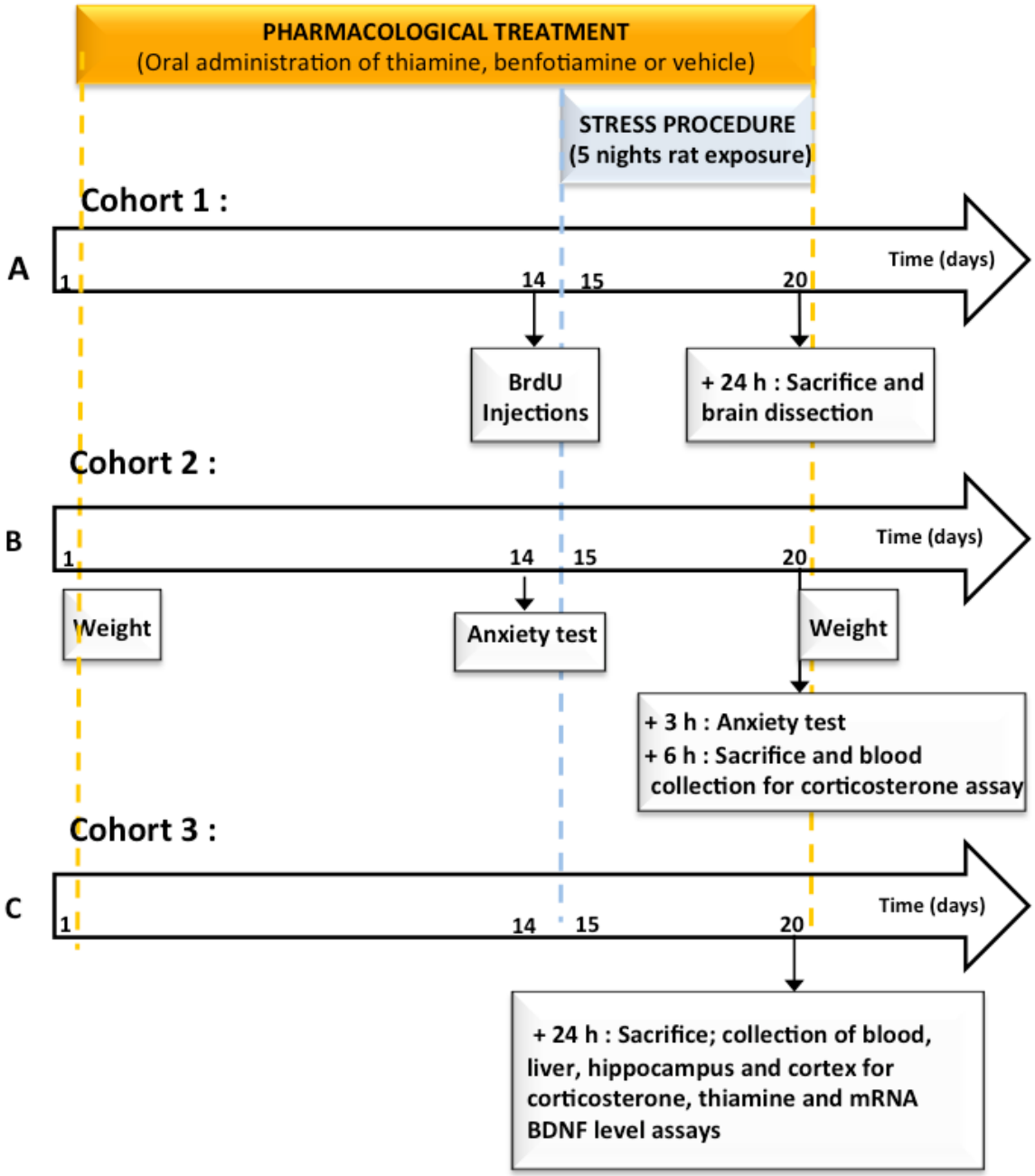

Figure 2

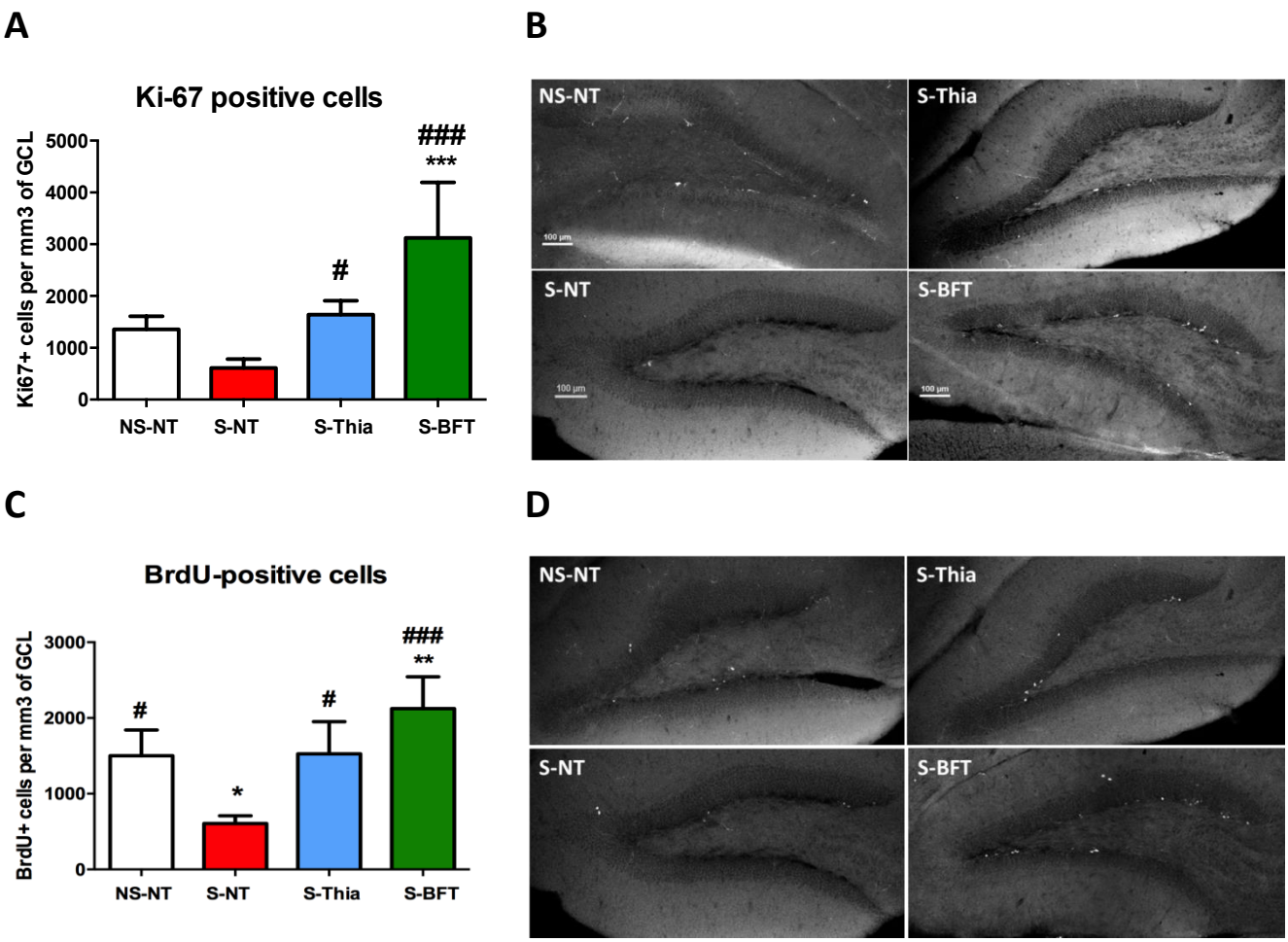
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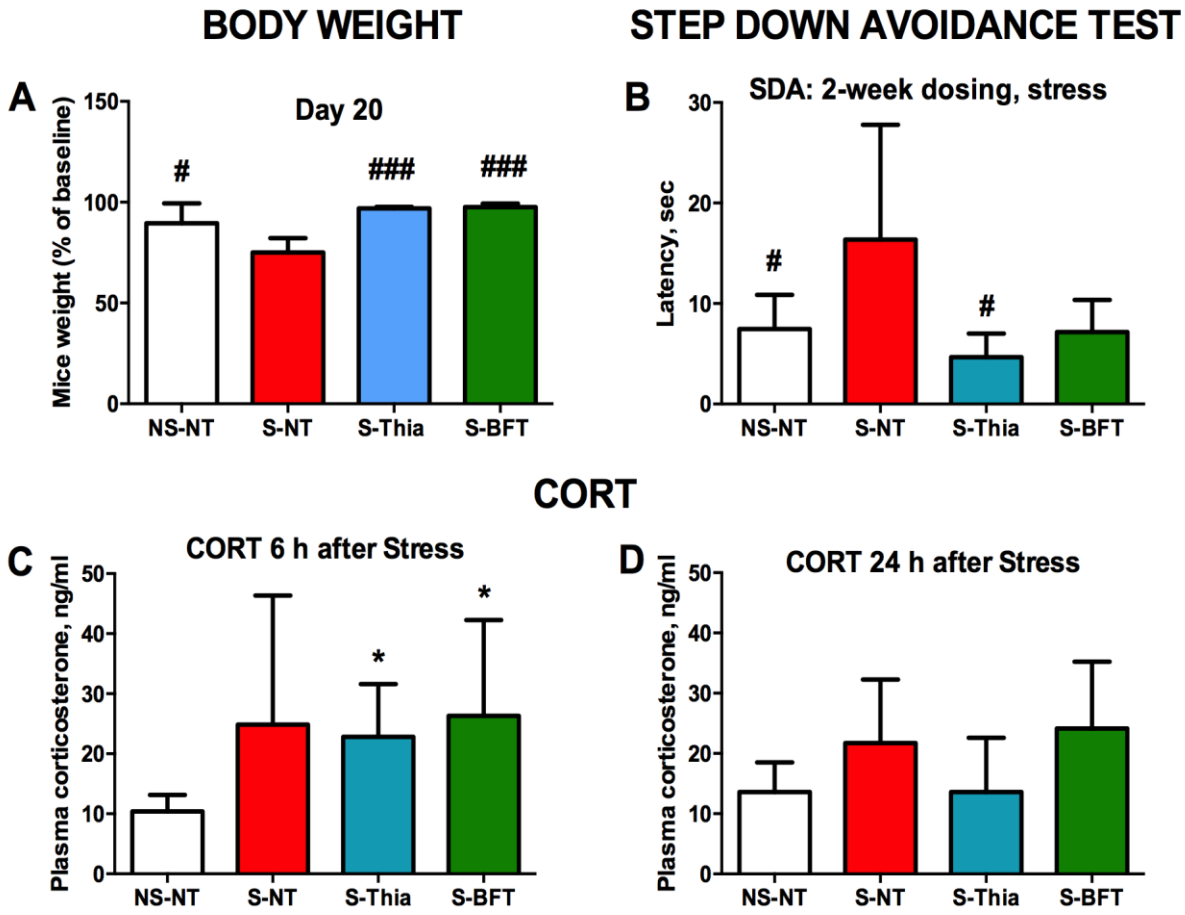
Figure 3


BODY WEIGHT
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S-BFT
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CORT 6 h after Stress
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S-BFT

Figure 4

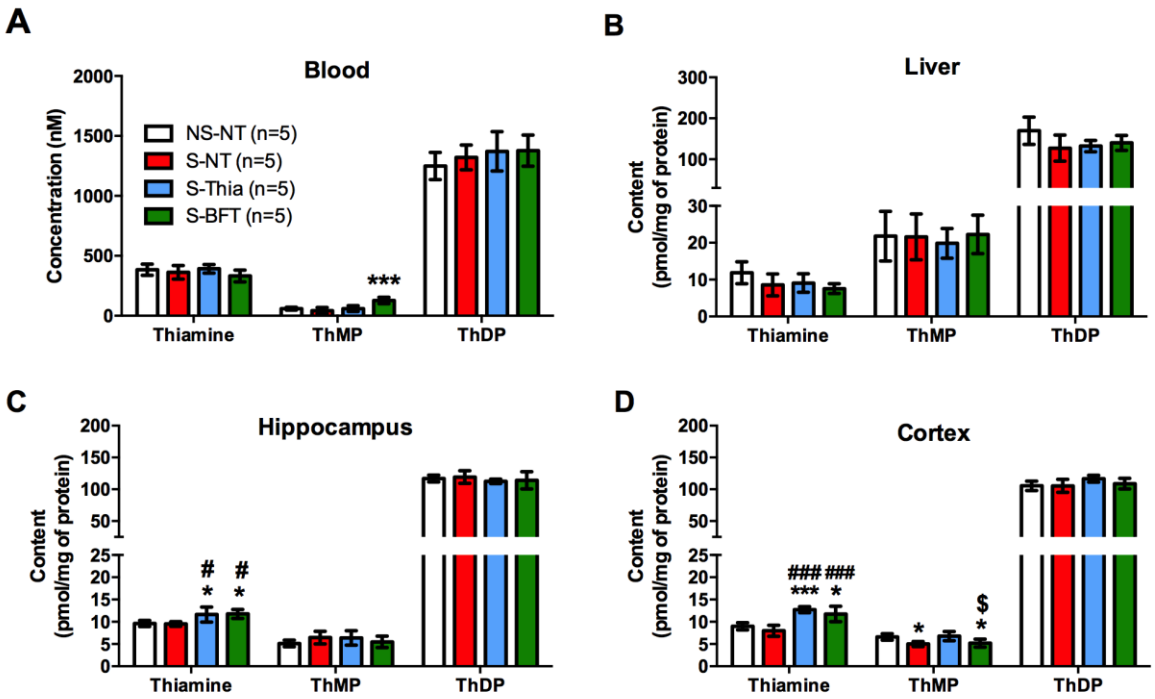


Figure 5

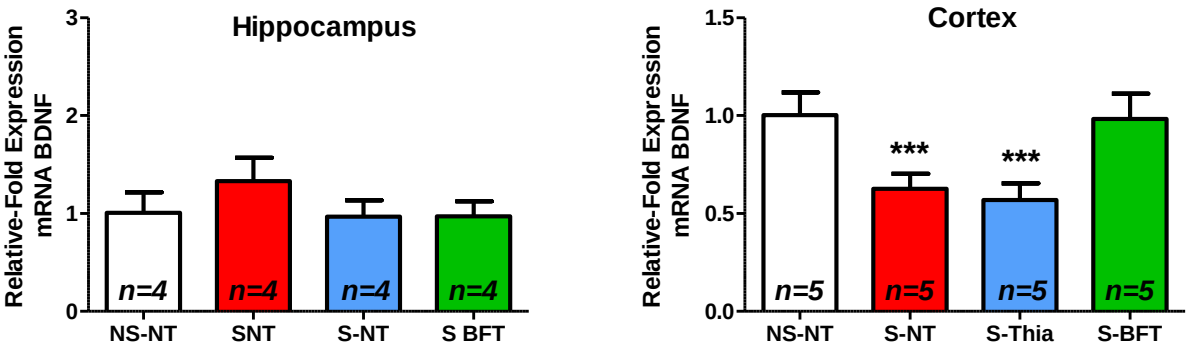
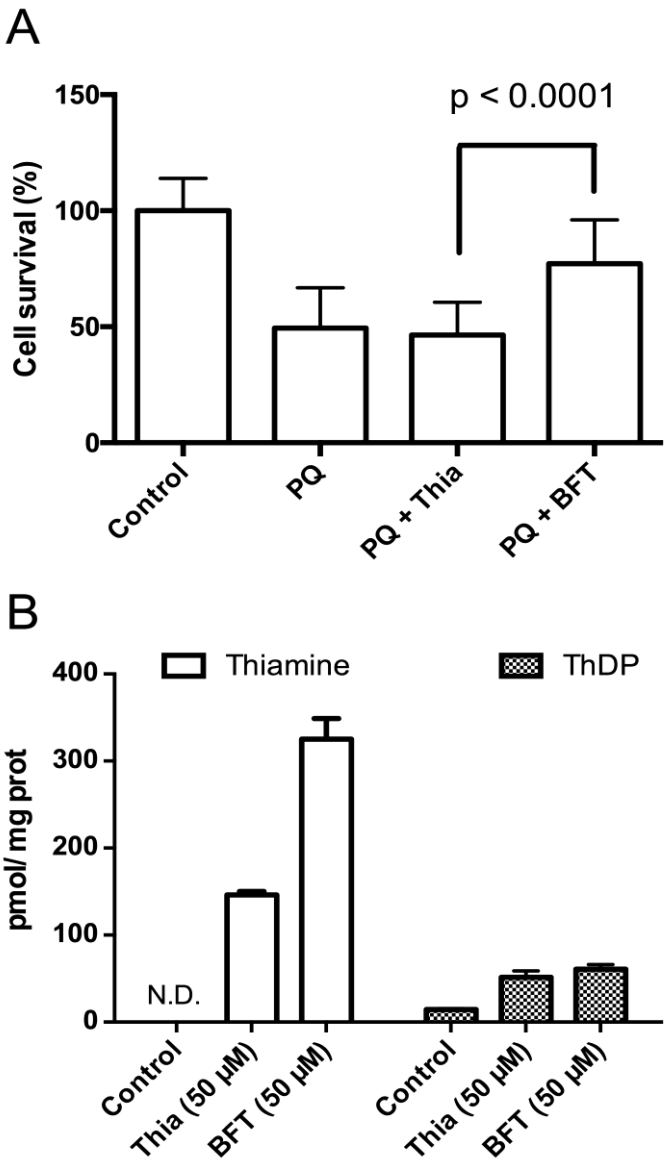


Figure 6


A
p < 0.0001
Cell survival (%)
150
100
50
0
Control
PQ
PQ + Thia
PQ + BFT
B
Thiamine
ThDP
pmol/ mg prot
400
300
200
100
0
N.D.
Control
Thia (50 µM)
BFT (50 µM)
Control
Thia (50 µM)
BFT (50 µM)