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Perspective

Upregulation of neurotrophins by S 47445, a novel positive allosteric modulator of AMPA receptors in aged rats

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ABSTRACT

At molecular levels, it has been shown that aging is associated with alterations in neuroplastic mechanisms. In this study, it was examined if the altered expression of neurotrophins observed in aged rats could be corrected by a chronic treatment with S 47445 (1–3–10 mg/kg, p.o.), a novel selective positive allosteric modulator of the AMPA receptors. Both the mRNA and the protein levels of the neurotrophins Bdnf, NT-3 and Ngf were specifically measured in the prefrontal cortex and hippocampus (ventral and dorsal) of aged rats. It was found that 2-week-treatment with S 47445 corrected the age-related deficits of these neurotrophins and/or positively modulated their expression in comparison to vehicle aged rats in the range of procognitive and antidepressant active doses in rodents.

Collectively, the ability of S 47445 to modulate various neurotrophins demonstrated its neurotrophic properties in two major brain structures involved in cognition and mood regulation suggesting its therapeutic potential for improving several diseases such as Alzheimer's disease and/or Major Depressive Disorders.

1. Introduction

Although aging is often accompanied by a decline of memory and executive functions, the neurobiological mechanisms that contribute to age-related cognitive decline remain largely unknown.

Several evidence showed that aging is associated with a reduction of the number as well as the plasticity of dendritic spines in the prefrontal cortex [1] and with alterations in neuroplastic mechanisms [2,3]. Indeed, while exposure to chronic stress reduces dendritic complexity at all ages in rat models, young adult (3 month-old) animals are capable of reversing these structural changes with a 3-week recovery period. Conversely, 12 month-old and 20 month-old rats fail to reverse these changes within the same stress-free recovery time [2]. Thus, since aging represents a period of vulnerability to environmental stressors, test novel therapeutics directed at increasing the capacity for adaptive neuronal responses during this period may provide interesting information on their potential to promote neuroprotection against insults.

The neurotrophins family, that includes Nerve Growth Factor (NGF), Brain-Derived Neurotrophic Factor (BDNF), neurotrophin 3 (NT3), and neurotrophin 4 (NT4), plays a key role in the development, maintenance, repair, and survival of specific neuronal populations and several lines of evidence indicate that decreased functioning of neurotrophins and their receptors can lead to neuronal injury and contribute to the pathogenesis of neurodegenerative as well as psychiatric diseases [4–6]. BDNF is the most widely distributed neurotrophin in the adult brain and, in humans, BDNF levels in plasma decrease with increasing age [7] and in patients affected by Alzheimer's disease [8–10]. Moreover, it has been hypothesized that neurotrophins and/or compounds increasing the activation of neurotrophin receptors have great potential for management of neurodegenerative as well as psychiatric diseases [11]. For example, it has been shown that in aged primates and in mouse models of Alzheimer's disease, BDNF delivery in the entorhinal cortex improves cognitive performance [12]. Furthermore, BDNF has emerged as a major regulator of synaptic transmission and plasticity at adult synapses in many regions of the CNS [13]. Since BDNF is released from neurons in an activity-dependent manner and depends on NMDAR and AMPAR activation [14,15], an interesting strategy to increase BDNF signaling in the adult brain is to modulate the AMPA receptors.

Indeed, several papers describe a regulation of BDNF by AMPA modulators in vitro [16,17], ex vivo [18] as well as in vivo [16,19,20]. Interestingly, BDNF, in turn, is implicated in the insertion and stabilization of AMPA receptors in the membrane [21,22].

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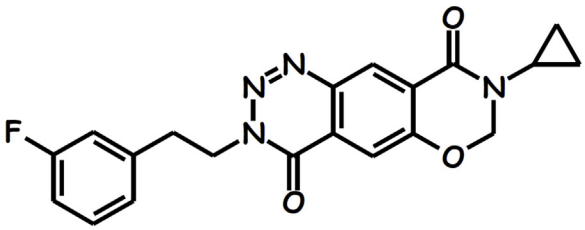

Fig. 1. S 47445 chemical structure.

Moreover, positive allosteric modulators of AMPA (AMPA-PAM) have proven to be effective in *in vitro* models of MPP+-induced toxicity as well as in *in vivo* models of Parkinson's disease via an increase of BDNF [23–26]. Recent data have also shown that treatment of animal models of Huntington's disease with an AMPA-PAM brought to an almost complete reversion of motor symptoms, striatal loss, BDNF levels, cognitive and LTP deficits and a reduction of huntingtin deposits [27–29].

On these bases, the aim of the present study was to establish if the altered expression of neurotrophic factors observed in aged rats could be corrected by a chronic treatment with S 47445 (8-cyclopropyl-3-[2-(3-fluorophenyl)ethyl]-7,8-dihydro-3H-[1,3]oxazino[6,5-*g*][1,2,3]benzotriazine-4,9-dione), a novel selective positive allosteric modulator of the AMPA receptors (AMPA-PAM) [30]. We investigated the effect of the chronic treatment with S 47445 on both the gene expression and the protein levels of the neurotrophins Bdnf, NT-3 and Ngf in the prefrontal cortex and hippocampus (ventral and dorsal) of aged rats.

2. Material and methods

2.1. Animals

Adult (3-month old; n = 10) and old (18-month old; n = 40) Wistar Han male rats (Harlan Laboratories, Horst, Holland) were used throughout the experiment. Rats were singly housed under 12 h/12 h light/dark cycle, constant temperature (22 ± 2 °C) and humidity (50 ± 5%) condition with food and water available ad libitum. Even if we are aware that the single housing may represent a stress for rats and may alter BDNF expression [31], the size and the attitude of aged rats make very difficult and uncomfortable the group-housing.

All animal handling and experimental procedures were performed in accordance with local ethical committees and the European Communities Council Directive (2010/63/UE), the Italian legislation on animal experimentation (Decreto Legislativo 116/92), and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Pharmacological treatment

Animals (10 for each group) were treated for 14 days with vehicle (1% Tween 80, 1% hydroxyethylcellulose in purified water) or S 47445 at 3 different doses (1, 3, 10 mg/kg). S 47445 (micronized form) was synthetized by Servier (France) (Fig. 1).

For this purpose, animals were divided into 5 experimental groups:

- 3 months/Vehicle (n = 10): adult rats (3 months) treated with vehicle;
- 18 months/Vehicle (n = 10): old rats (18 months) treated with vehicle;
- 18 months/S 47445 1 mg/kg (n = 10): old rats (18 months) treated daily with 1 mg/kg of S 47445;
- 18 months/S 47445 3 mg/kg (n = 10): old rats (18 months) treated daily with 3 mg/kg of S 47445;
- 18 months/S 47445 10 mg/kg (n = 10): old rats (18 months) treated daily with 10 mg/kg of S 47445;

The drug and the vehicle were administered via oral gavage (per os) and all the animals were sacrificed 24 h after the last administration.

After sacrifice, the brain regions (prefrontal cortex and hippocampus) were immediately dissected, frozen on dry ice, and stored at −80 °C. The dorsal hippocampus corresponds to the Plates 25–33 according to the atlas of Paxinos and Watson (Paxinos and Watson, 1996), whereas the ventral hippocampus corresponds to the Plates 34–43. The prefrontal cortex (defined as Cg1, Cg3 and IL subregions corresponding to Plates 6–9 according to the atlas Paxinos & Watson) was dissected from 2-mm-thick slices, whereas the hippocampus (including both ventral and dorsal part) was dissected from the whole brain.

2.3. RNA preparation for real time RT-PCR analysis of gene expression

Total RNA was isolated using single step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories S.r.l; Segrate, Italy) according to the manufacturer's instructions and quantified using spectrophotometric analysis. After total RNA extraction, the samples were processed for retrotranscriptase real-time polymerase chain reaction (PCR) to assess Bdnf, NT-3 and Ngf mRNA levels.

Briefly, an aliquot of each sample was treated with DNase to avoid DNA contamination. RNA was analyzed using the TaqMan qRT-PCR instrument (CFX384 real time system, Bio-Rad Laboratories S.r.l) using the iScript 1-step reverse transcription PCR kit for probes (Bio-Rad Laboratories S.r.l). Samples were run in 384-well formats in triplicate as multiplexed reactions with a normalizing internal control (36B4).

Thermal cycling was initiated with an incubation at 50 °C for 10 min (RNA retrotranscription) and then at 95 °C for 5 min (TaqMan polymerase activation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95 °C for 10 s to enable the melting process and then for 30 s at 60 °C for the annealing and extension reactions. A comparative cycle threshold (Ct) method was used to calculate the relative target gene expression.

Probe and primer sequences were purchased from Eurofins MWG-Operon (Table 1).

Table 1
Sequences of Forward and Reverse Primers and Probe used in Real-time PCR Analyses.

Gene	*Forward primer*	*Reverse primer*	*Probe*
Total BDNF	AAGTCTGCATTACATTCCTCGA	GTTTTCTGAAAGAGGGACAGTTTAT	TGTGGTTTGTTGCCGTTGCCAAG
NT3	GCAAAACGTCGCAAACCTAC	GGGGACAGATGCCAATTCA	AGCGCCAGCCTACGAGTTTGTT
NGF	AAGGACGCAGCTTTCTATCC	CTATCTGTGTACGGTTCTGCC	CTCTGAGGTGCATAGCGTAATGTCCA
36B4	TCAGTGCCTCACTCCATCAT	AGGAAGGCCTTGACCTTTTC	TGGATACAAAAGGGTCCTGG

Table 2
Antibody conditions used in Western Blotting Analyses.

Protein	Primary antibody	Secondary antibody
proBDNF (32 kDa)	1:2000 (Genetex) 4 °C, O/N	Anti-rabbit, 1:5000, RT, 1 h
mBDNF (14 kDa)	1:500 (Santa Cruz), 4 °C, O/N	Anti-rabbit, 1:1000, RT, 1 h
NT3 (27 kDa)	1:1000 (Abcam), 4 °C, O/N	Anti-rabbit, 1:2000, RT, 1 h
NGF (13 kDa)	1:1000 (Abcam), 4 °C, O/N	Anti-rabbit, 1:500, RT, 1 h
β-ACTIN (43 kDa	1:10000 (Sigma), RT, 1 h	Anti-mouse, 1:10000, RT, 1 h

2.4. Preparation of protein extract and western blot analysis

Western blot analysis was used to investigate the precursor (proBDNF) and the mature form (mBDNF) of BDNF, NT-3 and NGF protein levels in the total homogenate and in the crude synaptosomal fraction. Tissues were manually homogenized using a glass–glass potter in a pH 7.4 cold buffer containing 0.32 M sucrose, 0.1 mM ethylene glycol tetraacetic acid (EGTA), 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) solution in presence of a complete set of protease (Roche) and phosphatase (Sigma-Aldrich) inhibitors. The total homogenate was centrifuged at 1000*g* for 10 min at 4 °C. The supernatant obtained was centrifuged at 10000*g* for 15 min at 4 °C to obtain a pellet corresponding to the crude synaptosomal fraction which was resuspended in a buffer (20 mM HEPES, 0.1 mM dithiothreitol (DTT), 0.1 mM EGTA) supplemented with protease and phosphatase inhibitors. Total protein content was measured using the Bradford protein assay procedure (Bio-Rad Laboratories S.r.l), using bovine serum albumin as the calibration standard.

Equal amounts of protein (10 μg) were run under reducing conditions on the criterion TGX precast gels (Bio-Rad Laboratories S.r.l) and then electrophoretically transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories S.r.l). The blots were blocked with 10% nonfat dry milk and then incubated with the primary antibodies summarized in Table 2. Membranes were then incubated for 1 h at room temperature with the opportune secondary antibody (Table 2), and immunocomplexes were visualized by chemiluminescence using the Western Lightning Plus ECL (PerkinElmer) and the Chemidoc MP imaging system (Bio- Rad Laboratories S.r.l). Results were standardized using β-actin as the internal standard because its expression is not regulated in the experimental paradigm used.

2.5. Statistical analyses

The effects of age and drug treatment were analyzed using a one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD). Significance for all tests was assumed for $p < 0.05$. For graphic clarity, data are presented as means percent ± standard error (SEM) of 3-month-old rats.

3. Results

3.1. Modulation of total bdnf gene expression by chronic oral S 47445 treatment in brain areas of aged rats

As previously showed [3], total Bdnf mRNA levels were significantly reduced in the prefrontal cortex (Fig. 2a) of 18 months aged rats (−23%, $p < 0.05$ vs 3 months/Veh). Interestingly, this effect was not found in aged rats chronically treated with S 47445 at all the tested doses, even if, only for the lowest, the observed increase reaches the statistical significance (+43%, $p < 0.05$ vs 18 months/Veh).

In the ventral hippocampus (Fig. 2b), we found a slight, but not significant, decrease of the mRNA levels of the total form of Bdnf

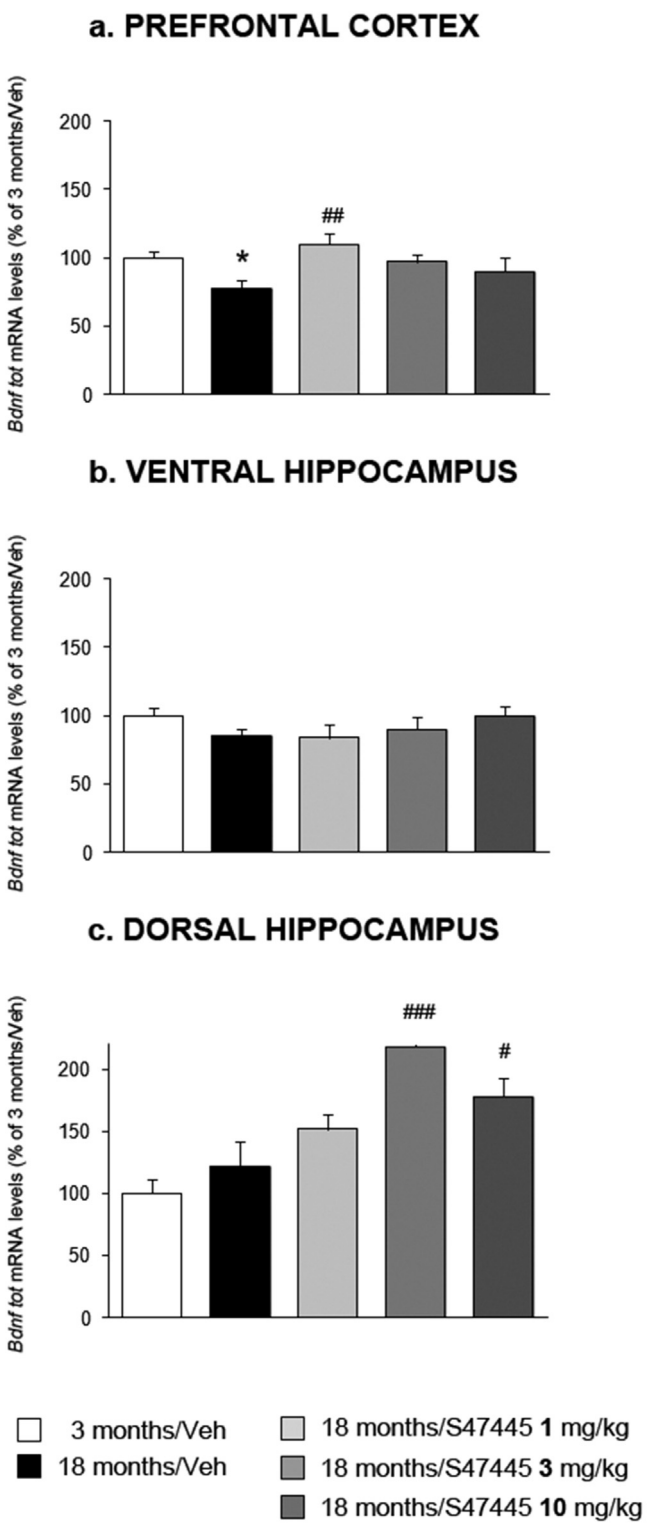

Fig. 2. Analysis of total Bdnf gene expression in 18 month-old rats exposed to chronic treatment with S 47445.
The mRNA levels of Bdnf were measured in the prefrontal cortex (a), ventral hippocampus (b) and dorsal hippocampus (c). The data are expressed as a percentage of 3 months/Vehicle rats (set at 100%). *$p < 0.05$ vs. 3 months/Vehicle; # $p < 0.05$, ## $p < 0.01$, ###$p < 0.001$ vs. 18 months/Vehicle (one-way ANOVA with Fisher PLSD).

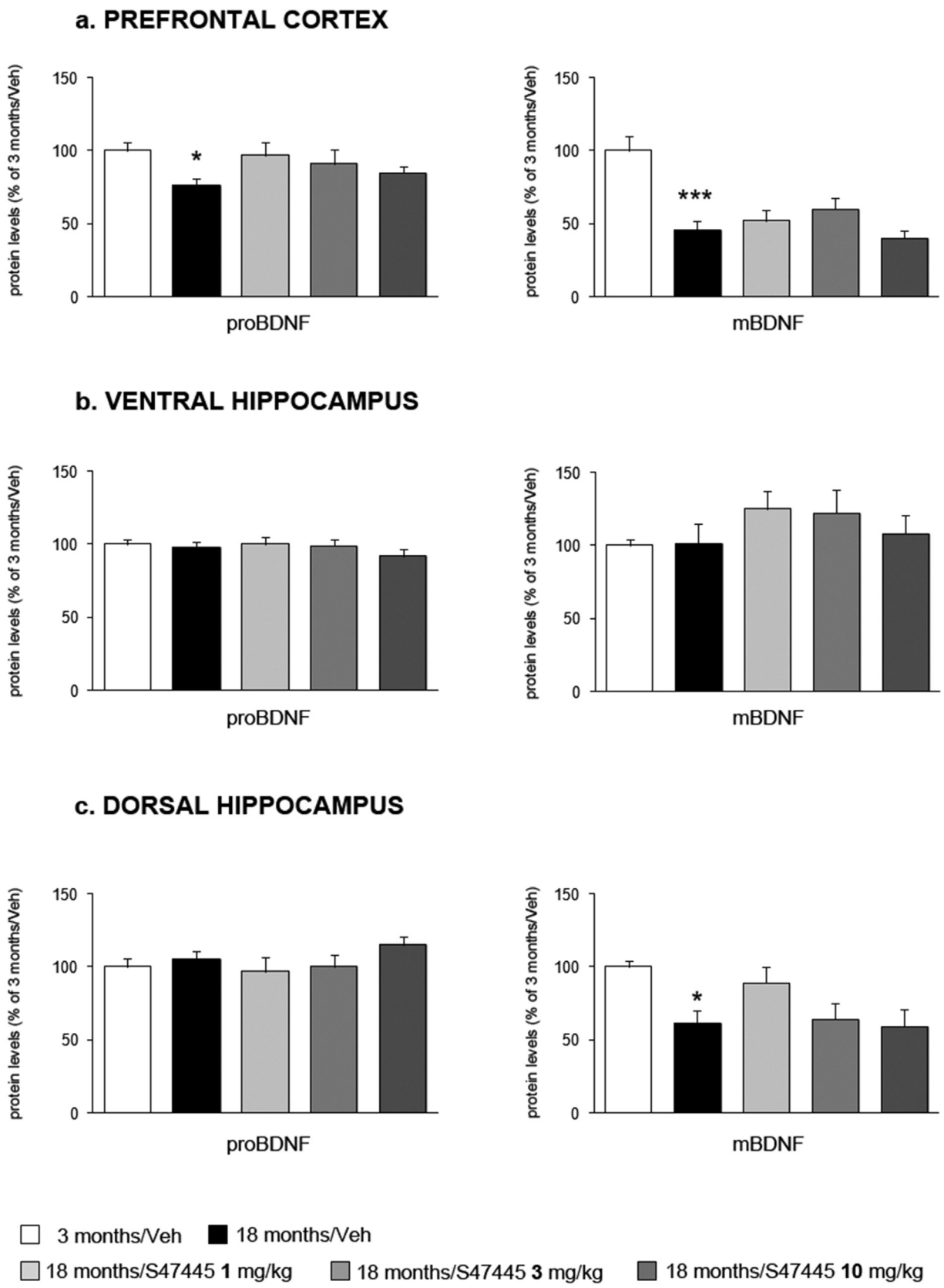

Fig. 3. Analysis of BDNF protein in 18 month-old rats exposed to chronic treatment with S 47445 in the total homogenate.
The protein levels of the precursor (proBDNF) and the mature form (mBDNF) of BDNF were measured in the whole homogenate of the prefrontal cortex (a), of the ventral hippocampus (b) and of the dorsal hippocampus (c). The data are expressed as a percentage of 3 months/Vehicle rats (set at 100%). *$p < 0.05$, ***$p < 0.001$ vs. 3 months/Vehicle (one-way ANOVA with Fisher PLSD).

Table 3
Modulation of Creb and Npas4 gene expression by chronic S47445 treatment in the brain of aged rats (PF: prefrontal cortex, VH: ventral hippocampus, DH: dorsal hippocampus).

	3 months Veh	18 months Veh	18 months S47445_1 mg/kg	18 months S47445_3 mg/kg	18 months S47445_10 mg/kg
PF					
Npas4	100 ± 6	93 ± 12	96 ± 8	92 ± 7	113 ± 16
Creb	100 ± 8	160 ± 12***	98 ± 7###	99 ± 8###	129 ± 14#
VH					
Npas4	100 ± 12	62 ± 8*	79 ± 11	90 ± 14	98 ± 8#
Creb	100 ± 8	100 ± 6	95 ± 11	100 ± 10	100 ± 9
DH					
Npas4	100 ± 6	72 ± 7*	98 ± 13#	102 ± 10#	121 ± 7###
Creb	100 ± 10	122 ± 10	99 ± 5#	96 ± 4#	93 ± 6#

(−15%, $p > 0.05$ vs 3 months/Veh) that was not observed in aged rats treated for 14 days with S 47445 at 10 mg/kg (+17%, $p > 0.05$ vs 18 months/Veh).

Conversely, in the dorsal hippocampus (Fig. 2c), even if total Bdnf gene expression was not affected by aging (+22%, $p > 0.05$ vs 3 months/Veh), chronic S 47445 treatment at 3 and 10 mg/kg doses induced a significant upregulation of Bdnf gene expression (3 mg/kg: +79%, $p < 0.001$ vs 18 months/Veh; 10 mg/kg: +46%, $p < 0.05$ vs 18 months/Veh).

3.2. Modulation of BDNF protein levels by chronic oral S 47445 treatment in brain areas of aged rats

To understand if the alteration in gene expression reflected a similar effect at translational levels, we next investigated the protein levels of BDNF (precursor (proBDNF) and the mature form (mBDNF) in the total homogenate. In the prefrontal cortex (Fig. 3a), in line with the changes observed for the mRNA levels, we found a reduction of BDNF protein due to the aging process. Indeed, both proBDNF and mBDNF protein levels were significantly reduced (−24%, $p < 0.05$ vs 3 months/Veh; −54%, $p < 0.001$ vs 3 months/Veh; respectively) in the prefrontal cortex of aged rats. Interestingly, the down-regulation of the precursor form was not observed when the old rats were chronically treated with S 47445 at all the tested doses, while the chronic administration of the drug did not influence the levels of mBDNF.

No effect was observed in the ventral hippocampus (Fig. 3b), while in the dorsal hippocampus (Fig. 3c), we observed a significant reduction of mBDNF levels in aged rats treated with vehicle (-39%, $p < 0.05$ vs 3 months/Veh), but not in those chronically treated with S 47445 at the lowest dose (1 mg/kg).

Seen that BDNF may be transported at synaptic levels, where it may be stored, forming a pool of protein ready to be released in case of demand, we then analyzed the levels of the precursor and of the mature form of the neurotrophin in the crude synaptosomal fraction.

In the prefrontal cortex, proBDNF levels (Fig. 4a) were reduced in the crude synaptosomal fraction of aged rats (−17%, $p < 0.05$ vs 3 months/Veh), and this deficit was normalized by the chronic treatment with S 47445 at 1 and 10 mg/kg doses (+21%, $p < 0.05$ vs 18 months/Veh; +26%, $p < 0.05$ vs 18 months/Veh). Moreover, similar changes were found for mBDNF protein levels that were significantly downregulated in aged rats (−43%, $p < 0.05$ vs 3 months/Veh). Interestingly, chronic treatment with S 47445, at all the three doses, restored mBDNF levels to those found in adult rats (1 mg/kg: +94%, $p < 0.01$ vs 18 months/Veh; 3 mg/kg: +96%, $p < 0.01$ vs 18 months/Veh; 10 mg/kg: +80%, $p < 0.05$ vs 18 months/Veh).

At a difference from the prefrontal cortex, the levels of BDNF protein (precursor and mature form) were not altered in the ventral and dorsal hippocampus of aged rats (Fig. 4b, c), although S 47445 treatment exerted a dose-dependent effect in the dorsal part (Fig. 4c). Indeed, while at 1 mg/kg, mBDNF was slightly increased without reaching statistical significance, the chronic administration of S 47445 at 3 and at 10 mg/kg significantly increased mBDNF protein levels (respectively +54%, $p < 0.01$ vs 18 months/Veh; +51%, $p < 0.05$ vs 18 months/Veh). Representative Western blot analyses of proBDNF, mBDNF and β-actin were shown in the Supplementary material.

3.3. Modulation of NT-3 and ngf mRNA levels by chronic oral S 47445 treatment in brain areas of aged rats

In order to establish the specificity of these modulatory effects, we next investigated the expression of other neurotrophins, namely NT-3 and NGF, both at transcriptional (Fig. 5) and translational levels (Fig. 6).

Interestingly, while aging did not alter the gene expression of both the neurotrophins, we observed a specific effect of S 47445 treatment. In particular, chronic S 47445 administration at 3 mg/kg produced a significant upregulation of NT-3 mRNA levels (+41%, $p < 0.01$ vs 18 months/Veh) in prefrontal cortex (Fig. 5a), whereas Ngf mRNA levels were significantly increased by the chronic treatment with S 47445 at all the doses investigated (1 mg/kg: +64%, $p < 0.001$ vs 18 months/Veh; 3 mg/kg: +63%, $p < 0.001$ vs 18 months/Veh; 10 mg/kg: +44%, $p < 0.01$ vs 18 months/Veh) in the dorsal hippocampus (Fig. 5f).

3.4. Modulation of NT-3 and NGF protein levels by chronic oral S 47445 treatment in the total homogenate in brain areas of aged rats

Unlike what observed at transcriptional levels, NT-3 protein levels were significantly reduced in the prefrontal cortex of 18 months old rats (−41% $p < 0.05$ vs 3 months/Veh) (Fig. 6a), defects that was normalized by chronic S 47445 administration at all the doses tested (1 mg/kg: +83%, $p < 0.01$ vs 18 months/Veh; 3 mg/kg: +71%, $p < 0.01$ vs 18 months/Veh; 10 mg/kg: +59%, $p < 0.01$ vs 18 months/Veh).

Moreover, in dorsal hippocampus (Fig. 6c) of 18 months old rats, compared to young rats, we found a decrease, even if not statistically significant, of NT-3 protein levels (-36%, $p = 0.06$ vs 3 months/Veh). This deficit was normalized by chronic administration of S 47445 at the dose of 1 mg/kg (+87% $p < 0.01$ vs 18 months/Veh) while the higher doses were ineffective.

NGF protein levels were specifically affected by aging in the prefrontal cortex (Fig. 6d). Indeed, we observed a significant decrease in aged rats treated with vehicle (−42% $p < 0.01$ vs 3 months/Veh), while this alteration was not detected after the chronic treatment with S 47445 at all the doses tested. Conversely, in the dorsal hippocampus (Fig. 6f), we found a significant increase of the protein levels of NGF in aged rats chronically treated with 1 mg/kg of S 47445 (+70% $p < 0.05$ vs 18 months/Veh).

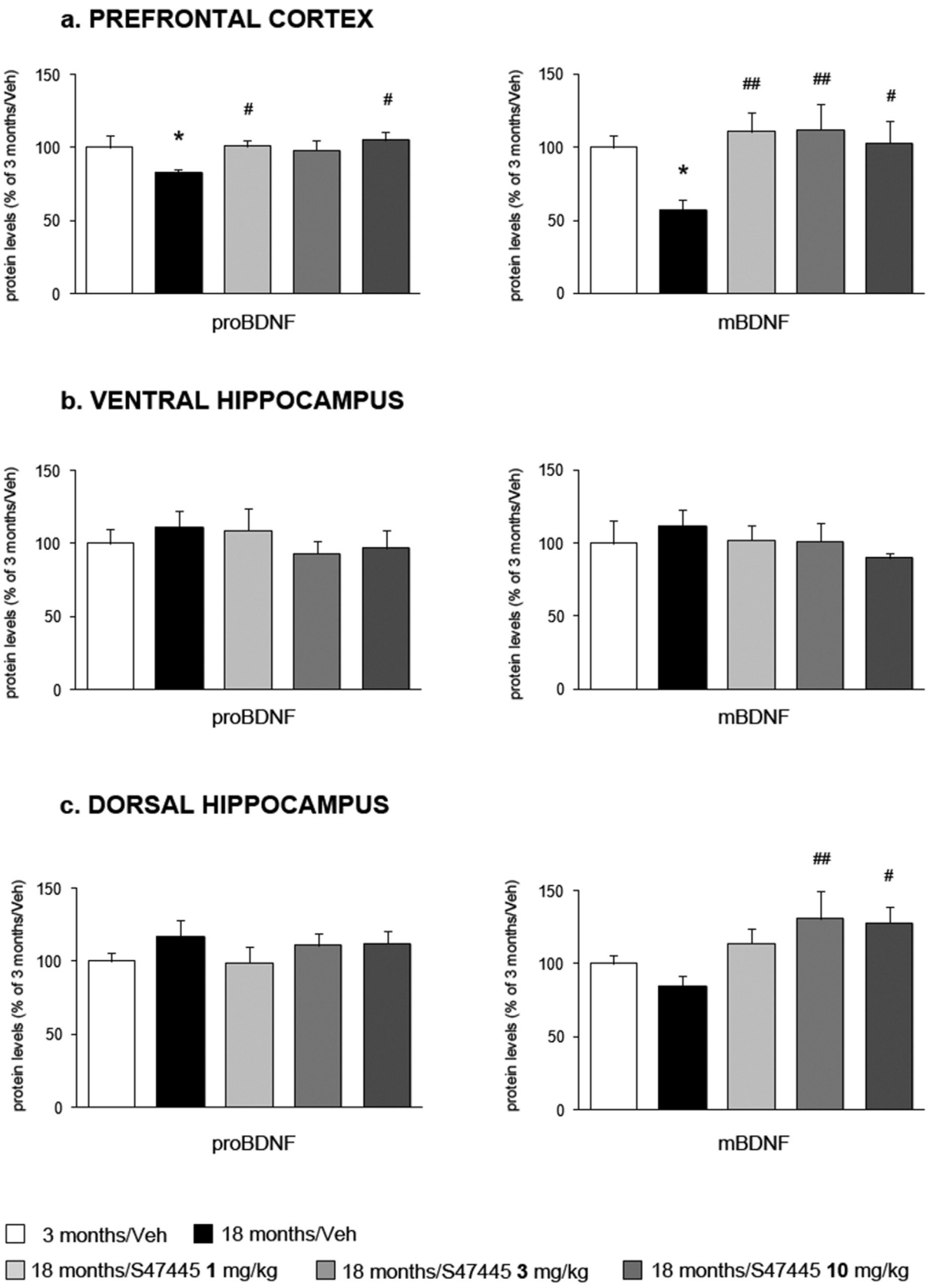

Fig. 4. Analysis of BDNF protein in 18 month-old rats exposed to chronic treatment with S 47445 in the crude synaptosomal fraction.
The protein levels of the precursor (proBDNF) and the mature form (mBDNF) of BDNF were measured in the crude synaptosomal fraction of the prefrontal cortex (a), of the ventral hippocampus (b) and of the dorsal hippocampus (c). The data are expressed as a percentage of 3 months/Vehicle rats (set at 100%). *$p < 0.05$ vs. 3 months/Vehicle; # $p < 0.05$, ## $p < 0.01$ vs. 18 months/Vehicle (one-way ANOVA with Fisher PLSD).

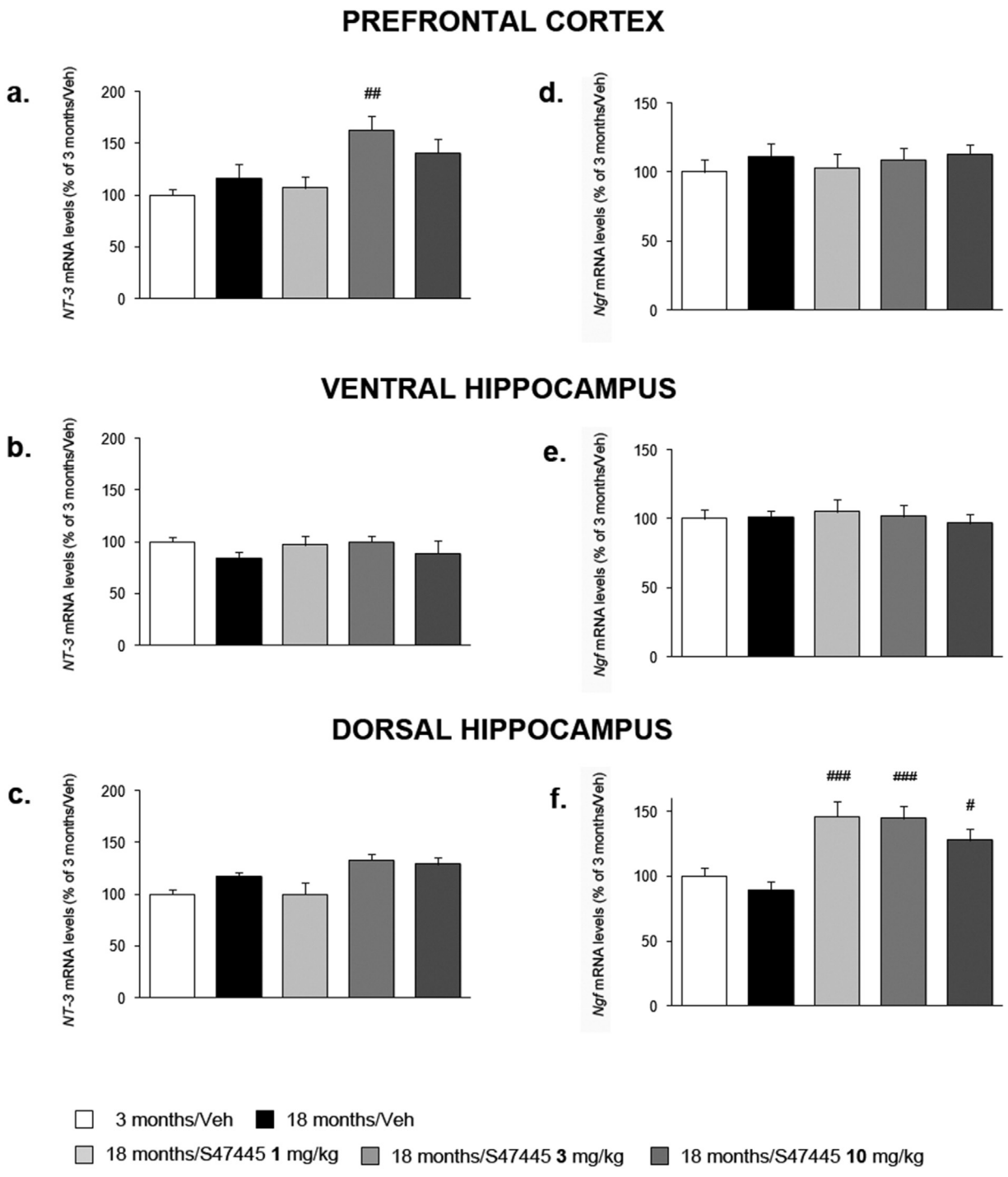

Fig. 5. Analysis of NT-3 and Ngf gene expression in 18 month-old rats exposed to chronic treatment with S 47445.
The mRNA levels of NT-3 (a, b, c) and Ngf (d, e, f) were measured in the prefrontal cortex (a, d), in the ventral hippocampus (b, e) and in the dorsal hippocampus (c, f). The data are expressed as a percentage of 3 months/Vehicle rats (set at 100%). # $p < 0.05$, ## $p < 0.01$, ###$p < 0.001$ vs. 18 months/Vehicle (one-way ANOVA with Fisher PLSD).

3.5. Modulation of creb and npas4 expression by chronic oral S 47445 treatment in the brain of aged rats

Since we previously demonstrated that the alterations observed on Bdnf mRNA levels in aged rats were associated with changes in the expression of specific transcription factors [3], here we investigated how S 47445 could affect the expression of these key factors for BDNF transcription in 18 month-old animals (Table 3).

According to what observed before [3], Npas4 expression was significantly reduced in the hippocampus of aged rats (ventral: −38%, $p < 0.05$ vs 18 months/Veh; dorsal: −28%, $p < 0.05$ vs 18 months/Veh), but not in the prefrontal cortex (−7%, $p > 0.05$ vs 3 months/Veh).

In the dorsal hippocampus, all the doses of S 47445 were able to correct the alterations due to the aging process (1 mg/kg: +36%, $p < 0.05$ vs 18 months/Veh; 3 mg/kg: +41%, $p < 0.05$ vs 18 months/Veh; 10 mg/kg: +68%, $p < 0.05$ vs 18 months/Veh), while in the ventral hippocampus only the 10 mg/kg dose was effective (+58% $p < 0.001$ vs 18 months/Veh).

Regarding Creb, we found a significant increase of its expression in the prefrontal cortex of aged rats (+60% $p < 0.001$ vs 3 months/Veh) that was corrected by all the three doses of S

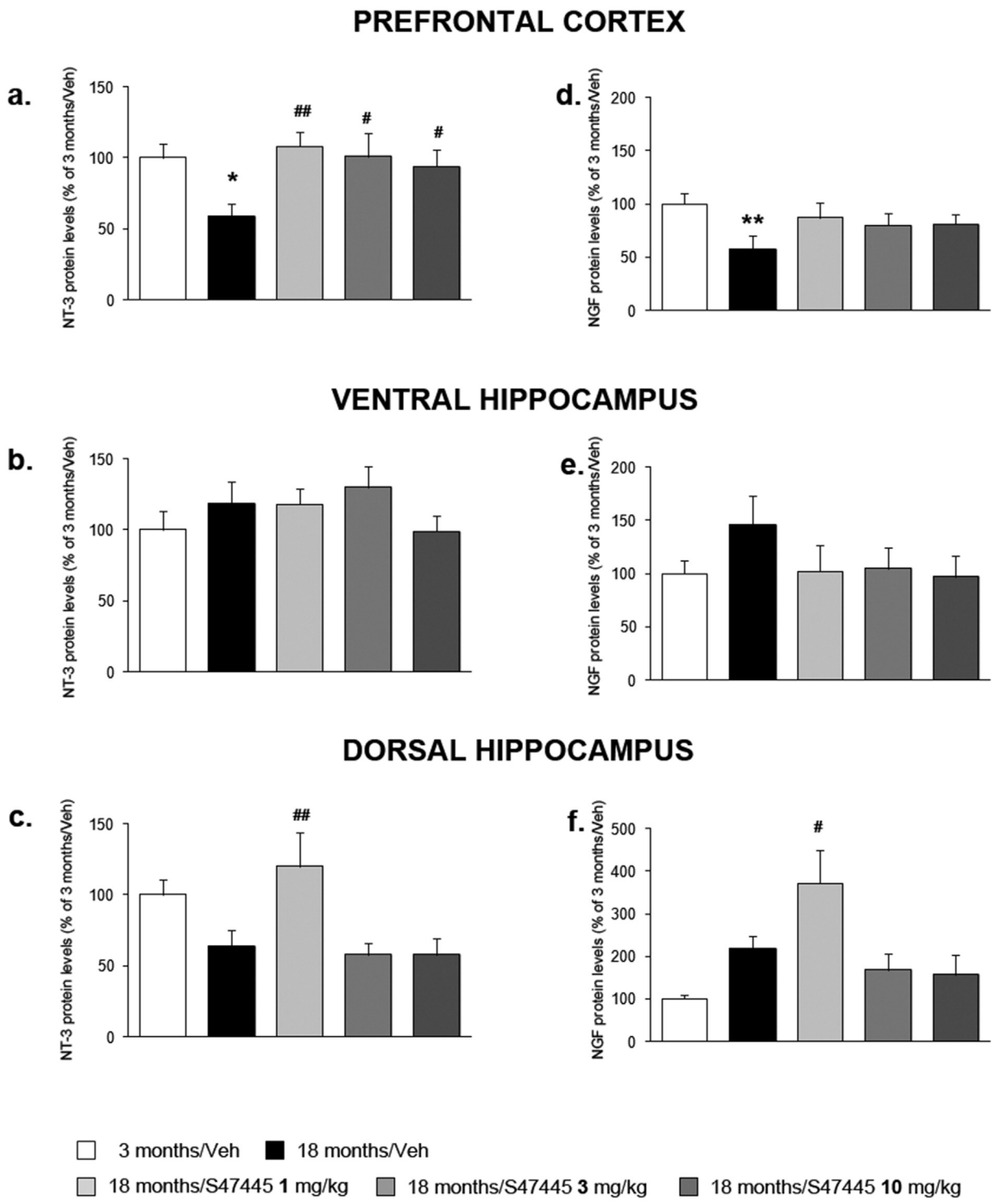

Fig. 6. Analysis of NT-3 and NGF protein in 18 month-old rats exposed to chronic treatment with S 47445 in the total homogenate.
The protein levels of NT-3 (a, b, c) and NGF (d, e, f) were measured in the whole homogenate of the prefrontal cortex (a,d), of the ventral hippocampus (b, e) and of the dorsal hippocampus (c, f). The data are expressed as a percentage of 3 months/Vehicle rats (set at 100%). *$p < 0.05$, **$p < 0.01$ vs. 3 months/Vehicle; # $p < 0.05$, ## $p < 0.01$ vs. 18 months/Vehicle (one-way ANOVA with Fisher PLSD).

47445 tested (1 mg/kg: +83%, $p < 0.001$ vs 18 months/Veh; 3 mg/kg: +71%, $p < 0.001$ vs 18 months/Veh; 10 mg/kg: +59%, $p < 0.01$ vs 18 months/Veh). Similarly, S 47445 normalized the mRNA levels of Creb in the dorsal hippocampus of aged rats (1 mg/kg: −19%, $p < 0.05$ vs 18 months/Veh; 3 mg/kg: −22%, $p < 0.05$ vs 18 months/Veh; 10 mg/kg: −24%, $p < 0.05$ vs 18 months/Veh).

4. Discussion

Our results provide evidence that prolonged treatment with the AMPA-PAM, S 47445, positively modulated neuroplastic mechanisms in aged rats by acting on different kind of neurotrophins in cerebral regions involved both in memory processes and in regulation of mood.

Accordingly, with our previous results [3] we found that 18 month-old rats shown a significant decrease of the Bdnf mRNA as well as protein levels in prefrontal cortex, while, regarding NT-3 and NGF we observed a specific decrease due to aging process only on the protein levels in the same region. Moreover, the mature form of BDNF was also reduced in the dorsal hippocampus.

Since the prefrontal cortex and the dorsal part of the hippocampus play a key role in cognitive functions [32], these alterations confirmed that aging is associated with reduced plasticity that may, in turn, underlie the cognitive decline observed in the elderly subjects [33]. Moreover, it is worth to mention that the reduction of BDNF protein levels is observed in the total homogenate but also in the crude synaptosomal fraction, indicating a specific reduction of the pool of neurotrophin at synaptic level, that may lead to the impairment in the mechanisms underlying BDNF, release and activation of downstream signaling events.

Interestingly, in the prefrontal cortex, chronic treatment with S 47445 completely restored the normal levels of BDNF protein in the synaptosomal fraction, with a profile that reflects the changes observed for total mRNA levels. Since specific BDNF exons can be targeted to dendrites [34], it may be inferred that potentiation of AMPA receptor activity can modulate these transcripts, which may contribute to local neurotrophin synthesis/storage. Moreover, these data provide 'in vivo' support for the increased dendritic mRNA translation obtained with other AMPA receptor potentiators [15] and is in good agreement with the evidence that positive modulation of AMPA receptors, through rapid phosphorylation of mTOR pathway, regulates local dendritic protein translation, presumably via BDNF changes [15].

Moreover since S 47445 administration did not alter mBDNF protein levels in total homogenate, these results do not only imply specificity in the regulatory mechanisms of the neurotrophin, but they are suggestive of a relevant impact of the AMPA modulator S 47445 in regulating mBDNF levels in a subcellular compartment that may hold implications for neuronal plasticity and synaptic function. Considering that the levels of proBDNF, the BDNF precursor, were only slightly (although significantly) affected by aging and drug treatment, it could be suggested that facilitation of AMPA transmission by S 47445 may enhance neurotrophin processing, which is to say the conversion of proBDNF to mBDNF [35]. The pro- and mature domains of BDNF showed that the pro-peptide is colocalized with mature BDNF in secretory granules in the presynaptic axon terminals, suggesting that the cleavage may occur intracellularly by pro-protein convertase 1/3 in secretory granules and by furin in *trans*-Golgi networks [36,37]. Under some experimental conditions, however, the processing of pro-BDNF into mature BDNF was found to occur extracellularly via the actions of metalloproteinases and the extracellular protease plasmin [35]. Indeed high frequency stimulation, which may also occur following treatment with drugs that potentiate AMPA transmission, can promote the release of tPA (tissue plasminogen activator) into the extracellular space, thus shifting the extracellular balance from proBDNF to mature BDNF. It is important to point out that the conversion of proBDNF to mature BDNF by tPA/plasmin is also necessary for hippocampal late-LTP [38].

Here, is described for the first time that an AMPA-PAM can increase NT3 and NGF protein levels in prefrontal cortex since chronic treatment with S 47445 was able to normalize also the alteration of NGF and NT3 induced by the aging processes. Thus, S 47445 by increasing different kind of neurotrophins suggests its ability to deeply modulate neuroplastic mechanisms as long term potentiation, synapse density and dendrite morphogenesis [13].

The present effects of S 47445 in normalizing neuroplasticity reflect positive effects of the drug on cognitive performance as observed in several memory-like tests [60]. Indeed, it has been demonstrated that neurotrophins levels in hippocampus and frontal cortex may relate to working memory proficiency in aged rats [39], and that NGF administration improves learning and memory in aged rats [40,41] possibly reverting the cholinergic atrophy [40,41] or, speaking of BDNF, through an initial effect on GABA presynaptic genes [42]. Accordingly, studies in rodents [43,44], monkeys [45] and human [43] have shown that the cognitive deficits associated with the aging processes is similar to the memory alterations due to a compromising cholinergic system and that growth factors are fundamental for the survival and maintenance of cholinergic neurons [46]. Further, NGF can quickly enhance acetylcholine release [47,48].

Moreover, our results are in line with evidence showing that AMPA-PAM treatment rescue synaptic plasticity and memory deficit in several animal models of pathologies characterized by cognitive deficits [27,49] and reduced neural plasticity [28,50]. Furthermore, these neurotrophic properties of S 47445 support the antidepressant-like activities observed at the same active doses with this drug in several rodent models [59].

Finally, as previously demonstrated [3], we found a significant reduction of Npas4 mRNA levels in the dorsal and ventral hippocampus of aged-rats, effects normalized by the chronic treatment with S 47445.

Npas4 is an immediate-early gene with unique features [51], for example, is expressed only in neurons, it is regulated selectively by neuronal activity, it directly controls the expression of a very large number of genes and it is important for both glutamatergic and GABAergic synapses development [52,53]. Functionally it is involved in neuronal plasticity [3,54], in maintaining circuit homeostasis and it is required for long-term memory formation [53,55].

Interestingly, despite the aging process do not modulate the markers investigated in the hippocampus, we found a specific effect of S 47445 administration in the dorsal but not in the ventral part of the hippocampus, probably due to the higher concentration of AMPA receptors in this brain subregion [56].

The effects we found are consistent with published studies investigating the effects of other AMPA-PAM on BDNF [16,18,28,57,58]. However, while these data are consistent in demonstrating the ability of such compound in increasing the neurotrophin expression, our study is novel as it demonstrated the effectiveness of S 47445 in up-regulating neurotrophin expression in aged rats.

In summary, taking into consideration the wide spectrum of functions controlled and/or that are regulated by the proteins investigated in this study, the ability of S 47445 to modulate them provide promising indications on its potential efficacy in all those pathological conditions characterized by cognitive deficits and altered neuroplasticity such as Alzheimer's disease, Major Depressive Disorder, Parkinson's disease and/or Huntington's disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2017.04.019.

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