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TrkB/BDNF-Dependent Striatal Plasticity and Behavior in a Genetic Model of Epilepsy: Modulation by Valproic Acid

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In mice lacking the central domain of the presynaptic scaffold Bassoon the occurrence of repeated cortical seizures induces cell-type-specific plasticity changes resulting in a general enhancement of the feedforward inhibition within the striatal microcircuit. Early antiepileptic treatment with valproic acid (VPA) reduces epileptic attacks, inhibits the emergence of pathological form of plasticity in fast-spiking (FS) interneurons and restores physiological striatal synaptic plasticity in medium spiny (MS) neurons. Brain-derived neurotrophic factor (BDNF) is a key factor for the induction and maintenance of synaptic plasticity and it is also implicated in the mechanisms underlying epilepsy-induced adaptive changes. In this study, we explore the possibility that the TrkB/BDNF system is involved in the striatal modifications associated with the Bassoon gene (*Bsn*) mutation. In epileptic mice abnormal striatum-dependent learning was paralleled by higher TrkB levels and an altered distribution of BDNF. Accordingly, subchronic intrastriatal administration of k252a, an inhibitor of TrkB receptor tyrosine kinase activity, reversed behavioral alterations in *Bsn* mutant mice. In addition, *in vitro* manipulations of the TrkB/BDNF complex by k252a, prevented the emergence of pathological plasticity in FS interneurons. Chronic treatment with VPA, by reducing seizures, was able to rebalance TrkB to control levels favoring a physiological redistribution of BDNF between MS neurons and FS interneurons with a concomitant recovery of striatal plasticity. Our results provide the first indication that BDNF is involved in determining the striatal alterations occurring in the early-onset epileptic syndrome associated with the absence of presynaptic protein Bassoon.
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INTRODUCTION

In the striatum of mice lacking the central domain of Bassoon gene (*Bsn*) corticostriatal synaptic plasticity changes in response to early-onset epilepsy (Ghiglieri *et al*, 2009). In epileptic mice, striatal medium spiny (MS) neurons show morphological alterations and a reduced long-term potentiation (LTP) while fast-spiking (FS) parvalbumin (PV)-positive GABAergic interneurons are more numerous and express a short-term potentiation that is not observed in the wild-type mice. Interestingly, early therapeutic intervention with valproic acid (VPA), a broad spectrum antiepileptic drug (AED), is able to prevent the cell-type-specific functional alterations induced by chronic seizures. In the healthy brain, structural and functional changes represent the basis of plasticity and are strongly regulated by brain-derived neurotrophic factor (BDNF) (Lu, 2003; Poo, 2001). Corticostriatal neurons deliver BDNF through activity-dependent release into the striatum in which it binds to its high-affinity receptor, the tyrosin kinase tropomyosin-related kinase B (TrkB) receptor, leading to the activation of distinct intracellular pathways that control neurite growth, synaptic plasticity, proliferation, and survival (Zuccato and Cattaneo, 2009). It is noteworthy that BDNF is specifically required for the maturation of inhibitory GABAergic synapses (Bartrup *et al*, 1997; Marty *et al*, 1996; Yamada *et al*, 2002) and the regulation of interneuronal properties (Berghuis *et al*, 2004). BDNF also differently modulates development and function of excitatory and inhibitory synaptic transmission (Bolton *et al*, 2000).

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Besides the control of these constitutive aspects, BDNF has a pivotal role in the mechanisms underlying epilepsy-induced adaptive changes.

In fact, it has been reported that in experimental models and in epileptic patients both BDNF mRNA and protein levels are increased in the foci of seizures (Gall, 1993; Humpel *et al*, 1993; Isackson *et al*, 1991; Schmidt-Kastner *et al*, 1996; Takahashi *et al*, 1999; Jankowsky and Patterson, 2001). It has also been shown that striatal mRNA of TrkB is transiently elevated after seizures (Merlio *et al*, 1993; Salin *et al*, 1995) and that epileptiform activity induces a strong upregulation of cortical TrkB (Wyneken *et al*, 2001, 2003). All these data support the idea that TrkB/BDNF signaling pathway is a key factor in the development and control of seizures, although the question whether BDNF has a permissive or protective function in the epileptogenesis is still debated. Moreover, in spite of the potential relevance of these actions for synaptic plasticity and striatal-dependent learning, the possible role of BDNF in the corticostriatal plasticity during epilepsy has not been considered yet. To analyze whether TrkB/BDNF system is specifically involved in the striatal modifications associated with *Bsn* mutation, we first analyzed behavioral and plastic changes in *Bsn* mutants after *in vivo* inhibition of TrkB receptor activity. Moreover, we performed quantification of striatal TrkB levels on immunoblots and immunohistochemical analysis of BDNF protein expression pattern in striatal MS neurons and FS interneurons in both wild-type and epileptic mice. Then, to explore whether these modifications may depend on chronic seizure activity, we measured TrkB levels and evaluated BDNF distribution in the striatum of epileptic mice after chronic VPA treatment.

MATERIALS AND METHODS

Animals

Mice lacking part of the Bassoon gene (*Bsn*ΔEx4/5) were generated as described earlier (Altrock *et al*, 2003), backcrossed into C57/bl6 mouse strain, and then crossed with SV129 strain yielding mice with a mixed C57/bl6-SV129 genetic background for mutants and wild types. Genotyping was performed by polymerase chain reaction as described earlier (Altrock *et al*, 2003); age-matched wild-type littermates were used as controls. At the beginning of the experiment, animals were 2–3 months old and their weights ranged from 23 to 28 g. They were housed in a temperature-controlled room (22 °C) with a light–dark 12 : 12 cycle (lights on 0700–1900 h). Food and water were given *ad libitum*. All experiments were carried out according to the guidelines on the ethical use of animals from the European Communities Council Directive of 24 November 1986 (86/609/EEC) and from the National Ministry of Health (DM 222/2008).

Surgery Procedures

Adult *Bsn* mutants and wild-type controls weighing 28–30 g were anesthetized by i.p. injection of chloral hydrate (400 mg/kg) (Merk KGaA, Darmstadt, Germany) and placed into a stereotaxic frame (Stoelting, Co, USA). A guide cannula (inner diameter 0.25 μm, outer diameter 0.50 μm) (Unimed, Lausanne, Switzerland) was placed into the dorsal striatum according to Paxinos and Franklin (2007) at + 1.1 mm from bregma, 2.0 mm lateral to the midline, and 2.5 mm from *dura mater*. The cannula was permanently secured to the skull using cranioplastic cement (Glasionomer Cement CX Plus, Shofu, Kyoto, Japan) and closed with a dummy cannula (Unimed). After the surgery, mice were allowed to recover for 3 days before drug administration. Only animals showing normal neurological functions and no evidence of infection were randomized into experiments. Brain sections were examined under a light microscope to allow visual checking of cannula placement and mice without proper positioning of cannula were excluded from analysis.

Behavioral Measurements

Eight mice per group were used in the training experiments. Active avoidance task requires to learn that an explicit conditional stimulus (CS: light) predicts the delivery of a negative unconditioned stimulus (US: footshock). The test was carried out in a battery of six two-way shuttle boxes (40 × 10 × 15 cm). Each box was divided into two compartments by a partition with an opening at the floor level. The boxes had a transparent cover with a light bulb (10 W) attached above each compartment. The floor was made of a stainless steel grid. Mice were subjected to one active avoidance session (duration 30 min, 60 avoidance trials) for 5 consecutive days. Each trial consisted of a 30 s light signal (CS) presented in one compartment 5 s before the onset of an electric foot shock (7 mA, 25 s) in the same compartment (US). Two dependent variables were recorded: escape responses (crossings during US presentation), and conditioned responses (crossings occurring within 5 s of CS). Foot-shock sensitivity was evaluated by placing mice in a Plexiglas cage (28 cm long × 28 cm wide × 10 cm high) with a grid floor connected to a shock producer. Mice were individually placed in the cage and their pain thresholds were evaluated by increasing current intensity from 0 to a maximum of 0.6 mA. The minimal intensity eliciting vocalization and jumping was retained as the score. Mice failing to squeak were given the maximum score of 0.6 mA. Two-way ANOVA was performed to compare means with one factor between groups (genotype) and one factor within groups (day). Mutants showing episodic thigmotaxis and stereotypies were not included in the statistical analysis to avoid bias in scoring motor behaviors.

Electrophysiological Experiments

Eight adult mice (2–3 months old, weighting 23–28 g) were used per groups for all the electrophysiological experiments. Animals were killed by cervical dislocation to obtain coronal corticostriatal slices for electrophysiological recordings (Calabresi *et al*, 1992). The slices (200–300 μm) were prepared from tissue blocks of the brain with the use of a vibratome. A single slice was transferred to a recording chamber and submerged in a continuously flowing Krebs' solution (33 °C, 2–3 ml/min) gassed with 95% O_2 and 5% CO_2. The composition of the control solution was (in mM): 126 NaCl, 2.5 KCl, 1.2 MgCl2, 1.2 NaH2PO4, 2.4 CaCl2, 11 glucose, and 25 NaHCO3. In all the experiments, the

intracellular recording electrodes were filled with 2 M KCl (30–60 MΩ). Signals were recorded using an Axoclamp 2B amplifier (Molecular Devices, Sunnyvale, CA, USA), showed on a separate oscilloscope, stored, and analyzed on a digital system (pClamp 9, Axon Instruments). For synaptic stimulation, bipolar electrodes were located in the white matter between the cortex and the dorsolateral striatum to activate corticostriatal fibers. For LTP protocol, at the beginning of intracellular recordings, magnesium ions were omitted from the medium to increase the NMDA-mediated component of the excitatory postsynaptic potential (EPSP). As conditioning tetanus (high-frequency stimulation, HFS), we used three trains (3 s duration, 100 Hz frequency, at 20-s intervals). During tetanic stimulation, the intensity was increased to suprathreshold levels. Quantitative data on EPSP modifications induced by tetanic stimulation are expressed as a percentage of the controls, the latter representing the mean of responses recorded during a stable period (15–20 min) before the tetanus. Values given in the text and in the figures are mean ± SEM of changes in the respective cell populations. Two-way ANOVA, followed by Bonferroni *post hoc* test, and Student's *t*-test were used to compare the means between genotypes and pre- *vs* post-tetanic, respectively.

Immunohistochemistry and Quantitative Analysis

For the histological analysis three animals for each group were used. Forty-micrometer-thick coronal sections were cut on a microtome (Microm, Walldorf, Germany) and collected in sequence in 24-well plates containing 1 ml of PB. Striatal sections were processed free-floating for parvalbumin (PV) and Calbindin (Calb) immunofluorescence, incubated with a rabbit anti-parvalbumin (1 : 500; Chemicon, CA, USA) and a mouse anti-Calb antibody (1 : 500; Sigma Aldrich, Mi, Italy) and subsequently with a secondary Cy2-conjugated antibody donkey anti-mouse IgG and Cy3- conjugated donkey anti-rabbit IgG (1 : 100; Jackson Immunoresearch Laboratories, West Grove, PA, USA). Specificity of the antibodies was tested by primary omission controls, blocking peptide immunoreaction, and dilution series. Sections were then mounted in gelatin-coated slides, air-dried, and coverslipped with GEL/MOUNT (Biomeda, Foster City, CA, USA). All the sections were batch-processed at the same time. For each section, three equally spaced photomicrographs of the dorsolateral part of the striatum were taken using the 40 × magnification of a confocal microscope CLSM (Zeiss LSM 510). For each mouse, PV and Calb-positive cells were counted at four different rostro-caudal levels of the striatum. The coordinates according to Paxinos and Franklin (2007) were +1.10, +0.62, +0.14, and −0.34 mm from bregma. For each brain, we examined three confocal images of dorsolateral striatum, using as landmark the *corpus callosum*. A total of 24 confocal images for each one animal was examined. A reconstructed striatal area in Supplementary Figure S1 describes the three subfields into which the striatum was subdivided that were used for the cell counts.

The number of cells was evaluated in each picture acquired using Image J software (http://rsb.info.nih.gov/ij). The ratio of the total amount of cells for each labeling was calculated following the formula: PV-positive cells/Calb-positive cells; and compared with a one-way ANOVA. Furthermore, to analyze the abundance of BDNF protein in PV-positive interneurons, the sections were double labeled with an antibody against BDNF (mouse anti-BDNF, 1 : 200, Immunological Sciences, Italy) and rabbit anti-PV (1 : 500, Chemicon). Sections were examined under an epi-illumination fluorescent microscope (Zeiss Axioskop 2) and a confocal Laser scanning microscope (CLSM) Zeiss LSM510. Digital images were acquired using the Zeiss LSM510 computer program. The number of PV-interneurons that were labeled for BDNF, and devoid of it, was counted. The density of PV-positive cells that were BDNF immunoreactive was thus obtained. The levels of colocalization in each field were averaged across all fields and a statistical analysis by means of one-way ANOVA was used setting $p < 0.05$.

Western Blotting Analysis

Six wild-type and six mutant mice with ages of 2–3 months were killed by cervical dislocation. The striatum was prepared from the brains, immediately frozen in liquid nitrogen and stored at −80 °C. For analysis of protein levels in total homogenates, tissue from individual mice was homogenized in buffer H (5 mM TrisHcl, 0,5% TritonX-100 with Complete protease inhibitors (Roche)) using Teflon homogenizer (900 r.p.m., 12 strokes). To isolate PSD-enriched protein fraction the initial homogenate was kept for 1 h at 4 °C to extract TritonX-100 soluble proteins and centrifuged at 100 000 r.p.m. at 4 °C for 1 h. To increase the purity of isolated PSD-enriched material the pellet was washed by rehomogenization in buffer H followed by centrifugation. The supernatants of both centrifugation steps were pooled. Proteins of both resulting fractions were precipitated with acetone. SDS-PAGE and western blotting were performed according to standard procedures. Antibody against alpha-tubulin (DM1A, Sigma) and TrkB (H-181) antibody (Santa Cruz) and secondary antibody coupled to Odyssey IRDye 680 or Odyssey IRDye 800 were used. The signals were scanned and quantified on Odyssey imaging system (LI-COR). The levels of TrkB in each probe were normalized to alpha-tubulin signal.

Drugs

For *in vivo* administration k252a (Alomone Labs, Jerusalem, Israel) was dissolved in dimethylsulfoxide and diluted to 9.34 ng/μl. (20 μM) in Krebs'solution. Intrastriatal infusion of either k252a or vehicle were given to hand-restrained conscious animals 20 min previous each session of active avoidance testing. Cannulas used for administration were attached by polyethylene tubing to a 10 μl Hamilton syringe (Sigma-Aldrich, Milano, Italy) which, in turn, was driven by a microinfusion pump (World Precision Instruments, Sarasota, FL, USA) at a rate of 0.2 μl/min (total volume of 1 μl). Injection cannulas were left in place for 2 min after the infusion was completed.

For *in vitro* electrophysiological recordings k252a was applied by diluting it in Krebs'solution to the final concentration of 200 nM and by switching the perfusion from control to drug-containing solution. Sodium Valproate (Depakin, 200 mg/ml; Sanofi Aventis, Mi, Italy)

was added into drinking water at a concentration of 100 mg/100 ml to administer a daily dose of approximately 400 mg/kg.

RESULTS

Increased Active Avoidance Performances in *Bsn* Mutant Mice

Striatal-dependent learning abilities of *Bsn* mutant and wild-type mice were assessed in the two-way active avoidance test. The test is based on the capability to form a simple CS–US association and extensive findings indicate that the formation of such associations largely depend on the dorsolateral striatum (White and McDonald, 2002). In the present experiment, eight mice for each genotype were subjected to daily sessions consisting of 30 CS–US presentations, for 5 consecutive days. On each session, the number of conditioned responses and inter-trial crossings was scored. Our results show that performances increased in each group as training proceeded (significant effect of session: $F(4,56) = 11.56$; $p < 0.001$). Interestingly, *Bsn* mutant mice showed higher active avoidance scores than wild-type mice (significant effect of genotype: $F(1,14) = 12.60$; $p < 0.01$) (Figure 1a, left panel) while the number of inter-trial crossings, one main index of sustained motility, was similar in both genotypes (Figure 1a, right panel).

Inhibition of TrkB Tyrosine Kinase Activity Reduces Procedural Learning of *Bsn* Mutant Mice to Control Levels

To analyze the role of TrkB/BDNF system in the striatal-dependent procedural learning, wild-type mice and *Bsn* mutants receiving intrastriatal administration of the TrkB inhibitor k252a were subjected to a battery of active avoidance testing. Fifteen minutes after the injection of drug or vehicle, animals were subjected to a 30-min session, once a day, for 5 days. Also in these groups the performances increased as training proceeded (significant effect of session: $F(4,28) = 12.80$; $p < 0.001$) (Figure 1b, left panel) and the number of inter-trial crossings was similar (Figure 1b, right panel). However, after administration of k252a (20 μM, intrastriatum), task performances were worsened in wild-type mice (data not shown) while scores of treated *Bsn* mutant mice were similar to vehicle-injected wild-type controls (Figure 1b, left panel).

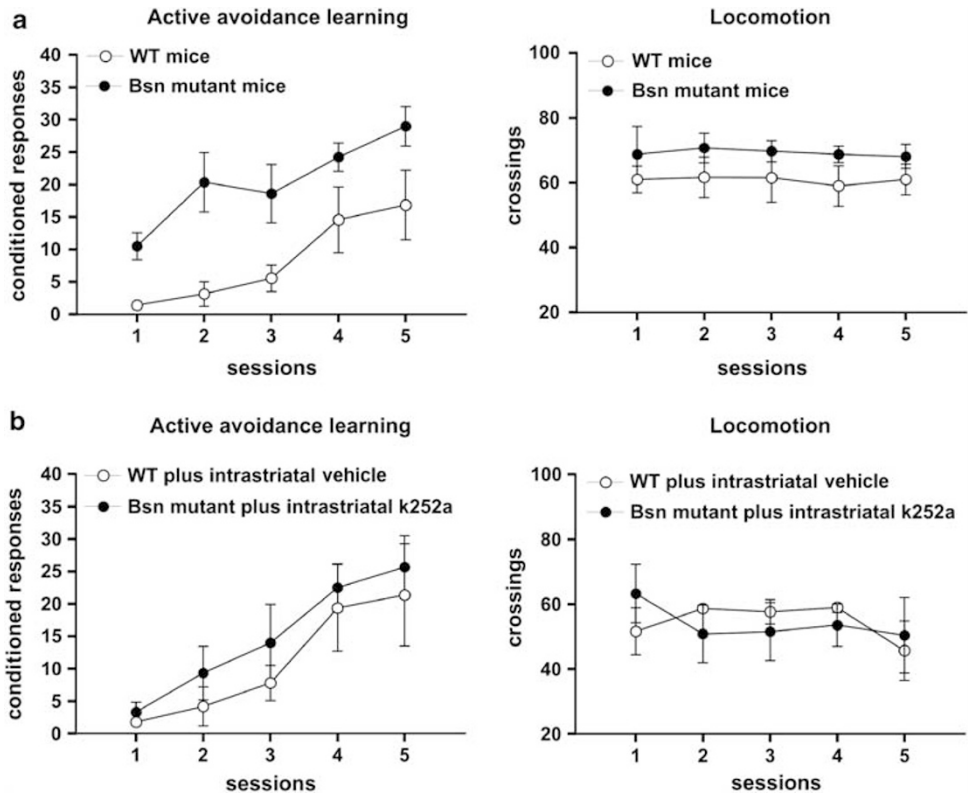

Figure 1 TrkB dependent increased procedural learning in *Bsn* mutant mice. Corticostriatal-dependent learning capabilities of *Bsn* mutants and wild-type littermates were tested using a two-way active avoidance test. (a) Graph on the left shows the mean number of avoidance responses per day ± SEM recorded for each group. Two-way ANOVA revealed an effect of genotype ($F(1,14) = 12.60$; $p < 0.01$), indicating that performances were higher in *Bsn* mutants than in wild-type mice. Mean number of crossings between the two compartments per day ± SEM are shown on the right panel. Two-way ANOVA revealed no significant group main effect, indicating that locomotion did not significantly differ between genotypes. (b) Subchronic intrastriatal administration of k252a (20 μM), an inhibitor of TrkB tyrosine kinase activity, in epileptic mice, caused a reduction of task performances to control values, as shown by the graph on the left. Statistical comparisons between the two genotypes show that subchronic k252a did not affect the number of crossing, which was similar in the two experimental groups (right panel).

In Vitro TrkB Antagonism Abolishes Short-Term Plasticity in FS Interneurons of *Bsn* Mutant Mice

We have previously observed that, after HFS, FS interneurons recorded from *Bsn* mutant mice show an NMDA-dependent short-term increase in synaptic strength (Ghiglieri *et al*, 2009). In the present experiments, intracellular recordings of FS interneurons recorded from corticostriatal slices of *Bsn* mutants showed that synaptic plasticity was completely blocked by a 20 min bath *in vitro* application of k252a (200 nM) ($p<0.01$, 5 min, $p<0.05$, 10 min after the tetanus, *Bsn* mutant + k252a *vs Bsn* mutant) (Figure 2a). We also tested whether mutants receiving subchronic k252a and showing normal procedural learning performances also showed a reversal of short-term potentiation in FS interneurons. As expected, after *in vivo* TrkB antagonism, the plasticity was no longer expressed by FS interneurons of epileptic-treated mice ($p<0.001$, 5 min, $p<0.01$, 10 min after the tetanus, *Bsn* mutant + vehicle *vs Bsn* mutant + k252a) (Figure 2b).

Synaptic Trkb Levels and PV/BDNF Colocalization are Increased in the Epileptic Striatum

To measure the BDNF abundance in the striatum, with particular focus on FS interneurons, we performed an immunoblot quantification of TrkB levels and a double PV/BDNF and Calb/BDNF immunohistochemistry in the dorsolateral striatum. Levels of TrkB were quantified by western blot analysis of striatal samples obtained from wild-type and *Bsn* mutant mice. Interestingly, while the TrkB expression in striatal homogenates obtained from mutant mice was similar to wild-types (97 ± 6.8% of wild-type level), the amount of TrkB in the PSD-enriched fraction (TIF) of mutant striata was 1.5-fold higher (156 ± 6.8% of wild-type level, $p<0.01$) (Figure 3). Increase in PSD association of TrkB suggests that changes in BDNF/TrkB signaling pathway may also occur in the epileptic striatum.

Comparison of striatal PV/BDNF-positive cells quantification between the two genotypes showed a significantly higher PV/BDNF-colocalization in *Bsn* mutant mice than observed in wild-type littermates ($p<0.01$) (Figures 4a and b). Interestingly, the BDNF immunoreactivity was not changed in the MS neurons of epileptic mice, as striatal Calb/BDNF colocalization was similar to wild-types. These findings indicate that, in physiologic conditions, BDNF protein is found preferentially in association with MS neurons rather than with FS interneurons, while in the epileptic condition this trend is reversed (Figure 4b, see also Figure 5a). The increased BDNF localization in PV-positive interneurons in the mutant mice may account for the observed short-term plasticity in this neuronal subtype.

Chronic Antiepileptic Treatment with VPA Rebalances TrkB Levels and Restores BDNF Distribution Between MS Neurons and FS Interneurons

Chronic exposure to VPA, a broad-spectrum AED, has been reported to prevent the occurrence of short-term plasticity

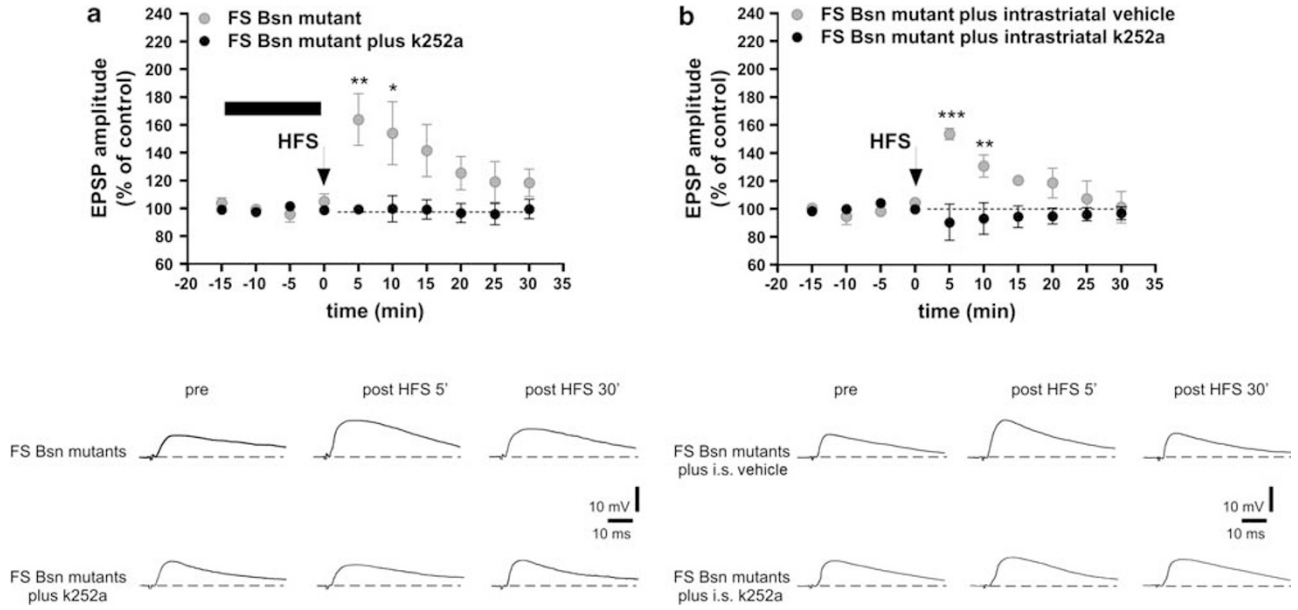

Figure 2 TrkB-dependent short-term plasticity in FS interneurons of *Bsn* mutant mice. (a) High-frequency stimulation (HFS, arrow) of corticostriatal pathway induced a synaptic potentiation that lasted 10–15 min in FS interneurons recorded intracellularly from corticostriatal slices of *Bsn* mutant mice. Conversely, 20-min bath application of the TrkB receptor antagonist k252a (200 nM), fully blocked the short-term potentiation (**$p<0.01$, 5 min, *$p<0.05$, 10 min after the tetanus, *Bsn* mutant + k252a *vs Bsn* mutant, $n=6$). Bottom panels show example traces of excitatory postsynaptic potential (EPSP) from FS interneuron recorded in *Bsn* mutants corticostriatal slices (upper traces) and *Bsn* mutant slices with application of k252a (lower traces). (b) EPSP amplitudes of FS interneurons recorded *ex vivo* from corticostriatal slices of mice that received intrastriatal administration of k252a (20 μM) or vehicle are shown in the upper panel. Similarly to what observed in naïve mutants, HFS was able to induce a short-term potentiation in mutants injected with vehicle but not in epileptic mice that received intrastriatal k252a (***$p<0.001$, 5 min, **$p<0.01$, 10 min after the tetanus, *Bsn* mutant + vehicle *vs Bsn* mutant + k252a, $n=5$). Traces on the bottom represent examples of EPSP of FS interneurons recorded corticostriatal slices from vehicle-injected *Bsn* mutants (upper traces) and *Bsn* mutant that received intrastriatal administration of k252a (lower traces).

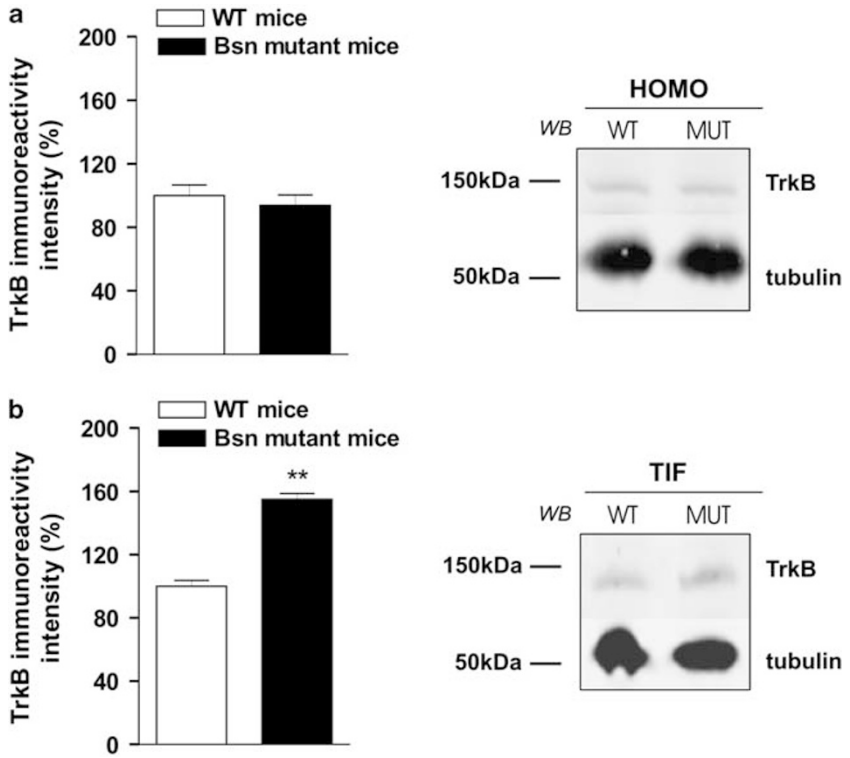

Figure 3 Striatal TrkB levels in *Bsn* mutant mice. Histogram shows the quantification of results of western blotting (WB) performed in homogenates (HOMO) (panel a) and Triton-insoluble fractions (TIF) (panel b) ($p > 0.05$, HOMO; $^{**}p < 0.01$ TIF). Representative immunoblots of striatal homogenates and TIFs from striata of wild-type (WT) and *Bsn* mutant mice are shown on the right of panels (a) and (b), respectively. The same amount of protein was loaded per lane, and tubulin was used as loading control in both homogenates and TIF fractions.

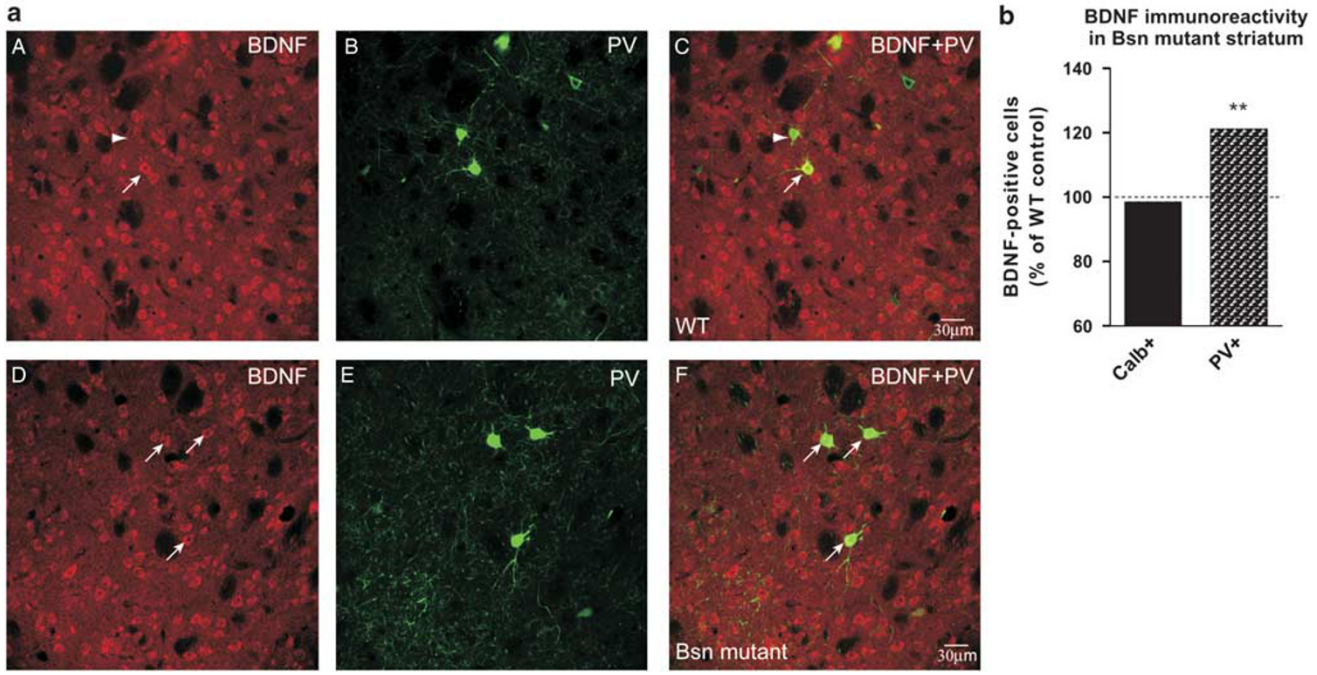

Figure 4 Striatal BDNF distribution in epileptic mice. (a) Comparison of striatal PV/BDNF and Calb/BDNF-positive cells quantification between the two genotypes showed a higher number of FS interneurons expressing BDNF protein in *Bsn* mutants than observed in wild-type controls ($^{**}p < 0.01$). (b) Confocal laser scanning microscopy (CLSM) images show double-labeled immunofluorescence for BDNF and PV in wild-type (A–B–C) and *Bsn* mutant (D–E–F) mice. BDNF immunolabeling is visualized in red-cy3 fluorescence and PV is visualized in green-cy2 fluorescence. The merged image is shown as a yellow fluorescence, which coincides with the colocalization between BDNF and PV. The arrows show the colocalization of PV-positive interneurons with BDNF. The arrowheads show the no BDNF-containing PV interneurons.

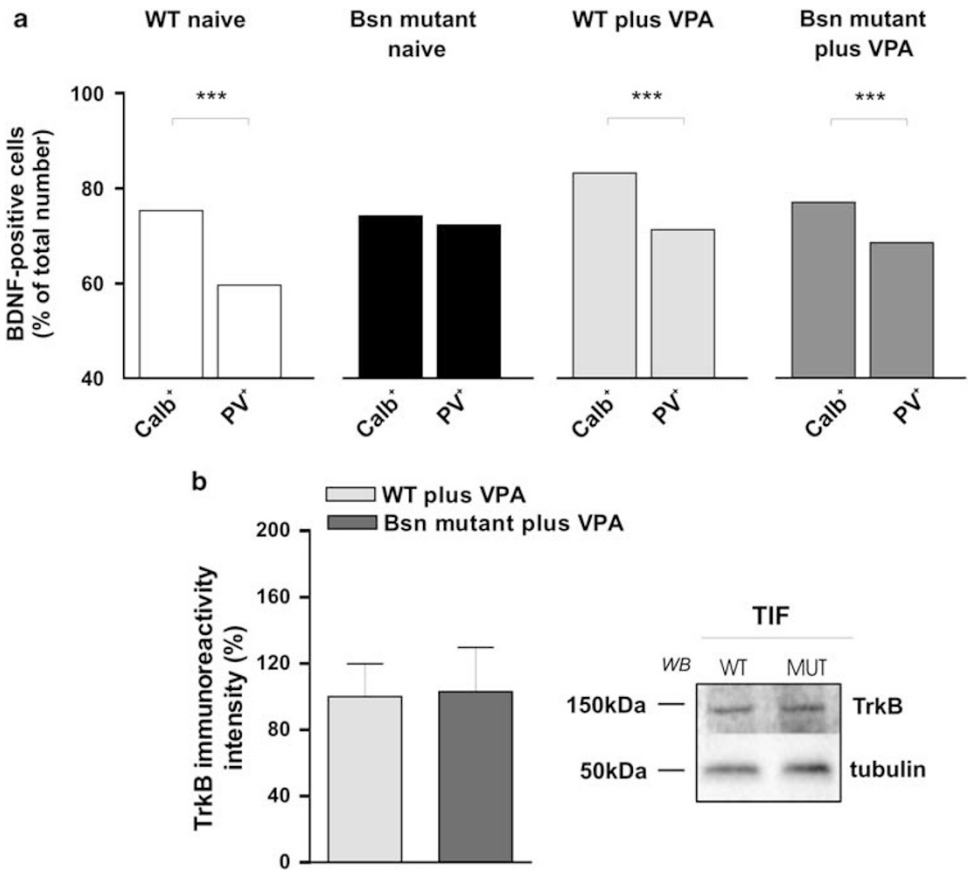

Figure 5 VPA effects on striatal BDNF distribution pattern. (a) The histograms show the percent of BDNF-positive FS interneurons and MS neurons in the striatum of naïve and VPA-treated wild types and *Bsn* mutants. Although in wild-type mice, the proportion of MS Calb-positive neurons that express BDNF protein was higher than the relative number of PV/BDNF-positive interneurons (***$p<0.001$), in epileptic mice the immunoreactivity of BDNF was similar in both neuronal subtypes. Chronic early treatment with VPA restored the ratio between PV/BDNF and Calb/BDNF cells in the striatum of epileptic mice (***$p<0.001$) to control levels. Early exposure to VPA in wild types increased the neurotrophin immunoreactivity in both neuronal subtypes, preserving the ratio between BDNF-positive MS neurons and FS interneurons that was similar to the one observed in naïve controls (***$p<0.001$). (b) After chronic treatment with VPA synaptic levels of striatal TrkB were similar in both *Bsn* mutant and wild-type mice ($p>0.05$). Histograms show the quantification of western blotting performed in TIF fractions.

in FS interneurons of *Bsn* mice by reducing the frequency of the epileptic seizures (Ghiglieri *et al*, 2009). To clarify whether changes of BDNF distribution may depend on chronic seizure activity, we analyzed the expression of BDNF protein into PV-positive (FS interneurons) and calb-positive (MS neurons) cells by means of double-label immunofluorescence in mice receiving chronic antiepileptic treatment. VPA (400 mg/kg/die) was given orally to heterozygous females, to treat pups by maternal breast milk, from postnatal day (P)0 to weaning (P21). Treatment was then extended into adulthood until the day of killing of wild-type and mutant mice.

Our results show that while the mutant condition was associated with a similar BDNF immunoreactivity in FS interneurons and MS neurons, because of increased colocalization with PV, early treatment of epileptic mice with chronic VPA was able to restore the ratio of BDNF distribution between the two neuronal subtypes ($p<0.001$) (Figure 5a, last graph on the right). In wild-type mice, although VPA exerted an increased protein expression in both PV and Calb, the pattern of BDNF distribution was preserved as the number of MS neurons found positive to BDNF was significantly higher than the number of PV-positive interneurons colocalizing with the neurotrophin ($p<0.001$) (Figure 5a, third graph). In line with this, after early chronic VPA treatment striatal synaptic levels of TrkB were similar in the two genotypes (Figure 5b).

DISCUSSION

The main finding of this study is that changes of TrkB/BDNF system underlie striatal behavioral and plastic alterations observed in epileptic mice lacking the presynaptic protein Bassoon.

In *Bsn* mutants, striatal neuronal subtypes are differently sensitive to recurrent paroxysmal activity (Ghiglieri *et al*, 2009). However, the behavioral correlates of neuronal abnormalities in this model of epilepsy have not been further characterized. In this study, we have shown that such reorganization of plasticity between MS neurons and FS interneurons was associated with an abnormal enhancement of procedural learning in epileptic mice. The first issue that arises from these data concerns the behavioral correlate of striatal plasticity. In *Bsn* mice, in fact, frequent cortical seizures alter synaptic plasticity of striatal MS

neurons and NMDA receptor subunit composition while striatal FS interneurons are more abundant and express an adaptive form of NMDA-dependent plasticity (Ghiglieri *et al*, 2009). In this scenario, given that MS projecting neurons constitute the large majority of neuronal cells in the striatum, one would expect to observe poor procedural learning performances in mutants rather than increase in conditioned responses. One possible explanation of this discrepancy might be that the enhanced feedforward inhibition, because of FS interneuronal plasticity, may somehow result in an improvement of striatal functions. This advantage may compensate the hippocampal deficit that characterizes this model. In fact, it has been shown that *Bsn* mutants show impaired synaptic functions in the hippocampus, that is, lower excitability, probably because of a reduced number of active excitatory synapses (Altrock *et al*, 2003). This is associated with a decrease in LTP and an impaired acquisition of hippocampus-dependent non-spatial memory (Sgobio *et al*, 2010). Under these conditions, it is possible that competitive roles of corticostriatal and hippocampal systems in learning behaviors may occur according to converging evidences from experimental and clinical studies (Gabrieli, 1995; McDonald and White, 1993; Packard and McGaugh, 1996; Packard and White, 1991). In particular, in animals in which hippocampal functions are compromised, procedural learning, which relies on striatal memory system, is improved and a competition of the two hippocampal and striatal memory systems may become evident when activated simultaneously to provide a correct learned solution in a specific task (Poldrack and Packard, 2003). Thus, the enhancement of procedural learning performances in this epileptic model can be interpreted as a result of the imbalance between striatal and hippocampal memory systems. The first interesting aspect of this study is that changes in BDNF/TrkB system seem to underlie neuronal modifications leading to altered behavioral response. In fact, *in vivo* intracerebral subchronic administration of TrkB receptor inhibitor was able to reset striatal-dependent learning to control conditions. Interestingly, *ex vivo* intracellular recordings, showed that abnormal plasticity of FS interneurons was absent in corticostriatal slices obtained from *Bsn* mutants that received intrastriatal k252a. Consistent with these results, *in vitro* acute application of k252a on corticostriatal slices obtained from epileptic mice was sufficient to inhibit the induction of pathologic short-term plasticity in FS GABAergic interneurons. Along this line, if plasticity of FS interneurons relies on TrkB receptor activation one would expect that in epileptic condition BDNF levels may also be changed in this striatal population. To clarify this point, we performed an immunohistochemical study of BDNF distribution in the dorsolateral striatum of epileptic mice. Our results revealed that while in control mice the neurotrophin is more abundant in MS neurons than in FS interneurons, in epileptic condition the relative amount of FS PV-positive interneurons colocalizing with BDNF was significantly higher than observed in wild-type mice, suggesting that a specificity of action of BDNF on FS interneurons may subsist.

Consistent with the idea that striatal neuronal subtypes are differently sensitive to recurrent paroxysmal activity, these data seem to suggest that BDNF preferentially interacts with TrkB receptors expressed on FS interneurons. Cell-type specificity is a common feature for neurotrophins, since effectiveness of BDNF is critically influenced by neuronal activity and synaptic potentiation induced by BDNF can be selective for active synapses. Accordingly, BDNF exerts opposite effects on cortical pyramidal neurons and GABAergic interneurons, depending on the level of neuronal activity (Rutherford *et al*, 1998). Moreover, several studies pointed out to the specific role of BDNF in the differentiation of GABAergic terminals (Bolton *et al*, 2000) and in particular on the development and maturation of GABAergic PV-positive interneurons (Berghuis *et al*, 2004). In this study, we have provided the first evidence that synaptic levels of TrkB are higher in the striatum of epileptic mice supporting the idea that also BDNF levels may increase during epilepsy as observed for other brain areas subjected to chronic seizure activity. In the striatum, low concentrations of BDNF, through its high-affinity receptor TrkB, activate different calcium-dependent intracellular pathways in a very precise mode, resulting in accurate tuning of MS neuronal activity. It is then possible that in these epileptic mice excessive discharge activity during early cortical seizures may induce increases of BDNF levels. Consequently, an alternative pattern of BDNF distribution may be established to favor an enhancement of inhibitory control within the striatum as adaptive mechanism. On this view, the increased presence of the neurotrophin at interneuronal level may account for the emergence of plasticity in FS interneurons with the concomitant reduction in LTP observed in the projecting neurons (Ghiglieri *et al*, 2009). Such adaptive response may protect MS neurons from excessive activity and preserve striatal functions. Our results are in line with a recent report showing that BDNF preferentially stimulates active synapses (Nagappan and Lu, 2005). In fact, in epileptic mice, while MS neurons show reduced LTP, FS interneurons, which are normally able to sustain higher neuronal activities, show a form of synaptic plasticity that critically requires the activation of NMDA receptor (Ghiglieri *et al*, 2009). It is then possible that this particular feature makes FS interneurons a preferential target for the neurotrophin. Early treatment of *Bsn* mutants with chronic VPA, by reducing seizures, rescues LTP in MS neurons and prevents the induction of adaptive plasticity in FS interneurons (Ghiglieri *et al*, 2009). However, the mechanism by which the reduction of seizures may induce a rebalance of synaptic plasticity has been difficult to explore. In this study, we tested the hypothesis that early exposure to AED might rebalance TrkB levels and BDNF distribution to normalize the physiological synaptic plasticity within the striatal MS neurons and FS interneurons. Our results show that, after chronic antiepileptic treatment, mutant mice showed a more physiological distribution of striatal BDNF protein between MS neurons and FS interneurons, similar to the one observed in wild-type controls. These data suggest that, by decreasing the frequency of seizures, VPA may also restore striatal synaptic TrkB and BDNF to control levels bringing to a physiological action of BDNF. The reduced effect of BDNF on FS interneurons may then clarify the restorative effect of chronic VPA on striatal synaptic plasticity (Ghiglieri *et al*, 2009). This study provides a first indication that BDNF is involved in the development of

striatal alterations underlying in the early-onset epileptic syndrome associated with the absence of presynaptic protein Bassoon. *In vivo* direct or indirect interferences on TrkB/BDNF system by either inhibition of TrkB tyrosine kinases or chronic VPA treatment showed that behavioral alterations of mutants are paralleled by higher synaptic TrkB levels, emergence of interneuronal plasticity and increased BDNF immunoreactivity at interneuronal level. These evidences clearly suggest that in epileptic mice profound alterations of TrkB/BDNF levels occur, leading to a pathological redistribution of BDNF between the two neuronal subtypes. Although such adaptive response may protect MS neurons from excessive activity and originally preserve striatal functions, alterations of TrkB/BDNF system may lead to pathological actions of BDNF underlying recurrence of seizures. Early treatment with VPA, able to limit the seizures, prevents this cell-type-specific alteration and restores striatal plasticity to control levels. These effects argue for a strong association between TrkB/BDNF changes and altered corticostriatal plasticity. At present it is, however, unclear whether *Bsn* deficiency directly affects BDNF secretion. Moreover, given the complex role of growth factors in the induction and maintenance of long-lasting adaptive changes, the question whether BDNF alterations are causative of epilepsy rather than being a result of recurring seizures is far from being resolved. It is worth mentioning that because of its involvement in activity-dependent synaptic plasticity BDNF has been recently suggested as a new therapeutic target in the treatment of other neurological disorders, such as neurodegenerative diseases (Zuccato and Cattaneo, 2009), drug abuse (Russo *et al*, 2009), and depression (Castren *et al*, 2007). Interestingly, all these pathologies share the common feature of maladaptive learning. In particular, recent evidences have suggested that abnormal regional-specific BDNF expression contributes to the functional defects observed in drug addiction (Fumagalli *et al*, 2007) and in depression (Molteni *et al*, 2009). In this scenario, our data shed new light on the complex role of BDNF in epilepsy and within the striatal microcircuit. However, additional studies are required to unravel these interesting effects of BDNF regulation on striatal activity.

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DISCLOSURE

The authors declare no conflict of interest.

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