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Age-related changes in STriatal-Enriched protein tyrosine Phosphatase levels: Regulation by BDNF

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ABSTRACT

Recent results indicate that STriatal-Enriched protein tyrosine Phosphatase (STEP) levels are regulated by brain-derived neurotrophic factor (BDNF), whose expression changes during postnatal development and aging. Here, we studied STEP ontogeny in mouse brain and changes in STEP with age with emphasis on the possible regulation by BDNF. We found that STEP expression increased during the first weeks of life, reaching adult levels by 2–3 weeks of age in the striatum and cortex, and by postnatal day (P) 7 in the hippocampus. STEP protein levels were unaffected in $BDNF^{+/-}$ mice, but were significantly reduced in the striatum and cortex, but not in the hippocampus, of $BDNF^{-/-}$ mice at P7 and P14. In adult wild-type mice there were no changes in cortical and hippocampal $STEP_{61}$ levels with age. Conversely, striatal STEP levels were reduced from 12 months of age, correlating with higher ubiquitination and increased BDNF content and signaling. Lower STEP levels in older mice were paralleled by increased phosphorylation of its substrates. Since altered STEP levels are involved in cellular malfunctioning events, its reduction in the striatum with increasing age should encourage future studies of how this imbalance might participate in the aging process.

1. Introduction

STriatal-Enriched protein tyrosine Phosphatase (STEP), encoded by the *PTPN5* gene, is a neural-specific phosphatase that opposes the development of synaptic strengthening by dephosphorylating key signaling molecules, including the glutamate receptor subunits GluN2B and GluA2, the kinases ERK1/2 (extracellular signal-regulated kinase 1/2), p38, Fyn, and Pyk2 (proline-rich tyrosine kinase 2) at regulatory tyrosine (Tyr) residues within their activation loop, thereby inactivating them and controlling the duration of their signal (reviewed by Goebel-Goody et al., 2012a). STEP also negatively regulates protein tyrosine phosphatase α, preventing its translocation to synaptic membranes and blocking its ability to interact with and activate Fyn (Xu et al., 2015).

Ptpn5 mRNA is alternatively spliced into distinct isoforms (Sharma et al., 1995; Bult et al., 1997) that are differentially targeted to the post-synaptic density (Oyama et al., 1995), extra-synaptic, and cytosolic compartments (Xu et al., 2009; Goebel-Goody et al., 2009). The major isoforms are $STEP_{46}$, a cytosolic isoform, and $STEP_{61}$, which is membrane-associated through the additional 172 amino acids at the N-terminus (Bult et al., 1997). Both isoforms are expressed in the striatum, whereas neurons of the hippocampus, cortex and spinal dorsal horn only express $STEP_{61}$ (Boulanger et al., 1995; Pelkey et al., 2002).

Initial studies indicated that in the developing rat striatum STEP is first expressed in neurons within patches, and subsequently localizes to both patches and matrix (Raghunathan et al., 1996; Okamura et al., 1997). In addition, STEP protein expression increases sharply from 2 to

Abbreviations: BDNF, brain-derived neurotrophic factor; IP, immunoprecipitation; MSNs, medium-sized spiny neurons; P, postnatal day; Q-PCR, quantitative polymerase chain reaction; STEP, STriatal-Enriched protein tyrosine Phosphatase; UPS, ubiquitin-proteasome system.

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4 weeks postnatally, when it reaches adult levels (Okamura et al., 1997). Accumulated evidence indicates that STEP levels/activity are altered in several pathological conditions, including Alzheimer's disease, Huntington's chorea, Parkinson's disease, schizophrenia, and fragile X syndrome (Carty et al., 2012; Goebel-Goody et al., 2012b; Kurup et al., 2010, 2015; Saavedra et al., 2011; Zhang et al., 2010). On the other hand, studies about alterations in STEP levels with age report disparate results. $STEP_{61}$ levels rise in mouse cortical synaptosomes with increasing age (Zhang et al., 2013), and its levels and activity are increased in the hippocampus of memory-impaired aged rodents (Brouillette et al., 2014). In contrast, we reported that $STEP_{61}$ levels are reduced with age in the lumbar spinal cord in both male and female mice (Azkona et al., 2016), and a recent study describes increased dimerization and loss of STEP activity, primarily in the cortex and hippocampus of aged rats (Rajagopal et al., 2016).

Brain-derived neurotrophic factor (BDNF) plays a key role in regulating neuronal survival, differentiation and function (Vicario-Abejón et al., 2002; Park and Poo, 2013; Huang and Reichardt, 2001). Similar to STEP, BDNF levels also increase during early postnatal stages in the striatum (Yurek et al., 1998; Katoh-Semba et al., 1997; Maisonpierre et al., 1990; Checa et al., 2000). Conversely, data on BDNF expression levels during aging vary depending on the rat strain (Tapia-Arancibia et al., 2008). We have recently reported that BDNF induces $STEP_{61}$ degradation in cultured cortical, hippocampal and striatal neurons through the ubiquitin-proteasome system (UPS) (Saavedra et al., 2016). Furthermore, $BDNF^{+/-}$ mice display elevated levels of $STEP_{61}$ in the cortex and hippocampus (Xu et al., 2016).

In the present work we sought to analyze both the postnatal developmental profile of STEP expression in mouse striatum, cortex and hippocampus, as well as STEP levels during aging in male and female mice, and their possible regulation by BDNF. We demonstrate that although STEP ontogenic profile is similar to that described for BDNF in the three brain regions analyzed, STEP levels are unaffected in the hippocampus of $BDNF^{-/-}$ mice during early postnatal development, whereas they are significantly reduced in the striatum and cortex in BDNF null mice. We suggest that this effect is indirect and likely related to impaired neurogenesis/reduced neuronal maturation. Remarkably, we describe a striatal-specific downregulation of STEP protein levels with age in mice from both sexes that is accompanied by higher BDNF levels and increased STEP ubiquitination. We propose that such a reduction of a striatal-enriched protein likely promotes neuronal dysfunction in medium-sized spiny neurons (MSNs) during aging, and we believe that the impact of reduced striatal STEP levels with age deserves further investigation.

2. Material and methods

2.1. Mice

$BDNF^{+/-}$ mice (Ernfors et al., 1994) were crossed to obtain wild-type ($BDNF^{+/+}$), $BDNF^{+/-}$ and BDNF knockout ($BDNF^{-/-}$) animals in a B6CBA background that were used for studies during development. C57BL/6J wild-type male and female mice were analyzed at 3, 6, 12 and 15 months of age, and adult male $BDNF^{+/-}$ mice and their wild-type littermates were evaluated at 8 months of age. For genotyping, DNA was obtained from tail biopsy and processed by PCR as previously described (Agerman et al., 2003). Mice were housed under a standard 12:12 h light/dark cycle with access to food and water *ad libitum* in a colony room kept at 19–22 °C and 40–50% humidity. All animal related procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the local animal care committee of the Universitat de Barcelona and the Generalitat de Catalunya, following European (2010/63/UE) and Spanish (RD 53/2013) regulations.

2.2. Western blot analysis

The brain was quickly removed and the striatum, cortex, and hippocampus were dissected out and homogenized in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 100 mM NaF, 5 μM $ZnCl_2$ and 10 mM EGTA plus protease inhibitors [phenylmethylsulphonyl fluoride (PMSF, 2 mM), aprotinin (1 μg/ml), leupeptin (1 μg/ml) and sodium orthovanadate (1 mM)]. The lysates were centrifuged at $16100 \times g$ for 20 min, supernatants were collected, and protein concentration measured using the Dc protein assay kit (Bio-Rad, Hercules, CA). Western blot analysis was performed as previously described (Saavedra et al., 2011). The primary antibodies used were (1:1000 unless stated otherwise): anti-STEP, anti-BDNF (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-panERK (BD Biosciences; San Diego, CA), anti-pGluN2B^{Tyr1472}, anti-GluN2B, anti-pPyk2^{Tyr402}, anti-Pyk2 and anti-pERK1/2$^{Thr202/Tyr204}$ (Cell Signaling, Beverly, MA) and anti-ubiquitin (1:4000; DakoCytomation, Denmark). Loading control was performed by reprobing the membranes with an anti-α-tubulin antibody (1:50,000; Sigma-Aldrich, St Louis, MO) for 20 min at room temperature. Then, membranes were washed with TBS-T (Tris-buffered saline containing 0.1% Tween 20), incubated for 1 h (20 min for loading controls) at room temperature with the corresponding horseradish peroxidase-conjugated antibody (1:2000; Promega, Madison, WI), and washed again with TBS-T. Immunoreactive bands were visualized using the Western Blotting Luminol Reagent (Santa Cruz Biotechnology), and quantified by a computer-assisted densitometer (Gel-Pro Analyzer, version 4, Media Cybernetics; Warrendale, PA).

2.3. Quantitative polymerase chain reaction (Q-PCR) assay

Total RNA was isolated from the striatum and hippocampus of $BDNF^{+/+}$ and $BDNF^{-/-}$ mice at P7 using the Total RNA Isolation Nucleospin RNA II Kit (Macherey-Nagel, Düren, Germany). Purified RNA (500 ng) was reverse transcribed using the PrimeScript™ RT reagent Kit (Perfect Real Time; Takara Biotechnology (DALIAN) CO., LTD, Japan). The cDNA synthesis was performed at 25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 min in a final volume of 20 μl. The cDNA was then analyzed by quantitative real-time-PCR using a TaqMan® Gene Expression Assay (Applied Biosystems, Foster City, CA) for PTPN5 (Mm00479063_m1). Q-PCR was performed in a final volume of 12.5 μl using the Premix Ex Taq™ (Probe qPCR) (Takara Bio INC). Reactions included 40 cycles of a two-step PCR: 95 °C for 5 s and 60 °C for 20 s, after initial denaturation at 95 °C for 30 s. All Q-PCR assays were performed in duplicate. To provide negative controls, and exclude contamination by genomic DNA, the reverse transcriptase was omitted in the cDNA synthesis step. The data were analyzed and quantified using the Comparative Quantitation Analysis program of the MxProTM Q-PCR analysis software version 3.0 (Stratagene) with the 18S gene expression (PrimeTime Std qPCR Assay; Integrated DNA Technologies; Coralville, IA) as internal loading control. Results were expressed relative to wild-type values.

2.4. Immunoprecipitation assay

Striatal protein extracts (200 μg) from 3- and 12-month-old male and female mice were diluted in 200 μl ice-cold immunoprecipitation (IP) buffer containing 40 mM Hepes (pH 7.5), 150 mM NaCl, 10 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 1 mM EDTA, 0,3% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 2 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mM

sodium orthovanadate. Samples were incubated overnight at 4 °C on a tube rotator with 2 μg anti-STEP antibody (Santa Cruz Biotechnology) or AffiniPure mouse anti-human IgGs (Jackson ImmunoResearch) as a negative control. The immune complexes were precipitated overnight at 4 °C by the addition of 25 μl protein A/G plus-Agarose (Santa Cruz Biotechnology). Beads were collected by centrifugation (5 min, 3300 × *g* at 4 °C) and washed three times with IP buffer and once with wash buffer (50 mM Hepes, pH = 7.5, 40 mM NaCl, 2 mM EDTA). Then bound proteins were eluted with SDS sample buffer, samples were boiled for 5 min at 100 °C and subjected to SDS-PAGE.

2.5. Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis was performed by using the unpaired Student's *t*-test (95% confidence), the one- or two-way ANOVA, as appropriate and indicated in the text/figure legends. Values of $p < 0.05$ were considered as statistically significant.

3. Results

3.1. STEP levels increase significantly during the first postnatal weeks

To characterize STEP protein expression during postnatal development we performed Western blot of striatal, cortical and hippocampal protein extracts obtained from wild-type mice at P3, P7, P14, P21 and 3 months of age (adult). In the striatum, $STEP_{61}$ protein levels were very low at P3 and P7, and then progressively increased until reaching adult levels at P21 (Fig. 1A). $STEP_{46}$ immunoreactivity was hardly detected at P7 (Fig. 1A), but then rapidly increased until adulthood. Similarly, in the cortex, $STEP_{61}$ levels were low at P3 and gradually increased during the first 2 weeks of postnatal development, when they reached adult levels (Fig. 1B). In contrast, in the hippocampus, $STEP_{61}$ protein levels at P3 were already half of those detected in adult mice, and adult-like levels were observed from P7 (Fig. 1C).

The expression profile of STEP found in the striatum, cortex and hippocampus is similar to that described for BDNF during rat postnatal development (Checa et al., 2000; Das et al., 2001; Katoh-Semba et al., 1997; Yurek et al., 1998; Maisonpierre et al., 1990). In line with these findings, we observed that BDNF levels also increased during the first postnatal weeks in mouse striatum, cortex and hippocampus (Fig. 1).

3.2. STEP protein levels are reduced in the striatum and cortex of $BDNF^{-/-}$ mice

The previous results suggested a possible correlation between BDNF and STEP expression patterns. To address the possibility that BDNF regulates STEP expression during postnatal development, we analyzed STEP levels in the striatum, cortex and hippocampus of $BDNF^{+/-}$ and $BDNF^{-/-}$ mice at P7 and P14. We could not analyze older $BDNF^{-/-}$ mice as these animals die during the first weeks after birth (Ernfors et al., 1994; Jones et al., 1994; Rauskolb et al., 2010). STEP levels were significantly reduced in the striatum and cortex of mice lacking BDNF both at P7 (Fig. 2A) and P14 (Fig. 2B). In contrast, STEP levels were unchanged in the hippocampus of $BDNF^{-/-}$ mice at both ages (Fig. 2A and B). STEP levels in $BDNF^{+/-}$ were similar to wild-type mice in all brain regions and ages analyzed (Fig. 2A and B).

To determine whether the alterations found in STEP protein levels in mice lacking BDNF correlated with changes in *Ptpn5* mRNA levels, we performed Q-PCR analysis of cDNA samples obtained from the striatum of 7-day-old $BDNF^{+/+}$ and $BDNF^{-/-}$ mice. We found that *Ptpn5* mRNA levels were significantly reduced in the striatum of BDNF null mice compared with wild-type littermates. Conversely, no differences were found in hippocampal *Ptpn5* mRNA levels between genotypes (Suppl. Fig. 1).

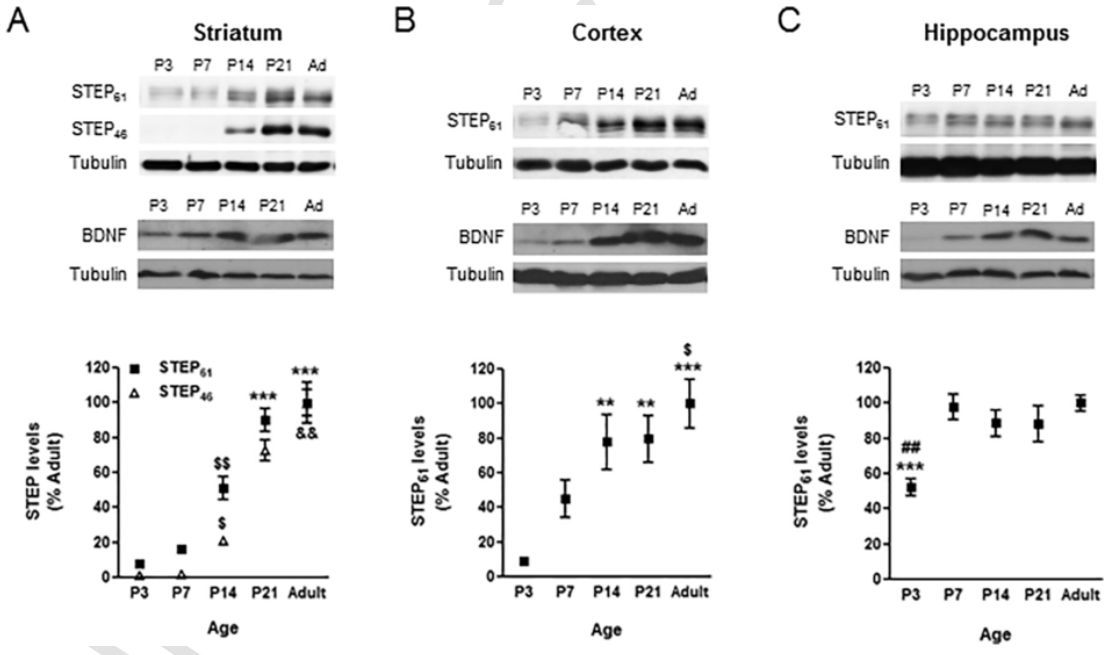

Fig. 1. Time course of STEP and BDNF protein levels during mouse postnatal development. STEP and BDNF protein levels were analyzed by Western blot of protein extracts obtained from the striatum (A), cortex (B) and hippocampus (C) of wild-type mice from P3 to 12 weeks of age (adult; Ad). Representative immunoblots are shown. Values (obtained by densitometric analysis of Western blot data) are expressed as percentage of adult values, and shown as mean ± SEM (n = 4–10). Data were analyzed by one-way ANOVA followed by Bonferroni's *post hoc* test. For the striatum: ***$p < 0.001$ compared with P3, P7 and P14; \$$p < 0.05$ and \$\$$p < 0.01$ compared with P3 and P7; &&$p < 0.01$ compared with P21 (only for $STEP_{46}$). For the cortex: **$p < 0.01$ and ***$p < 0.001$ compared with P3; \$$p < 0.05$ compared with P7. For the hippocampus: ##$p < 0.01$ compared with P14 and P21 and ***$p < 0.001$ compared with P7 and adult.

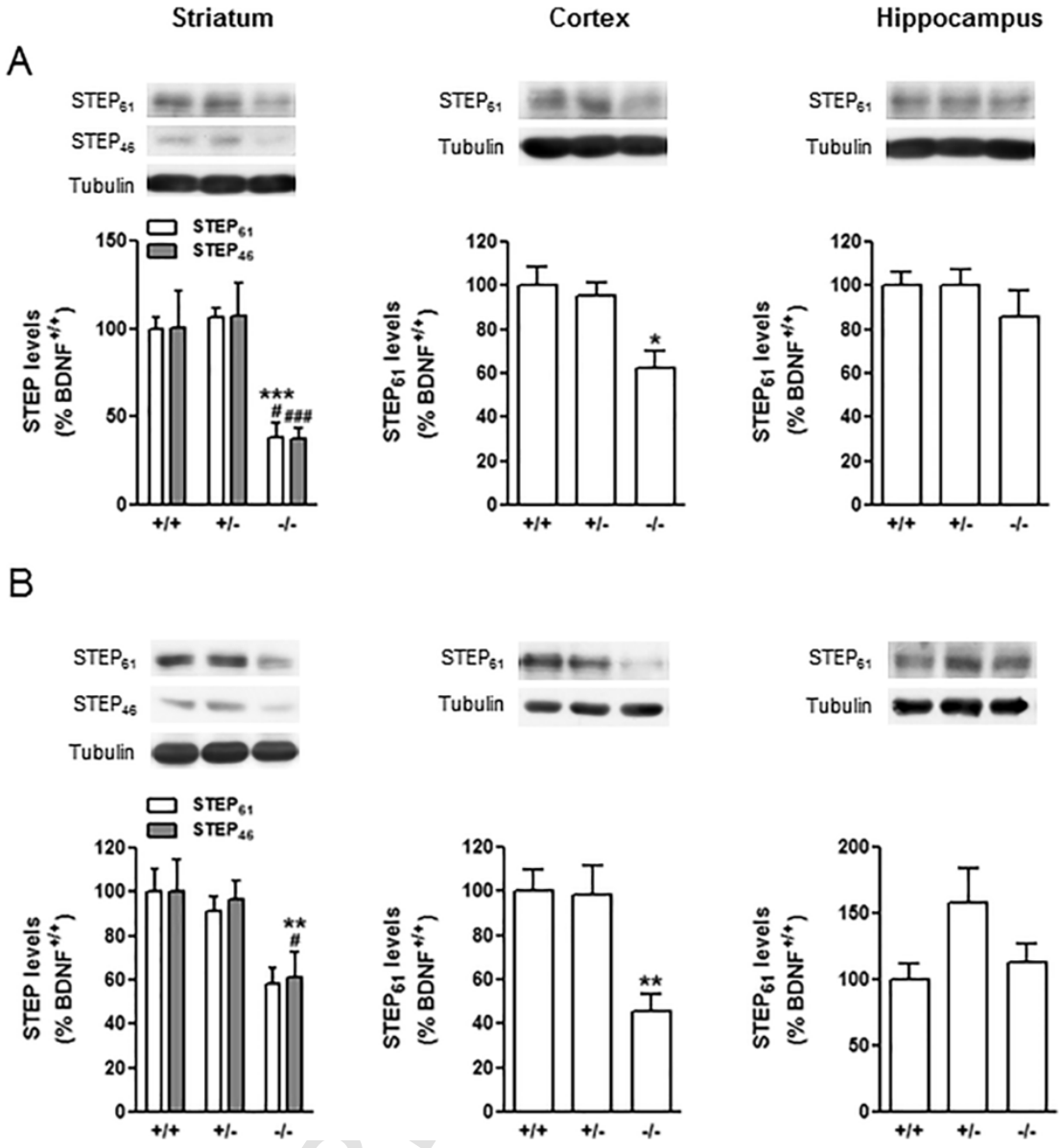

Fig. 2. STEP protein levels are reduced in the striatum and cortex, but not in the hippocampus, of $BDNF^{-/-}$ mice. STEP levels were analyzed by Western blot of protein extracts obtained from the striatum, cortex and hippocampus of wild-type (+/+), BDNF heterozygous (+/−) and BDNF knockout (−/−) mice at P7 (A) and P14 (B). Representative immunoblots are shown for each brain region and age analyzed. Values (obtained by densitometric analysis of Western blot data) are expressed as percentage of wild-type mice, and shown as mean ± SEM (n = 4–18 in A; n = 6–23 in B). Data were analyzed by one-way ANOVA followed by Bonferroni's *post hoc* test. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ compared with $BDNF^{+/+}$ mice; $\#p < 0.05$ and $\#\#\#p < 0.001$ compared with $BDNF^{+/-}$ mice.

3.3. STEP protein levels decrease with age in male and female mice specifically in the striatum

We next analyzed STEP protein levels in the adult striatum, cortex and hippocampus with increasing age. Since we reported sex differences in $STEP_{61}$ levels in the spinal cord of young mice (Azkona et al., 2016), we performed this study in mice from both sexes. Quantification of STEP levels in male and female mice at 3 months of age indicated no significant differences between sexes in any brain region analyzed (Fig. 3A–C). Temporal profiling of striatal STEP levels based on sex revealed that both $STEP_{61}$ and $STEP_{46}$ were significantly reduced with increasing age (Fig. 3D), with no difference between sexes (two-way ANOVA; for $STEP_{61}$: age effect: $F_{(3,60)} = 6.32$ and $p = 0.0009$; sex effect: $F_{(1,60)} = 3.35$ and $p = 0.072$; interaction effect: $F_{(3,60)} = 2.24$ and $p = 0.0929$; for $STEP_{46}$: age effect: $F_{(3,61)} = 7.2$ and $p = 0.0003$; sex effect: $F_{(1,61)} = 1.34$ and $p = 0.2459$; interaction effect: $F_{(3,61)} = 1.85$ and $p = 0.1482$). In contrast, no significant changes were detected in cortical $STEP_{61}$ levels with age (Fig. 3E), but there was a sex effect (two-way ANOVA; age effect: $F_{(3,39)} = 1.87$ and $p = 0.1513$; sex effect: $F_{(1,39)} = 9.14$ and $p = 0.0044$; interaction effect: $F_{(3,39)} = 1.56$ and $p = 0.2157$), whereas in the hippocampus no significant alterations were found in $STEP_{61}$ levels up to 15 months of age in either sex (Fig. 3F; two-way ANOVA; age effect: $F_{(3,34)} = 0.24$ and $p = 0.8701$; sex effect: $F_{(1,34)} = 0.13$ and $p = 0.7235$; interaction effect: $F_{(3,34)} = 0.32$ and $p = 0.8075$).

3.4. $STEP_{61}$ is more ubiquitinated in the striatum of older mice

STEP can be cleaved by calpains, generating a fragment of 33 kDa (Saavedra et al., 2017; Xu et al., 2009). However, the reduction of STEP levels in the striatum of older mice was not paralleled by signifi-

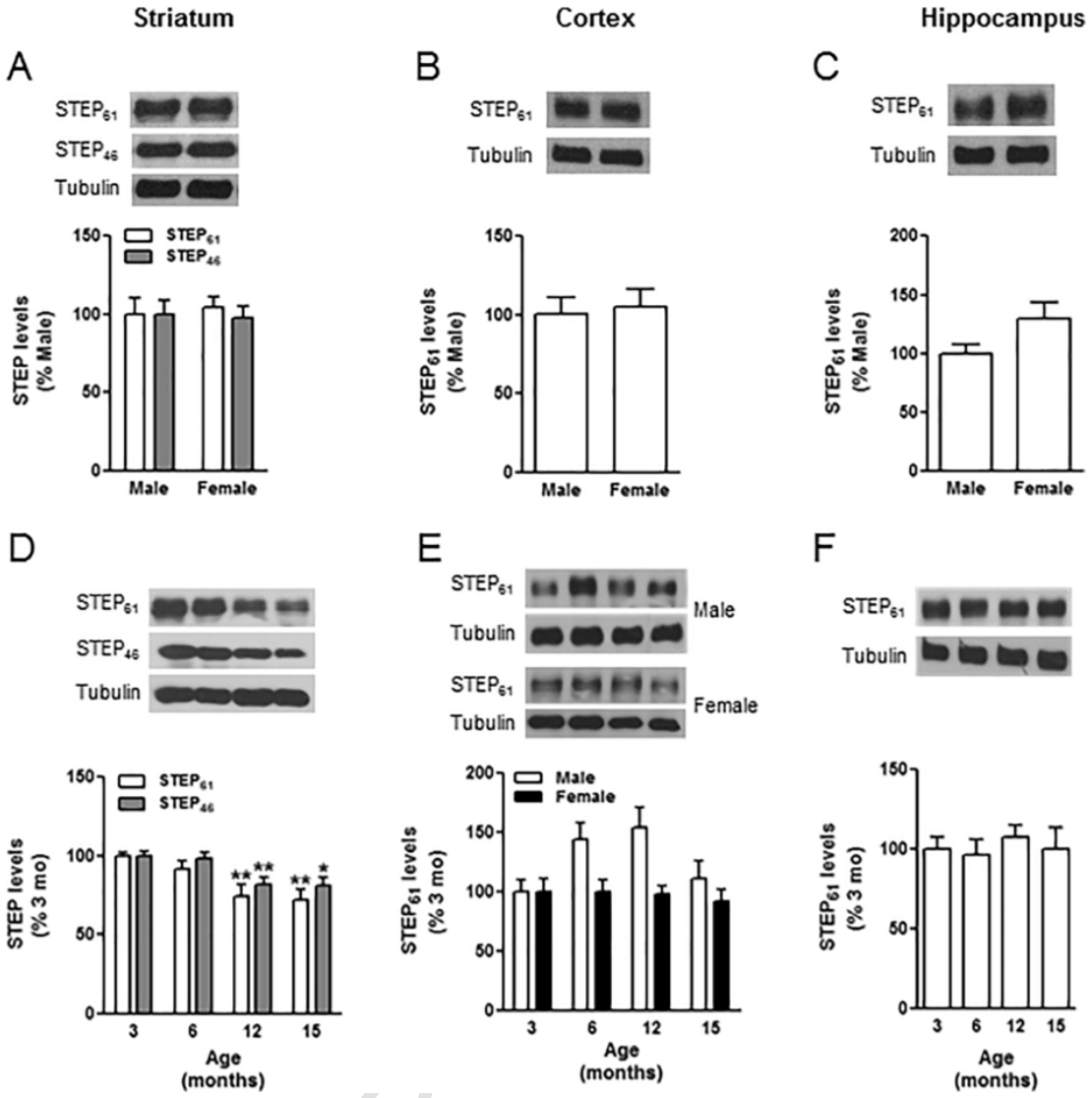

Fig. 3. Changes in STEP protein levels in the striatum, cortex and hippocampus with age. STEP protein levels were analyzed by Western blot of protein extracts obtained from the striatum (A and D), cortex (B and E) and hippocampus (C and F) of wild-type male and female mice at different ages (A–C, 3 months; D–F, from 3 to 15 months of age). Representative immunoblots are shown. Values (obtained by densitometric analysis of Western blot data) are expressed as percentage of male mice (A–C) or 3-month-old mice (D–F), and shown as mean ± SEM (n = 12–19 for the striatum, n = 10–15 for the cortex and n = 9–12 for the hippocampus). Data were analyzed by Student's t-test (A–C) and one-way ANOVA with Bonferroni's *post hoc* test (D-F). *$p < 0.05$ and **$p < 0.01$ compared with 3-month-old mice.

cant changes in $STEP_{33}$ levels (3 months: 100.04 ± 10.07% and 15 months: 82.89 ± 9.34%; n = 10–14, p = 0.2438, Student's t-test), suggesting that other mechanisms are responsible for lowering STEP levels in the striatum with increasing age.

In addition to calpain cleavage, STEP levels are also regulated by the UPS (Xu et al., 2009; Kurup et al., 2010; Mukherjee et al., 2011; Saavedra et al., 2016). To know whether reduced striatal STEP levels with increasing age were due to increased ubiquitination and subsequent proteasome degradation, we immunoprecipitated STEP from striatal extracts obtained at 3 and 12 months of age (Fig. 4A) and performed Western blot against ubiquitin. We found that the levels of ubiquitin-conjugated $STEP_{61}$ were higher in the striatum of 12-month-old mice compared with 3-month-old animals (Fig. 4B).

Since BDNF promotes STEP degradation through the UPS (Saavedra et al., 2016) and STEP levels decreased in the striatum from 12 months of age, we next examined striatal BDNF protein levels in 3-, 6- and 12-month-old male and female mice. At 3 months of age we did not find significant differences in BDNF protein levels between male and female striatum (male: 100.0 ± 18.6% and female: 106.4 ± 15.4%; n = 6/group; p = 0.8, Student's t-test). At 12 months of age, when STEP was found to be more ubiquitinated, BDNF levels were significantly increased in the striatum (Fig. 4C), with no differences between sexes (two-way ANOVA; sex effect: $F_{(1,41)}$ = 0.01708 and p = 0.8967; age effect: $F_{(2,41)}$ = 12.16 and $p < 0.0001$; interaction effect: $F_{(2,41)}$ = 0.4343 and p = 0.6507). Accordingly, $pTrkB^{Tyr816}$ levels were higher in the striatum of 12-month-old mice compared with young mice (3 months: 100.00 ± 8.25%; 12-months: 193.67 ± 17.79%; n = 13, $p < 0.0001$, Student's t-test). Conversely, in the hippocampus, where $STEP_{61}$ levels were unchanged, we did not detect significant changes in BDNF levels with age in either sex (two-way ANOVA; sex effect: $F_{(1,33)}$ = 0.02990 and p = 0.8638; age effect: $F_{(3,33)}$ = 1.226 and p = 0.3157; interaction effect: $F_{(3,33)}$ = 0.05674 and p = 0.9819). Although there is evidence that BDNF regulates $STEP_{61}$ levels *in vitro* (Saavedra et al., 2016) and in cortical and hippocampal neurons *in vivo* (Xu et al., 2016), we found that neither $STEP_{61}$ nor $STEP_{46}$ levels were

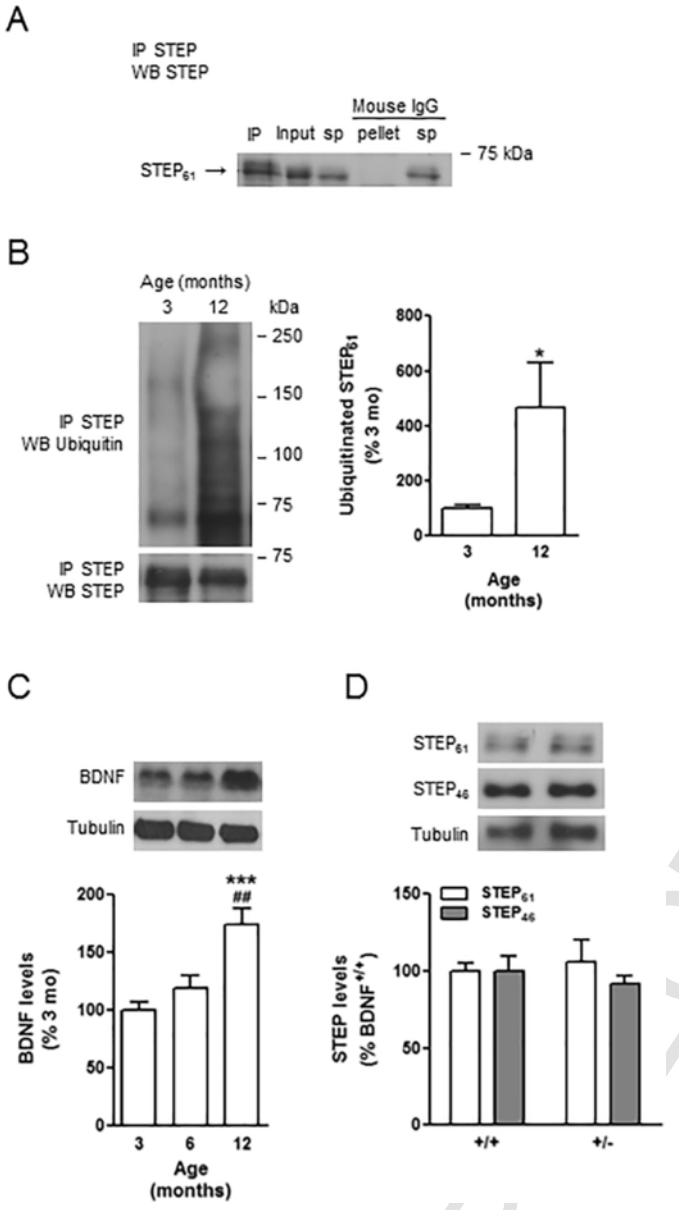

Fig. 4. Analysis of $STEP_{61}$ ubiquitination and BDNF levels with age. The levels of $STEP_{61}$-ubiquitin conjugates in the striatum were determined by performing immunoprecipitation (IP) of STEP and Western blot (WB) against ubiquitin. (A) The IP of STEP was confirmed by reprobing the membranes with the anti-STEP antibody. STEP immunoreactivity was absent in complexes obtained from samples incubated with mouse IgG, and was found in the supernatant (sp). Representative immunoblots are shown. (B) Levels of $STEP_{61}$-ubiquitin conjugate in striatal protein extracts obtained from 3- and 12-month-old male and female mice. Representative immunoblots are shown. The ratio of ubiquitin-conjugated $STEP_{61}$/IP $STEP_{61}$ was calculated for each sample and data were expressed as percentage of 3-month-old mice and shown as mean ± SEM (n = 14–15). The data from male and female samples were pooled together and analyzed by Student's t-test. *$p < 0.05$ compared with 3-month-old mice. (C) BDNF protein levels were analyzed by Western blot of protein extracts obtained from the striatum of male and female mice at different ages (mo, months). Representative immunoblots are shown. Values (obtained by densitometric analysis of Western blot data) are expressed as percentage of 3-month-old mice and shown as mean ± SEM (n = 15–16). Data were analyzed by one-way ANOVA followed by Bonferroni's *post hoc* test. ***$p < 0.001$ compared with 3-month-old mice and ##$p < 0.01$ compared with 6-month-old mice. (D) STEP protein levels were analyzed by Western blot of protein extracts obtained from the striatum of male wild-type (+/+) and BDNF heterozygous (+/−) mice at 8 months of age. Representative immunoblots are shown. Values (obtained by densitometric analysis of Western blot data) are expressed as percentage of wild-type mice and shown as mean ± SEM (n = 11–14). Data was analyzed by Student's t-test.

altered in total extracts obtained from 8-month-old $BDNF^{+/-}$ males compared with age-matched wild-type mice (Fig. 4D).

3.5. Increased phosphorylation of STEP substrates in the striatum of older mice

To evaluate the impact of reduced STEP levels in the striatum of older mice we analyzed the phosphorylation of several STEP substrates. Concomitant with lower levels of STEP, the phosphorylation level of $GluN2B^{Tyr1472}$ and $ERK1/2^{Thr202/Tyr204}$ was significantly increased in the striatum at 15 months of age compared with 3-month-old mice (Fig. 5A and C). In contrast, $pPyk2^{Tyr402}$ levels were similar at 3 and 15 months of age (Fig. 5B).

4. Discussion

In this work we found that STEP expression increases substantially during the first 1–3 postnatal weeks in mouse striatum ($STEP_{61}$ and $STEP_{46}$) and cortex ($STEP_{61}$), whereas in the hippocampus, $STEP_{61}$ expression peaks and stabilizes at P7. STEP protein levels are significantly lower in the striatum and cortex of $BDNF^{-/-}$ pups, whereas hippocampal STEP levels are not altered in the absence of BDNF.

Our data on STEP expression during postnatal development in mouse striatum are similar to previous findings in rat (Raghunathan et al., 1996; Okamura et al., 1997). As in the striatum, STEP levels increase significantly in the cortex and hippocampus during the first postnatal week(s) and, as far as we are aware, this is the first report about the postnatal profile of STEP in these brain regions. Interestingly, we found that the developmental profile of STEP parallels that of BDNF in the three brain regions analyzed as its levels also increase during the first postnatal weeks in mice (present results), and in the striatum (Maisonpierre et al., 1990; Katoh-Semba et al., 1997; Yurek et al., 1998; Checa et al., 2000), cortex (Das et al., 2001) and hippocampus (Das et al., 2001) of rats. Moreover, as for BDNF (Das et al., 2001), adult-like STEP levels were not observed until the second/third postnatal week. Our results show reduced STEP protein levels in the striatum and cortex, but not hippocampus, of $BDNF^{-/-}$ mice, suggesting that there is a brain region-dependent regulation of STEP levels during postnatal development in the absence of BDNF. Moreover, the reduction of STEP protein levels in the striatum of $BDNF^{-/-}$ mice was paralleled by diminished *Ptpn5* mRNA levels. Striatal STEP levels were not altered in $BDNF^{+/-}$ mice during early postnatal development and thus the classic 'gene-dosage' effect reported in other paradigms (Bianchi et al., 1996; Altar et al., 1997; Conover and Yancopoulos, 1997) was not observed.

The present results indicating that STEP protein levels were reduced in the striatum and cortex of $BDNF^{-/-}$ mice during postnatal development add a new element to the list of phenotypic markers reduced in these animals. Consistent with the requirement of BDNF for the maturation of a large subset of MSNs (Ivkovic and Ehrlich, 1999), $BDNF^{-/-}$ mice also show a marked decrease in the striatal expression of calbindin (Jones et al., 1994), DARPP-32 (Ivkovic et al., 1997; Ivkovic and Ehrlich, 1999), ARPP-2131 (Ivkovic and Ehrlich, 1999) and parvalbumin (Altar et al., 1997; Große et al., 2005). Selected brain areas require BDNF signaling for normal postnatal development (reviewed by Zagrebelsky and Korte, 2014). Namely, BDNF determines the survival of immature striatal neurons at their origin, their maturation and the establishment of striatal connections during brain development (reviewed by Baydyuk and Xu, 2014). For instance, there is a marked decrease in dendritic complexity and spine density in the striatum of conditional $BDNF^{-/-}$ mice (Rauskolb et al., 2010). On the other hand, BDNF is required for the maintenance of cortical dendrites (Gorski et al., 2003) and TrkB signaling is essential for neurogenesis and cell proliferation during cortical development (Bartkowska et al., 2007). Conversely, although BDNF levels also increase in the hippocampus during

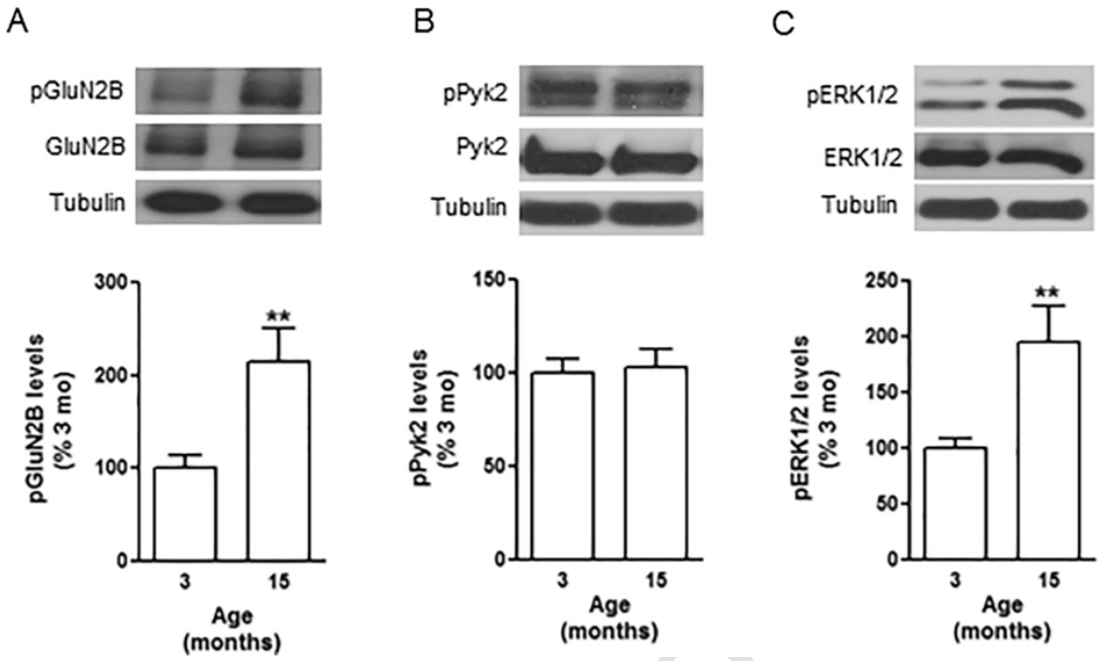

Fig. 5. Phosphorylation of STEP substrates in the striatum. pGluN2B (A), pPyk2 (B) and pERK1/2 (C) levels were analyzed by Western blot of protein extracts obtained from the striatum of male and female mice at 3 and 15 months of age. Representative immunoblots are shown. Values (obtained by densitometric analysis of Western blot data) are expressed as percentage of 3-month-old mice and shown as mean ± SEM (n = 10–13). Data were analyzed by Student's t-test. $^{**}p < 0.01$ compared with 3-month-old mice.

postnatal development in parallel with STEP, lack of BDNF did not affect STEP levels in this brain region. Interestingly, BDNF is dispensable for the growth of the hippocampus, and spine density remains unchanged in conditional mutant mice (Rauskolb et al., 2010). In fact, BDNF is specifically required for the activity-dependent maintenance of the mature spine phenotype of hippocampal neurons (Kellner et al., 2014). Thus, lower STEP levels found in the striatum and cortex of BDNF-deficient mice are likely related to impaired neurogenesis/reduced neuronal maturation in these animals resulting in minor expression of STEP and/or lower number of STEP-positive cells. However, distinction between these possibilities was hampered by the low expression level of STEP in the striatum and cortex at P7 and P14, and its further reduction in $BDNF^{-/-}$ mice, which prevented the analysis of STEP at the cellular level.

The present study also aimed to characterize STEP levels in mouse brain during aging and potential sex-related differences. In contrast to the findings in the spinal cord of 3-month-old mice, where female mice display lower $STEP_{61}$ levels (Azkona et al., 2016), here we did not observe differences in STEP levels between sexes in young mice in either brain region. However, our results showed that striatal STEP levels were reduced between 3 and 15 months of age in both sexes. Similarly, recent data from our group showed a reduction of $STEP_{61}$ protein levels in the spinal cord with age (Azkona et al., 2016). Here, we did not detect differences in STEP protein levels, either in the cortex or hippocampus up to 15 months of age. In contrast, a previous study has shown that STEP levels increase from 15 months of age in mouse cortical synaptosomes (Zhang et al., 2013). Moreover, elevated STEP levels occur with advancing age in human, rhesus monkey and rodent hippocampus (Brouillette et al., 2014). Since Zhang et al. (2013) also used mice in a C57BL/6 background a possible explanation for this discrepancy is that we analyzed total protein extracts and not synaptosomal fractions. Therefore, it is likely that increased cortical $STEP_{61}$ levels at advanced ages are detected earlier in this subcellular compartment. Altogether, we conclude that there are brain region-dependent changes in STEP levels during aging in mice, with changes in the striatum being opposite and occurring earlier than in the cortex and hippocampus. Conversely, a recent study performed in rats found that aging is associated with increased dimerization and loss of STEP activity primarily in the cortex and hippocampus, whereas no significant changes were reported in the striatum (Rajagopal et al., 2016).

In the present study we found that reduced STEP levels in the striatum at 12 months of age in both males and females were paralleled by high BDNF content. These findings are in line with recent studies showing that BDNF/TrkB signaling promotes $STEP_{61}$ degradation through the UPS *in vitro* (Saavedra et al., 2016; Xu et al., 2016). Accordingly, although there is evidence of an age-related decrease in proteasome function in the brain (Keller et al., 2002; Keller et al., 2004), here we showed that the possible mechanism responsible for reduced STEP levels was its increased proteasomal degradation as demonstrated by the presence of more ubiquitin-$STEP_{61}$ conjugates in the striatum of older mice. As BDNF promotes STEP degradation we speculated that STEP levels could be increased in the striatum of $BDNF^{+/-}$ mice. However, analysis of STEP levels in total extracts at 8 months of age showed no significant differences between genotypes. This could be due to a dilution effect since in frontal cortex and hippocampus of $BDNF^{+/-}$ mice $STEP_{61}$ levels are elevated in synaptosomal membrane fractions (Xu et al., 2016). As BDNF also stimulates STEP degradation in cultured striatal neurons (Saavedra et al., 2016), another possibility is that further mechanisms operate *in vivo* to control STEP protein levels in the striatum. In fact, chronic downregulation of TrkB in mature MSNs promotes a reduction of *Ptpn5* mRNA levels in the striatum of male and female mice (Unterwald et al., 2013).

Studies on BDNF levels in the aging brain have generated quite inconsistent results. Here we found increased BDNF levels in the striatum of 12-month-old mice from both sexes compared with mice at 3 months of age. In contrast, other studies report either no significant differences up to 20 months of age (Schulte-Herbrüggen et al., 2008), or reduced BDNF protein levels from 6 (Ma et al., 2015) or 9 (Arumugam et al., 2010) months of age in male mice. The difficulty in clarifying the course of age-related BDNF alterations likely relies on the use of different species and strains, together with the sex of the subjects analyzed and differences in the age of young and old groups. For instance, a study using Fisher 344 rats revealed that the effects of age

on neurotrophin levels depend on the specific age of the young and aged groups, which affects the extent, and even the recognition of the age effect (Bimonte-Nelson et al., 2008).

Consistent with a reduction of striatal STEP levels we found increased phosphorylation of STEP substrates in older mice. However, not all STEP substrates analyzed were significantly more phosphorylated. This is likely due to their regulation by mechanisms other than altered STEP phosphatase activity with age. Reduced spinal $STEP_{61}$ levels with increasing age correlate with thermal hyperalgesia in mice (Azkona et al., 2016), and loss of STEP activity due to cleavage contributes to ischemia-induced brain damage (Deb et al., 2013). On the other hand, elevated STEP levels are also detrimental, as evidenced by the finding that higher hippocampal STEP levels are associated with age-related cognitive deficits in several species (Brouillette et al., 2014), and that both genetic deletion (Zhang et al., 2010) and pharmacological inhibition (Xu et al., 2014) of STEP improve cognitive deficits in Alzheimer's disease mouse models. In addition, *Ptpn5* loss of function delays recovery from stress and increases the development of stress-related cognitive and morphological changes, while overexpression of a constitutively active form enhances resilience to stress in rats (Yang et al., 2012). Thus, it becomes evident that altered STEP levels are accompanied by neuronal dysfunction. Since STEP is highly expressed in the striatum we propose that the reduction of striatal STEP levels during aging likely promotes neuronal dysfunction in MSNs. Both unpublished data from our group and results in the literature indicate that STEP knockout mice have unaltered performance in the rotarod task (Sukoff Rizzo et al., 2014; Venkitaramani et al., 2011), suggesting that striatal-dependent functions other than motor control might be affected by this alteration. For instance, it has been shown that knockdown of STEP expression in the dorsal hippocampus increases neuronal vulnerability to glutamate-induced toxicity (Yang et al., 2012). Thus, the impact of reduced striatal STEP levels with age deserves further investigation. In fact, the increased dimerization and loss of STEP activity in the cortex and hippocampus of aged rats was proposed as a contributing factor for the susceptibility to age-associated neurodegenerative disorders (Rajagopal et al., 2016). Moreover, changes in the levels/activity of other phosphatases in the aging brain have been shown to participate in the hyperphosphorylation of neurofilaments (Veeranna et al., 2011), to be associated with increased phosphorylation and oligomerization of α-synuclein (Liu et al., 2015), and proposed to impact in signal transduction (Agbas et al., 2005).

In conclusion, in the present work we demonstrate that STEP postnatal profile in the striatum, cortex and hippocampus parallels that described for BDNF and that there is a significant reduction of STEP levels in the striatum and cortex, but not in the hippocampus, of $BDNF^{-/-}$ mice during early postnatal development. We propose that these effects are indirect and likely related to impaired neurogenesis/reduced neuronal maturation. On the other hand, we describe a striatal-specific downregulation of STEP protein levels with age in adult male and female mice, paralleled by higher BDNF levels and correlating with increased ubiquitination and higher phosphorylation level of its substrates.

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Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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