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Research article

Sleep deprivation changes thimet oligopeptidase (THOP1) expression and activity in rat brain

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ABSTRACT

The consequences of sleep deprivation on memory, cognition, nociception, stress, and endocrine function are related to the balance of neuropeptides, with peptidases being particularly essential. Thimet oligopeptidase (THOP1) is a metallopeptidase implicated in the metabolism of many sleep-related peptides, including angiotensin I, gonadotropin releasing hormone (GnRH), neurotensin, and opioid peptides. In the present study, we evaluated the effect of sleep deprivation and sleep recovery in male rats on THOP1 expression and specific activity in the central nervous system. In the striatum and hypothalamus, THOP1 activity decreased following sleep deprivation and a recovery period. Meanwhile, THOP1 activity and immunoexpression increased in the hippocampal dentate gyrus during the sleep recovery period. Changes in THOP1 expression after sleep deprivation and during sleep recovery can potentially alter the processing of neuropeptides. In particular, processing of opioid peptides may be related to the known increase in pain sensitivity in this model. These results suggest that THOP1 may be an important player in the effects of sleep deprivation.

1. Introduction

Neuropeptides play fundamental roles in control of the physiological sleep-wake cycle, and have been shown to be altered under conditions of sleep deprivation [1, 2]. In particular, several studies have reported roles in sleep control for gonadotropin releasing hormone (GnRH), somatostatin, neurotensin, substance P, neuropeptide Y, growth hormone, adrenocorticotropic hormone, and corticotropin-releasing hormone [2, 3, 4, 5, 6]. However, during and after sleep deprivation, physiological changes occur in both humans and animal models with respect to cardiovascular parameters, immune function, pain perception, inflammatory response, learning and memory, and insulin resistance and obesity [7, 8, 9]. Many of these effects are related in some manner to changes in the metabolism of neuropeptides in the central nervous system (CNS) [2].

Peptidases play essential roles in the generation and degradation of peptides within the CNS [10]; they directly control the release of active peptides from non-active precursors, the generation of new ligands from already active precursors, and the inactivation of both endogenous and exogenous peptides. However, the modification of proteolytic activities in the CNS as a possible consequence of sleep deprivation has not been a frequently-addressed subject [11].

Thimet oligopeptidase (EC 3.4.24.15; THOP1) is a metallopeptidase involved in the metabolism of several neuropeptides expressed in neurons and glial cells [12, 13]. THOP1 hydrolyzes peptides of 6–17 amino acids in length, including: angiotensin I, somatostatin, neurotensin, bradykinin [12, 14], GnRH [15], and opioid peptides [16]; it also participates in the presentation of major histocompatibility complex class I (MHC-I) peptides [17, 18]. Moreover, processing of neurotensin and opioid peptides by THOP1 emphasizes the importance of peptidases in the control of neuroendocrine and immunological systems. According to Simões and collaborators (2014), THOP1 levels are reduced in both the hippocampus of epileptic patients and in animal models of epilepsy, which effect is potentially related to the accumulation of inflammatory peptides in this area of the brain [19]. However, in vivo demonstration

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that THOP1 is required to metabolize neuropeptides and to modulate the nociception, immunological, and endocrine systems remains elusive.

A commonly accepted animal model for studying the consequences of and mechanisms associated with sleep loss is paradoxical sleep deprivation (PSD) in rodents [20]. Using this model, we have previously shown changes in angiotensin-converting enzyme (ACE) expression and activity in the CNS, which may correlate with changes in cardiovascular response, stress, and cognitive performance [21]. Furthermore, in the present study, we determined THOP1 expression and activity in different areas of the CNS in male rats subjected to PSD and sleep rebound (SR).

2. Materials and methods

2.1. Animals

Male Wistar rats (3 months of age) were obtained from the Centro de Desenvolvimento de Modelos Experimentais para Biologia e Medicina at the Universidade Federal de São Paulo (UNIFESP). The animals were housed inside standard polypropylene cages in a colony maintained at 22 °C on a 12:12 h light–dark cycle (lights on at 07:00 h and off at 19:00 h). All procedures used in the present study complied with the Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Ethics Committee of UNIFESP (#0144/09).

2.2. Experimental protocol

Animals were divided into five groups (n = 7–10). The PSD group was subjected to paradoxical sleep deprivation over a 96 h period using the modified multiple platform method [20]. During the PSD period, rats were placed inside a water tank (123 × 44 × 44 cm), containing 14 circular platforms, each 6.5 cm in diameter. The tank was filled to within 1 cm of the upper borders of the platforms; the animals could thus move around inside the tank by jumping from one platform to another. When an animal entered PSD, muscle atonia set in and it fell into the water, thus waking up. Immediately after completing the 96 h PSD period, the animals were euthanized, and tissues collected. The three sleep recovery (SR) groups were similarly submitted to PSD, then were allowed 24, 48, or 96 h of SR before being euthanized, generating respectively the SR24, SR48, and SR96 groups. During SR periods, the animals were left to sleep freely. Finally, a home-cage control (CTRL) group was maintained for the duration of the experiment in the same room and sleeping *ad libitum*. Food and water were provided *ad libitum*. Water in the tank was changed daily throughout the study period.

2.3. Tissue samples for evaluation of activity and gene expression

All rats were euthanized by decapitation in the same day between 8:00 and 10:00 AM. The brain tissues were hand-dissected on ice according to stereotaxic coordinates atlas and histological anatomy [22] and removed, either for protein or RNA total extraction (Trizol, Thermo Fisher Scientific, USA). The brain was removed from skull and placed ventral side up to dissect hypothalamus using curved forceps. Then the location and shape of the tissues of total cerebral cortex, striatum (caudado-putamen and nucleus accumbens), hippocampus (included CA1-3 hippocampal regionals), and brainstem (pons-medulla and midbrains) were dissected from thick coronal slides based on the atlas. For protein extraction, tissues were homogenized in Tris-HCl 50mM buffer, pH 7.4, containing 100 mM NaCl and 0.1% Triton X-100. Total homogenates were centrifuged at 1,000 *g* for 15 min at 4 °C and the supernatant was frozen at -20°C until THOP1 activity measurements were taken. Protein content was measured by the method of Lowry using bovine serum albumin as a standard.

2.4. THOP1 activity measurement

THOP1 activity in total homogenate was determined using a fluorogenic substrate, Abz-GFDPFRQ-EDDnp (10 μM) [23], and the specific inhibitor JA-2 (N-[1-(R,S)-carboxy-3-phenylpropyl]-Ala-Aib-Tyr-*p*-aminobenzoate) at 2 μM final concentration [24]. In order to differentiate neurolysin activity from that of THOP1, a specific neurolysin inhibitor, Pro-Ile (0.5 mM), was added to the incubations [25]. Assays were performed in a buffer containing 50 mM Tris-HCl, pH 7.4, and 100 mM NaCl at 37 °C. A 5 min incubation with DTT (0.5 mM) at assay temperature was used to activate THOP1 before adding the substrate. Reactions were continuously followed by measuring fluorescence at λex = 320 nm and λem = 420 nm with a fluorometer (Gemini XS, Molecular Devices Company, USA). Measurements were performed in triplicate. THOP1 activity values were reported as nanomoles of substrate hydrolyzed per minute per milligram of protein ($nM.min^{-1}.mg^{-1}$).

2.5. Real-time PCR

Tissue expression levels of *THOP1*, *β-actin* and *GAPDH* mRNA were independently assessed by real-time PCR using 10 ng total cDNA, SYBR Green Universal Master Mix (Thermo Fisher Scientific, USA), and the following sets of primers: *THOP1*, 5'-TGATGTGGA-GATGAGCATGAGGCA-3' and 5'-AGGCTTCAGTGAGTCTTTCGGGAT-3', NM_172075.2; *β-actin*, 5'AGGCCAACCGTGAAAAGATG-3' and 5'-CCA-GAGGCATACAGGGACAAC-3', NM_017008; and *GAPDH*, 5'-TGCCCCCATGTTTGTGATG-3' and 5'-GCTGACAATCTTGAGGGAGTTGT-3', NM_017008. Gene expression was determined from the cycle threshold (Ct) associated with exponential growth of the PCR products. Measurements were performed in triplicate and quantitative values for *THOP1* mRNA expression were obtained by the $2^{-\Delta\Delta Ct}$ method, in which ΔCt represents subtraction of the *β-actin* or *GAPDH* Ct values from that of *THOP1*.

2.6. Immunofluorescence staining

The rats were anesthetized with a combination of ketamine (120 mg/kg) and xylazine (20 mg/kg), then subjected to transcardiac perfusion with 0.1 M phosphate buffer solution (PBS), pH 7.4 (15 ml/rat, infusion rate 15 ml/min) followed by a solution of 4% paraformaldehyde, pH 7.4 (150 ml/rat, infusion rate 15 ml/min). After perfusion, the brain was carefully removed from the skull, post-fixed in 4% paraformaldehyde for 48 h, and cryoprotected by immersion in 30% sucrose for 48 h. Coronal slices (40 μm) were obtained using a microtome and stored in PBS. We performed a double-labeling procedure to colocalize THOP1 (specific anti-THOP1, able to distinguish from neurolysin [13, 26], Proteimax Company, Brazil) with NeuN, a neuronal nuclei marker (1:100, anti-NeuN, Chemicon International, USA). THOP1-positive cells stained green (secondary antibody goat-anti-rabbit Alexa Fluor 488, Thermo-Scientific, USA), cells positive for NeuN were red (secondary antibody Alexa fluor 594, Thermo-Scientific, USA), nuclei were stained with DAPI (blue – Thermo-Scientific, USA), and co-localizations were indicated with white arrows. The characterization and distribution of THOP1 in the CNS using a specific antibody was reported by previous studies [13, 26]. Samples were imaged using a Leica Confocal TCS SP5 microscope (Leica Microsystems, Solms, Germany).

2.7. Statistical analysis

Data were analyzed using one-way ANOVA followed by post-hoc Sidak correction, with $P < 0.05$ being considered statistically significant. Data are expressed as means ± standard error of mean (SEM). Statistical analyses were performed using Graphpad Prisma software (version 7.0).

3. Results

3.1. Specificity of enzyme activity detection

Specific enzymatic activity of THOP1 was determined across multiple

regions of the rat brain (Fig. 1). In all tissues, hydrolysis rates after the addition of inhibitors (JA-2 and Pro-Ile) were close to zero, equivalent to the background rate of peptide alone (data not shown). THOP1 activity in the striatum was at least seven-fold higher than in other measured regions of the brain (Fig. 1).

3.2. THOP1 activity and protein expression is decreased in striatum following sleep deprivation and recovery periods

Similar profiles in the striatum were observed for relative mRNA amounts, immunoexpression, and protein specific activities (Fig. 2). *THOP1* expression levels were not statistically different between groups (F (4,35) = 2.503; *P* = 0.06; Fig. 2A). A significant decrease in THOP1 activity was detected for SR48 when compared to CTRL (F (4,34) = 3.378; *P* = 0.02). In addition, a similar result was obtained for THOP1-positive neurons in the caudate-putamen (Fig. 2C), while no difference of immunoreactivity could be demonstrated for neurons in the nucleus accumbens (data not shown).

3.3. THOP1 activity changes in hypothalamus, brainstem, and total cortex

In the hypothalamus, no significant changes in mRNA expression were observed (F (4,35) = 2.112; *P* = 0.102; data not show). Nonetheless, enzyme activity after PSD was distinctly reduced relative to CTRL and that difference was maintained until SR48 (F (4,34) = 13.95; *P* < 0.0001). Enzymatic activity only returned to CTRL values at SR96 (Fig. 3).

In the brainstem, a statistically significant increase in THOP1 activity (Fig. 3) was observed at SR48 when compared to CTRL (F (4,34) = 6.856; *P* = 0.004). In the cerebral cortex, a significant reduction in THOP1 specific activity was observed at SR48 relative to CTRL, PSD, and SR24 alike (F (4,35) = 18.08; *P* < 0.0001). No statistically significant differences were demonstrated for real-time PCR results from any experimental condition in the hypothalamus, total cortex, or brainstem.

3.4. THOP1 activity increases in hippocampus during the sleep recovery period

No significant changes in mRNA expression (Fig. 4A) were observed in the hippocampus. However, a statistically significant increase in THOP1 activity was attained at SR48 and SR96 (F (4,34) = 8.179; *P* < 0.001) in relation to the control group (Fig. 4B). The increase in the dentate gyrus is noteworthy in that it shows an increase of immunoreactivity of the positive neurons for SR48 and SR96 when compared to control group (Fig. 4C). Inherently, THOP1 immunoexpression in CA3 compared to qualitative visual scoring of FITC was not different from among the groups (data not shown).

4. Discussion

The demonstration of specific activity for a given enzyme is not a simple task, especially for proteases in biological samples; ideally, a combination of specific substrates and inhibitors is used to strengthen the evidence. The activity of THOP1 is frequently difficult to distinguish from that of neurolysin (EC 3.4.24.16), as both enzymes are for the most part able to hydrolyze the same neuropeptides [14]. In this study, we followed THOP1 activity in total tissue homogenate from the striatum, hypothalamus, cerebral cortex, hippocampus, and brainstem through using the combination of a widely accepted specific inhibitor for THOP1, JA-2, that is about 20-fold more specific for THOP1 [24] than neurolysin, and a 20-fold more specific substrate, Abz-GFDPFRQ-EDDnp [23]. In addition, this substrate also has a Km value 10^3 higher (~17 μM) than the Ki of JA-2 (22 nM); consequently, competition between substrate and inhibitor is practically eliminated. In all studied tissues, initial concentrations of 2 μM JA-2 and 10 μM Abz-GFDPFRQ-EDDnp reduced enzyme activity to nearly zero. Additionally, we have used 0.5 mM Pro-Ile, a specific neurolysin inhibitor [25], and observed no changes in the activity over the fluorogenic substrate (data not shown), assuring that under these conditions we were only measuring THOP1 activity. Therefore, using the combination of a sensible substrate and specific inhibitors, we were able to quantify THOP1 activity in the cerebral cortex, brainstem, hippocampus, striatum, and hypothalamus.

As shown in Fig. 1, the minimum and maximal activities of THOP1 in all studied tissues of control animals spanned a seven-fold range. These ratios are approximately the same as those observed in prior studies carried out in the early 1990s, which compared endopeptidase baseline activities in different brain areas of control rodents [27, 28]. These studies measured THOP1 relative activities using different fluorogenic substrates. In all studies, THOP1 activity was highest in the striatum (nucleus accumbens and caudate-putamen), followed by the hippocampus, cortex, and hypothalamus in decreasing order in the presence of inhibitors (CFP-Ala-Ala-Phe-pAB) and phosphoramidon [27, 28]. The immunolocalization using the same antibody as described in our study demonstrated a high intensity of THOP1 labeling in the rostral portion of the CNS, such as cortical areas (cortex and cerebellum), hypothalamus, amygdala, and thalamus. THOP1 expression was ubiquitously distributed in the midbrain. However, it appears to be more intense in some areas, such as periaqueductal gray, ventral tegmental area, colliculus, geniculate complex, dorsal raphe, pontine nuclei, and motor nucleus of vagus [13, 26]. Most of these areas contain peptides substrates of the enzyme *in vitro* [12], furthermore, changes to the distribution of THOP1 across brain regions may result in locally increased degradation of neuropeptides.

It has been a matter of discussion whether or not THOP1 processes neuropeptides, since the immunostaining was predominantly nuclear in
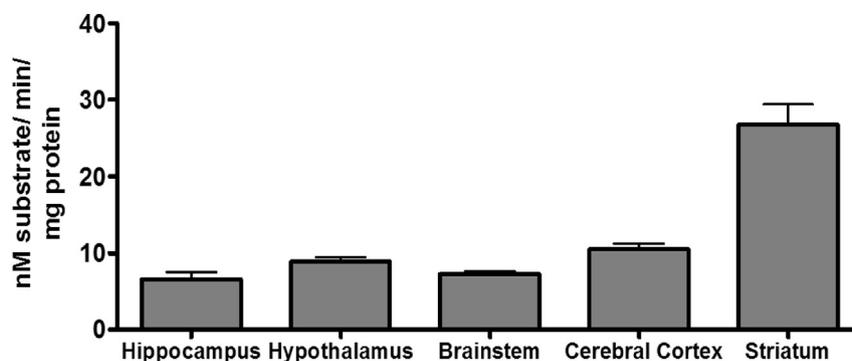

Fig. 1. THOP1 specific activities measured in different CNS tissues of control animals using a fluorogenic substrate. The striatum shows the highest THOP1 activity. Results are expressed as mean ± SEM (n = 10/group). Incubations were carried out in triplicate in 50 mM Tris-HCl, pH7.4, 100mM NaCl, 0.1% Triton X-100 buffer at 37 °C. JA-2 (2μM) and Pro-Ile were used for specific inhibition of THOP1.
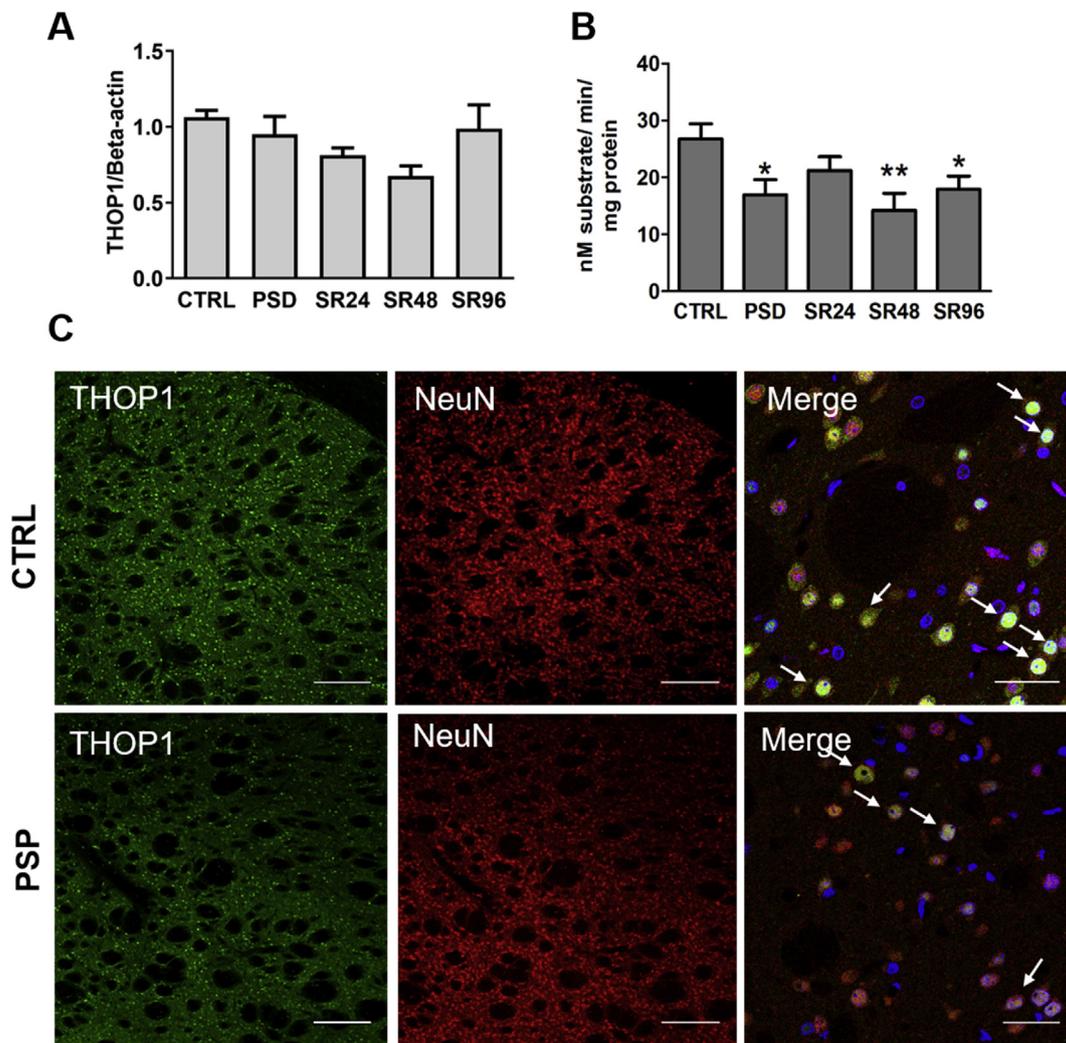
Fig. 2. *THOP1* expression in the striatum is decreased following sleep deprivation (PSD) and sleep recovery. (A) Relative *THOP1* mRNA amounts determined by real-time PCR and (B) specific activity. Results are expressed as mean ± SEM. Images are representative of 4 rats in each group. (A) No differences in gene expression were observed. (B) *P < 0.01 relative to CTRL. (C) Immunofluorescence labeling from caudate-putamen of control and PSD groups were obtained using a 10x and 63 x objectives. Cells that label for THOP and NeuN antibodies, as well as colocalization in nuclei neurons are indicated by white arrows. Of note, the less intensity observed in the striatum of PSP group, when compared with control tissue. Representative immunofluorescence images of THOP1, NeuN, and cell nuclei (DAPI) in striatum (63x objective) are presented in Supplementary Data Fig. 1. Scale bars: 200 μm and 50 μm.

the brain areas [13, 26, 29]. Nevertheless, the major activity (>80%) is detected in soluble fractions of tissue or cell extracts, while the remainder is distributed between the nuclear form and membrane bound [16, 30, 31, 32]. Previous studies have shown that the amount of THOP1 in the nucleus is inversely correlated to the observed in the cytosol and the protease might be mobilized from one intracellular compartment to the other [13, 33]. Furthermore, THOP1 can be associated with lipid rafts in the plasma membrane of neurons, suggesting its participating in the bradykinin metabolism and maybe other biologically active peptides [34, 35, 36]. In addition, the interaction between THOP1 and calmodulin enhances the protease secretion to the extracellular environment in mouse brain [37]. To emphasize further the importance of THOP1 activity as a neuropeptide-processing enzyme, and parallel with our findings, classical studies confirmed that the administration, *in vivo*, of THOP1 inhibitors plays a significant role in the metabolism of peptides and the corresponding physiological effects [38, 39, 40, 41, 42, 43, 44].

The most striking changes in THOP1 activity and immunoexpression were observed in the striatum (Fig. 2). During sleep deprivation, gene expression, specific activities, and the THOP1-positive status of neurons

all differ in a time-dependent manner and seem to parallel one another. An extensive body of evidence indicates that in the mammalian brain, THOP1 and neurolysin are the main neurotensin-inactivating enzymes [31, 45, 46]. In the striatum, neurotensin has been reported to induce inhibition of dopamine receptor 2, which increases dopamine signaling [47]. Administration of haloperidol and other dopaminergic agents has been shown to alter THOP1 activity in the caudate-putamen and nucleus accumbens [48, 49]. Intraperitoneal administration of haloperidol decreased THOP1 activity (17%) in membranes isolated from the caudate-putamen, and this result correlated with the fragments generated from neurotensin (NT1-8 and NT9-13) [49]. Our results suggest a potential role for reduction of THOP1 activity within the striatum in this increase of dopaminergic activity, consistent with findings from previous studies using PSD models [50, 51].

Changes in THOP1 activity in the hypothalamus during and after PSD are also relevant, as they may affect GnRH levels. A significant reduction of THOP1 was evident after the sleep deprivation period, and it returned to initial values only after 96 h of normal sleep recovery (Fig. 3). Wu et al. summarized compelling evidence indicating that THOP1 is well-suited as
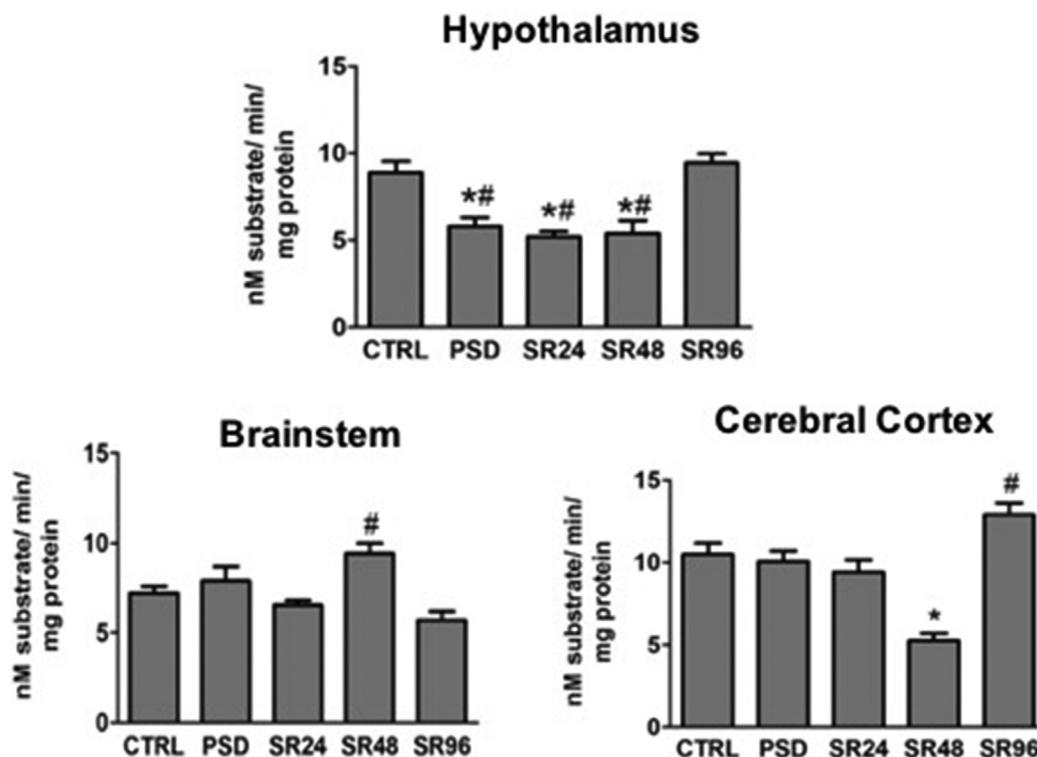
Fig. 3. THOP1 specific activities in hypothalamus, brainstem, and cerebral cortex. Results are expressed as mean ± SEM. Hypothalamus: *different from CTRL (P < 0.001); #different from SR96 (P < 0.0001). Brainstem: #different from SR24 and SR96 (P = 0.001). Cerebral cortex: *different from CTRL, PSD, SR24, and SR96 (P = 0.001); #different from SR24 (P = 0.006).

a physiological regulator of GnRH activity. Previous reports also demonstrate that progesterone and estradiol regulate THOP1 expression [39, 52]. It is noteworthy that for estrone and to a lesser extent testosterone serum concentrations, quite similar THOP1 hypothalamus activity profiles are observed in male rats submitted to the same PSD protocol [53]. Interestingly, serum progesterone levels show an inverse profile in the same model, with a five-fold increase during PSD that returns to control levels after 48 h of sleep recovery. It is tempting to consider that changes in progesterone, estrone, and testosterone levels may correlate with changes in THOP1 activity and GnRH processing in the hypothalamus.

Paradoxical sleep deprivation for 96 h decreased nociceptive thresholds, suggesting that PSD promotes hyperalgesia and reverts the antinociceptive effect of enkephalinase inhibition [54]. After periods of 72 h or 96 h of PSD, the increased nociception takes more than 24 h to return to control values, while an antinociceptive effect can be detected after 48 h of sleep recovery [55, 56]. Between SR24 and SR48, a continuous reduction in THOP1 activity becomes significant in the total cortex (Fig. 3C). Although displaced by 24 h, these results parallel the changes in nociception observed by Onen and collaborators. A decrease in THOP1 would account for reduced Met/Leu-enkephalin production with consequent increase in nociception. These results help us to understand the inability of enkephalinase inhibition, with its antinociceptive effects, to revert the effects of PSD [55, 56]. The reverse can be expected at SR96, when increases of THOP1 activity are observed in both brainstem and hippocampus.

The reduction in THOP1 enzymatic activity during PSD was not accompanied by a significant reduction of mRNA in the hypothalamus, hippocampus, total cortex, or brainstem. It is not uncommon to have no correspondence exist between RNA and protein abundance [57], and many different mechanisms could explain these results. For protein and mRNA, expression levels only fell after 24–48 h following PSD, during

the recovery period, and returned to control values at SR96. Beyond the obvious speculation of different biosynthesis rates for the protein, the different THOP1 activity rates observed for different tissue-specific roles in this study can also indicate that post-translational modifications like glutathionylation [58], and cell redox status [59], and/or phosphorylation [60], which are known to affect enzyme activity.

Significant increases in THOP1 activity and representative immunoexpression were also observed in the hippocampus dentate gyrus at SR48 and SR96 (Fig. 4), but not in the CA3 area (data not shown). It is important to note that sleep deprivation impacts memory consolidation and cognitive performance in hippocampus-dependent memory [61]. In addition, sleep deprivation affects differently genes responsible for increasing neural activity and learning in the hippocampal and cortical areas [62]. Previous studies have emphasized the importance of angiotensin peptides to promote enhance hippocampal of long-term potentiation [63, 64] and object recognition [65]. Furthermore, THOP1 in the hippocampus is mainly responsible for generating 50% of the angiotensin 1-7 formation from angiotensin I [66]. Qualitative changes in THOP1 expression in the dentate gyrus but not in the CA3 area (data not shown) may relate to the process of memory formation and cognition after sleep deprivation.

In this study, we have described changes in THOP1 activity after PSD and during subsequent sleep recovery. These changes could be associated to loss of memory and cognition, endocrine changes related to GnRH inactivation, and modification of pain sensitive linked to changes in the generation of opioid peptides. From a broader perspective, more detailed future experiments are needed in order to quantify neuropeptide levels and the consequences of THOP1 inhibition in PSD models. Nonetheless, to the best of our knowledge this is the first study showing changes in THOP1 enzymatic activity after sleep deprivation and sleep recovery. Together, these results corroborate previous suggestions that peptidases are important players in mediating the effects of sleep-related conditions.
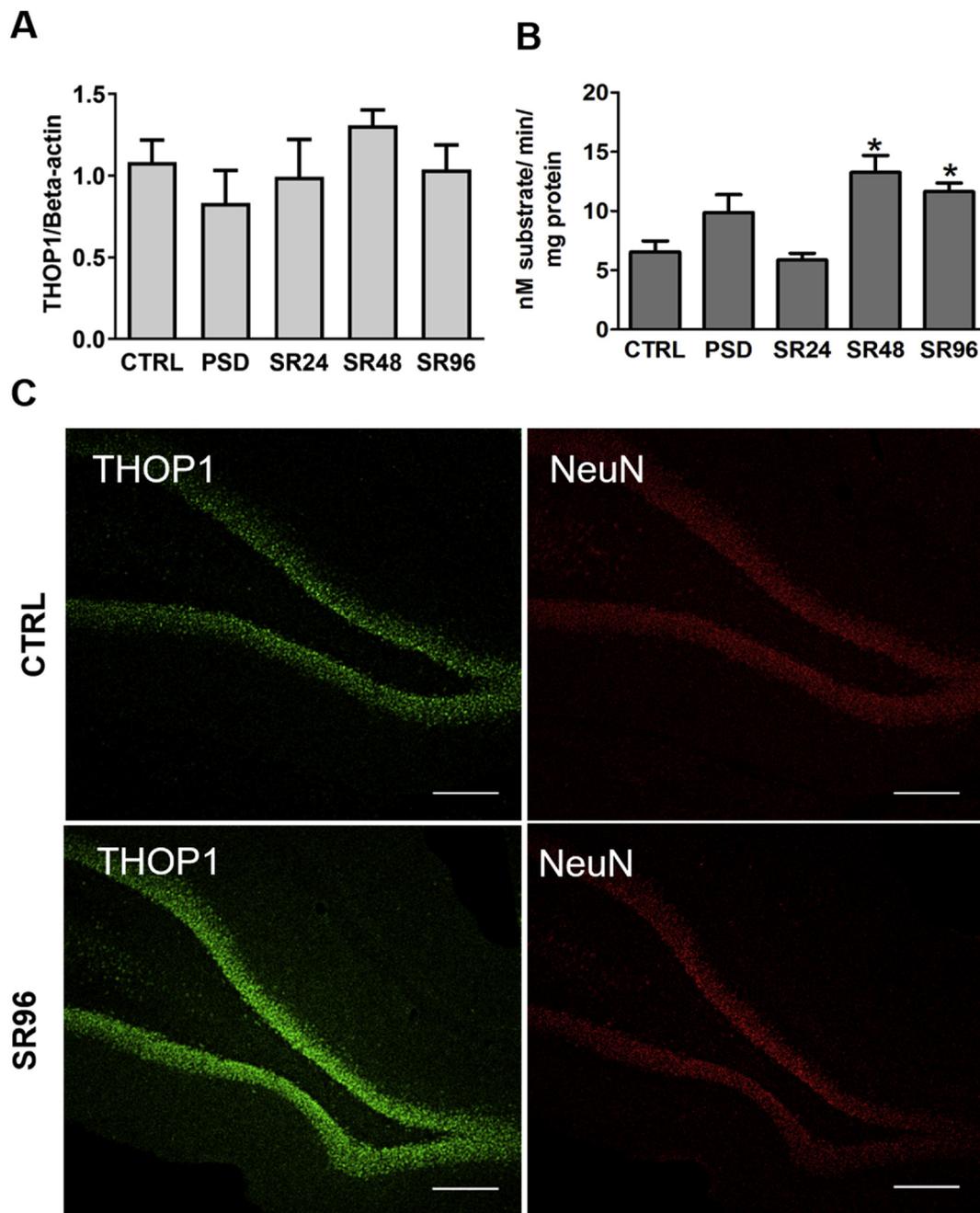
Fig. 4. THOP1 expression in hippocampus. (A) gene expression and (B) specific proteolytic activity. Results are expressed as mean ± SEM. Images are representative of 4 rats in each group. (A) No differences in gene expression were observed. (B) *Different from CTRL and SR24 (P = 0.01). (C) Representative microphotographs from the hippocampal dentate gyrus in control, PSD, and sleep recovery for 96 h (SR96) groups were obtained using a 10x objective. Of note, the strong labeling of THOP1-positive cells in dentate gyrus for the SR96 group. Scale bar: 100 μm.

Declarations

Author contribution statement

Bruna Visniauskas, Jair R. Chagas: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Priscila S.R. Simões, Fernanda M. Dalio: Performed the experiments.

Maria D.G. Naffah-Mazzacoratti, Vitor Oliveira, Sergio Tufik: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Competing interest statement

The authors declare no conflict of interest.

Additional information

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