Alpha2delta ligands act as positive modulators of adult hippocampal neurogenesis and prevent depressive-like behavior induced by chronic restraint stress.

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List of non-standard abbreviations:

α2δ, alpha2delta;
NPC, neural progenitor cells;
MDD, Major Depressive Disorder;
GAD, generalized anxiety disorder;
GBP, gabapentin;
PGB, pregabalin;
VGCC, voltage-gated calcium channels;
D.I.V., days in vitro;
PFA, paraformaldehyde;
Sox-2, SRY-related HMG-box gene 2;
MAP-2, Microtubule Associated Protein-2;
mpk, mg/kg;
BrdU, bromodeoxyuridine;
GFAP, Glial Fibrillary Acidic Protein;
NeuN, Neuronal Nuclei;
TST, Tail Suspension Test;
FST, Forced Swim Test;
EGF, Epidermal Growth Factor;
bFGF, basic Fibroblast Growth Factor;
SGZ, subgranular zone;
GCL, granular cell layer;
DG, dentate gyrus;
IKK2, IκB kinase 2;
PTSD, Posttraumatic Stress Disorder.
ABSTRACT

Although the role of adult hippocampal neurogenesis remains to be fully elucidated, several studies suggest that the process is involved in cognitive and emotional functions and deregulated in various neuropsychiatric disorders, including major depression. Interestingly, several psychoactive drugs, including antidepressants, can modulate adult neurogenesis. Here we show for the first time that the alpha2delta (α2δ) ligands gabapentin [1-(aminomethyl)cyclohexanecetic acid; GBP] and pregabalin [S-[+]3-isobutylGABA or (S)-3-(aminomethyl)-5-methylhexanoic acid; PGB] can produce a concentration-dependent increase in the number of newborn mature and immature neurons generated in vitro from adult hippocampal neural progenitor cells (NPC), and, in parallel, a decrease in the number of undifferentiated precursor cells. These effects were confirmed in vivo, since a significantly increased number of adult generated neurons was observed in the hippocampal region of mice chronically treated with PGB [10 mg/kg, i.p., 21 days] compared to vehicle-treated mice. Moreover, we demonstrated that PGB administration prevented the appearance of depression-like behaviours induced by chronic restraint stress and, in parallel, promoted hippocampal neurogenesis in adult stressed mice. Finally, we provided data suggesting the potential involvement of the α2δ1 subunit and NF-κB signaling pathway in the drug-mediated proneurogenic effects. The new pharmacological activities of α2δ ligands may help explaining their therapeutic activity as add-on therapy in major depression and on depressive symptoms in posttraumatic stress disorder and generalized anxiety disorders. Furthermore these data contribute to the identification of novel molecular pathways which may represent potential targets for pharmacological modulation in depression.
INTRODUCTION

A vast array of experimental work has established that new neurons are generated throughout the entire life in the hippocampus and the subventricular zone of the adult brain of mammals, including humans (Alvarez-Buylla et al., 2001; Kempermann, 2008; Kriegstein and Alvarez-Buylla, 2009; Ming and Song, 2011).

Although the role of adult hippocampal neurogenesis remains yet to be fully elucidated, several studies suggest that the process is involved in cognitive and emotional functions (Deng et al., 2010; Aimone et al., 2011; Couillard-Despres et al., 2011; Sahay et al., 2011) and deregulated in various neuropsychiatric disorders (Decarolis and Eisch, 2010; Samuel and Hen, 2011; Lazarov et al., 2010). Based on the evidence that hippocampal neurogenesis can be downregulated under stressful conditions, including those that result in animal models of depressive-like behaviors, and it can be upregulated by antidepressant drugs and treatments, the hypothesis has emerged that neurogenesis, together with other related aspects of hippocampal plasticity, may contribute to the pathophysiology of the major depressive disorder (MDD) and its effective treatment (Malberg and Blendy, 2005; Pittenger and Duman, 2008; Hanson, et al., 2011). In particular, several authors have suggested that neurogenesis may be necessary for some, although not all, of the behavioural effects of antidepressants (Santarelli et al., 2003; David et al., 2009). Recently, Boldrini et al. (2009) have demonstrated that antidepressants increase neural progenitors in hippocampi of depressed patients.

PGB and GBP are anticonvulsant, analgesic, and anxiolytic drugs whose effects have also been demonstrated in several preclinical models (Sills, 2006; Taylor et al., 2007). Although multiple sites and modes of action have been proposed for GBP and PGB, at present one mechanism is considered primary for their clinical efficacy, namely high affinity drug interaction with the
α2δ1/2 subunits of voltage-gated calcium channels (VGCC) (Bian et al., 2006; Gee et al., 1996). Studies have demonstrated that drug binding to the α2δ1 subunit is necessary for its antihyperalgesic effects in a preclinical model of pain (Field et al., 2006) as well as for its anxiolytic-like effects (Lotarski et al., 2011) in rodents.

Here we provide evidence, for the first time, that, like classical antidepressants, α2δ ligands, and in particular PGB, can elicit positive modulation of adult hippocampal neurogenesis both in vitro and in vivo. Moreover, chronic PGB administration results in prevention of depressive-like behavior and promotion of hippocampal neurogenesis in adult mice subjected to chronic restraint stress. Finally, based on in vitro data, we propose that the proneurogenic effects of PGB are mediated by interaction with the α2δ subunit and activation of the NF-κB signaling pathway, which has been recently involved in regulation of adult neurogenesis both in vitro and in vivo (Denis-Donini et al., 2005; Rolls et al., 2007; Denis-Donini et al., 2008; Koo et al., 2010; Meneghini et al., 2010).
MATERIALS AND METHODS

Animals. Adult (4-6 month-old) male CD1 and C57BL/6J mice were purchased from Charles River Laboratories (Calco, Italy). All animals were maintained in high-efficiency particulate air (HEPA)-filtered Thoren units (Thoren Caging System) at the University of Piemonte Orientale animal facility and, kept in number of 3-4/cage, had unlimited access to water and food. Care and handling of animals were performed in accordance with the NIH guidelines and also reviewed and approved by the local IACUC.

Drugs. Pregabalin was purchased from Qventas (Branford, Connecticut), gabapentin, L-(-)-isoleucine and L-(-)-α-phenylglycine from Sigma Aldrich (Milan, Italy). For in vitro experiments all drugs were dissolved in sterile water, while for in vivo treatments pregabalin was dissolved in saline.

Isolation and Cultures of Adult Hippocampal Neurospheres. For each neurosphere preparation, the brains from three adult (4-6 month-old) male mice were dissected. Hippocampi were isolated under a dissecting microscope and cell suspension was prepared as previously described (Meneghini et al., 2010). Primary (Passage 1, P1) neurospheres were dissociated after 7-9 days in vitro (D.I.V.), whereas P2-P16 neurospheres every 5 D.I.V. At each passage cells were plated in T25 flask at a density of 12,000 cells/cm² in growing medium. P3-P16 neurospheres were utilized for experiments.
Neural Progenitor Cell Differentiation and Proliferation. For NPC differentiation, detailed procedure was previously described (Meneghini et al., 2010). NPC were treated in the presence of indicated concentrations of drugs or vehicle for 24 h. For neutralization of PGB and GBP effects, NPC were pre-treated for 60 min, before addition of \( \alpha2\delta \) ligands, with L-isoleucine, L-\((+)-\alpha\)-phenylglycine or with vehicle. For NF-\(\kappa\)B inhibition, 10 \( \mu \)g/ml NF-\(\kappa\)B SN-50 or NF-\(\kappa\)B SN-50M peptide, 3 \( \mu \)M JSH-23, 3 \( \mu \)M SC-514 (Calbiochem-Merck KGaA, Darmstadt, Germany) were added to NPC culture medium 60 min before \( \alpha2\delta \) ligand or vehicle addition. After 24 h, cells were washed in PBS and fixed with ice-cold 4\% PFA for 20 min at RT for subsequent immunofluorescence analysis. In each experiment, 5 fields/well (corresponding to 100-150 cells/well) were counted using the fluorescence microscope ECLIPSE E600 (NIKON, Calenzano, Italy) with a X60 objective. All experiments were run in triplicates, using different cell preparations and repeated at least three times. Data represent the mean value ± standard deviation (S.D.). In parallel with differentiation analysis, necrotic and apoptotic rates in culture were also evaluated in each experiment, as previously described (Meneghini et al., 2010). For evaluation of cell proliferation, dissociated NPC were plated at a density of 10,000 cells/well onto LuminNUNC F96 MicroWell (Wiesbaden, Germany) in growing medium (Neurobasal-A medium containing B27 supplement, 2 mM L-glutamine, 20 ng/ml EGF and 100 U/100 \( \mu \)g/ml Penicillin/Streptomycin), and in presence of 1 nM PGB or vehicle for 6-96 h. Proliferation rate was determined by using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Milan, Italy), according to manufacturer instructions. All experiments were run in triplicates and data, expressed as counts per second (CPS), represent the mean value ± S.D.
Immunocytochemical Analysis. Adult mouse neurospheres were harvested onto super-frost microscope slides (Menzel-Glaser, Braunschweig, Germany) by cytospin centrifugation (235g x 5 min) (Thermo-Shandon Scientific, Waltham, Massachusetts) and fixed with ice-cold methanol (Sigma Aldrich) for 10 min at -20°C, for subsequent immunofluorescence analysis. The following primary antibodies were utilized: mouse anti-Ca₃δ₁ antibody (1:500, Alomone Labs, Jerusalem, Israel), chicken anti-nestin polyclonal (1:4,000, Neuromics, Edina, Minnesota), rabbit anti-SRY-related HMG-box gene 2 (Sox-2) (1:500, Chemicon, Temecula, California). After fixation, neurosphere-derived differentiated cells were incubated with antibodies against Microtubule Associated Protein-2 (MAP-2) (rabbit polyclonal, 1:600, Chemicon), nestin (mouse monoclonal, 1:1,200, Abcam, Cambridge, Massachusetts; or chicken monoclonal, 1:4,000, Neuromics). Secondary antibodies were as follows: Alexa Fluor 488-conjugated goat anti-mouse (1:1,600, Molecular Probes, Eugene, Oregon), Alexa Fluor 555-conjugated goat anti-rabbit (1:1,400, Molecular Probes) and Alexa Fluor 488-conjugated goat anti-chicken antibody (1:1,400, Molecular Probes). Nuclei were counterstained with Draq5 (1:2,000, Alexis Biochemicals, San Diego, California), diluted in PBS. Slides were coverslipped with Fluorescent Mounting Medium (DakoCytomation, Glostrup, Denmark) as anti-fading agent. Adobe Photoshop CS (Adobe Systems Inc., San Jose, California) was used for digital processing of the images. Only light intensity, brightness, and contrast adjustments were applied to improve information.

Protein Isolation and Western Blot Analysis. For protein isolation, neurosphere and tissue extracts were prepared as previously described (Meneghini et al., 2010). Immunoblots were carried out overnight in an antibody solution containing 3% (wt/vol) BSA in TBS-tween 0.1%
with the primary antibody anti-Ca,\(\alpha_{2}\delta_{1}\) (1:1,500, Alomone Labs). After washing, blots were incubated with peroxidase-conjugated goat anti-mouse antibody (1:10,000, Bio-Rad, Hercules, California) for 60 min at RT and the immunocomplexes were visualised by the Supersignal West Pico Chemiluminescent substrate (Pierce, Rockford, Illinois). Densitometric analysis was performed using the Quantity One software system (Bio-Rad Laboratories) and each band was normalized to the \(\alpha\)-tubulin signal (mouse monoclonal, 1:2,000, Sigma Aldrich) in each lane.

**In vivo Neurogenesis Studies.** Adult male CD1 mice (3-4 month-old) were randomly distributed into either vehicle and PGB treatment groups \((n = 6/group)\). PGB [1 mg/kg or 10 mg/kg body weight (mpk)] and corresponding vehicle (saline) were administered intraperitoneally (i.p), once daily, for twenty-one days. For the first five days of treatment, mice were also given a daily dose of bromodeoxyuridine (BrdU; 150 mpk, Sigma Aldrich, i.p). Twenty-one days after the last BrdU administration, mice were transcardially perfused. Brain tissue was prepared for subsequent analysis as previously described (Denis-Donini et al., 2008). The following primary antibodies were utilized: rat monoclonal anti-BrdU (1:200; Novus Biologicals, Littleton, Colorado); goat anti-Glial Fibrillary Acidic Protein (GFAP) (1:100; Santa Cruz Biotechnology, Santa Cruz, California); mouse anti-Neuronal Nuclei (NeuN) (1:150; Millipore, Billerica, Massachusetts). For quantification and phenotypic characterization of newborn cells, a modified unbiased stereology protocol was used, as previously described (Denis-Donini et al., 2008).

**Unpredictable Chronic Restraint Stress and PGB treatment.** Male adult (3 mo-old) C57BL/6J mice were subjected to restraint \((n = 12)\) by individually placing them in well-ventilated polypropylene-tubes (internal diameter 3 cm, length 11.5 cm). The test duration was 3h daily for
twenty-one days, and administration time changed every day so to be unpredictable. During immobilization stress mice did not have access to food and water. After restraint stress administration, mice were returned to their home cage environment. The unstressed controls (n = 6) were left undisturbed in their home cage for the entire duration of the stress experimental procedure. One observer who was not aware of the stress procedure or drug administration performed the behavioural tests. In the PGB chronic administration experiments, behavioural tests were performed 24 h after the last drug (or vehicle) injection. In the acute PGB experiments, tests were performed two hours after drug (or vehicle) injection, a time point described to correspond (Lotarski et al., 2011) to peak behavioral effects. The first five days of the experimental procedure, animals were administered BrdU (150 mpk i.p., once daily). Thirty days after the last BrdU injection and fourteen days after the last restraint, mice were transcardially perfused and their brains immunoprocessed for double BrdU/NeuN labelling, as previously described (Denis-Donini et al, 2008).

**Tail Suspension Test.** Duration of immobility was measured while each mouse was suspended by the tail in a 6-minute trial. Immobility was defined as hanging without struggling or attempting to climb. Because no changes over time were measured, the results are reported as a total duration of immobility out of the last 4 minute trial.

**Forced Swim Test.** Mice were individually placed into a transparent glass cylinder (13 cm diameter, 24 cm height), filled with 11.5 cm warm water (22-23°C). Immobility was defined as making only those movements necessary to keep the head above water. Immobility was measured during the last 4 min of a 6 min test session. After the swim session, mice were rapidly dried and placed back to their home cages.
Statistical analysis. Data were reported as mean ± S.D. or ± S.E.M, of at least three experiments and analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test or by Student’s t-test. Statistical significance level was set for p values less than 0.05.

RESULTS

GBP and PGB Effects on the Differentiation of Adult Mouse Neural Progenitor Cells. In our experimental setting adult murine NPC are grown in presence of Epidermal Growth Factor (EGF) and basic Fibroblast Growth Factor (bFGF) as floating neurospheres. Under these conditions, cells are phenotypically characterized by the expression of well-recognized markers of undifferentiated neural progenitors such as nestin and Sox-2, as shown in Fig. 1A, and by the absence of markers of committed neuronal, glia or oligodendrocyte markers (Meneghini et al., 2010). Upon removal of growth factors from the medium, NPC stop dividing and differentiate. By double immunolabelling with antibodies against nestin and MAP-2, we are able to evaluate in vitro different stages of neuronal differentiation of NPC. As shown in Fig. 1B, after 24 hours, hippocampus-derived NPC give rise to: a subpopulation of MAP-2+/nestin− cells (3.9 ± 0.6 % of total) that we regard as newly generated neurons; MAP-2+/nestin+ cells (32.7 ± 2.0 % of total), which we consider as a population of cells in a transition phase towards neuronal commitment; a small population of MAP-2/nestin+ undifferentiated cells (6.8 ± 0.7 % of total), indistinguishable from neurosphere-composing progenitors, and a large population (56.6 ± 2.4 % of total) of MAP-2/nestin− cells. Double negative cells still express Sox-2, marker of undifferentiated progenitors (Brazel et al, 2005), and mainly generate GFAP+ cells and NG2+ oligodendrocyte precursors (data not shown). Under these experimental conditions, we evaluated the effects of PGB and GBP, in a concentration range compatible with their binding affinities at the α2δ subunit, on
neuronal differentiation of adult mouse hippocampal NPC. Both drugs exhibited a remarkable ability to promote neuronal differentiation of adult NPC. In particular, when hippocampal NPC were incubated with GBP, we observed a concentration-dependent increase (ANOVA: \( p < 0.001 \)) in the percentage of MAP-2\(^+\)/nestin\(^-\) (Fig. 1C) mature neurons in comparison with vehicle, with a maximal increase elicited by 1 nM GBP (% increase over vehicle-treated cells: +154.6 ± 15.1; Student’s \( t \)-test: \( p < 0.001 \)). A smaller, but significant increase of MAP-2\(^+\)/nestin\(^+\) immature neurons, was produced by GBP at all tested concentrations (Fig. 1D). In parallel, compared to vehicle, the drug significantly decreased the percentage of MAP-2\(^-\)/nestin\(^-\) cells in a concentration-dependent manner (ANOVA: \( p < 0.001 \)) (Fig. 1F), with maximal inhibition elicited by 1 nM GBP (% decrease over vehicle-treated cells: -44.7 ± 6.4 %; Student’s \( t \)-test: \( p < 0.01 \)). The drug produced no effect on the MAP-2\(^-\)/nestin\(^+\) cell population (Fig. 1E).

We then evaluated PGB effects on hippocampal NPC neuronal differentiation. Similarly to GBP, the drug (0.1-10 nM) produced a dramatic concentration-dependent increase (ANOVA: \( p < 0.001 \)) both in the percentage of MAP-2\(^+\)/nestin\(^-\) (Fig. 1G) and of MAP-2\(^+\)/nestin\(^+\) (Fig. 1H) cells and a decrease in the number of MAP-2\(^-\)/nestin\(^-\) cells (Fig. 1J), compared to vehicle. In particular, maximal effects were elicited by 1 nM PGB (% change over vehicle-treated cells: +168.6 ± 15.9, +72.5 ± 4.3, -53.7 ± 2.9 for MAP-2\(^-\)/nestin\(^-\), MAP-2\(^+\)/nestin\(^+\) and MAP-2\(^-\)/nestin\(^-\) cell populations, respectively; Student’s \( t \)-test: \( p < 0.001 \)) (Fig. 1G, H, J). As demonstrated for GBP, PGB produced no effect on the MAP-2\(^-\)/nestin\(^+\) cell population (Fig. 1I). MAP-2 immunolabelling experiments revealed that cells treated with 1 nM GBP (Fig. 1L) and 1 nM PGB (Fig. 1M) increased neuritic arborizations, compared to vehicle-treated cells (Fig. 1K). We also demonstrated that, within the tested concentration range, neither GBP nor PGB affected survival of NPC and/or their progeny in our experimental conditions (\textit{data not shown}). Overall, these data suggested that GBP and PGB were endowed with the ability to promote neuronal differentiation.
of adult hippocampal NPC in vitro. To investigate whether α2δ ligands could also affect NPC proliferation, a time-course experiment was undertaken in the presence of vehicle or 1 nM PGB. As shown in Fig. 1N, no difference between vehicle and ligand-treated cells could be observed at any time point, suggesting that the drug does not affect cell proliferation of adult hippocampal neural progenitors.

**PGB Promotes Adult Hippocampal Neurogenesis in vivo.** The in vitro results prompted us to investigate if α2δ ligands had any effect on adult hippocampal neurogenesis in vivo. Adult male mice (n = 18) were injected i.p., once/daily, with vehicle (saline), 1 or 10 mpk PGB for a period of twenty-one days. During the first five days of treatment, mice were also administered with the thymidine analog BrdU (150 mpk, i.p), in order to label cells in S-phase. Twenty-one days after the last BrdU injection, mice were killed and their brain tissue analyzed for the presence and phenotype of BrdU+ cells in the hippocampus. The number of BrdU-labelled cells in the subgranular zone (SGZ) and in the granular cell layer (GCL) within the dentate gyrus (DG) of vehicle- and drug-treated mice was quantified by a modified unbiased stereology protocol as previously described (Denis-Donini et al., 2008). As shown in Fig. 2A, we observed no significant difference in the absolute number of surviving BrdU+ cells in the DG of mice treated with 1 and 10 mpk PGB compared to vehicle (number of BrdU+ cells in the DG: 2,218 ± 586, 2,082 ± 964, 2,266 ± 562 in vehicle-, 1 mpk and 10 mpk PGB-treated mice, respectively).

We then phenotypically characterized BrdU+ cells by performing a triple immunostaining experiment with antibodies raised against BrdU, NeuN (a marker of mature neurons) and GFAP (a marker of astrocytes). A total of one hundred BrdU+ cells were randomly selected from brain sections throughout the entire hippocampal DG extension in vehicle- and drug-treated animals. The percentage of BrdU+/NeuN+/GFAP+, BrdU+/NeuN+/GFAP, BrdU+/NeuN/GFAP+ cells was
calculated. Compared to vehicle-treated animals, the number of BrdU+/NeuN-/GFAP- cells was significantly reduced in the DG of mice chronically treated with 10 mpk PGB (53 ± 9.8% and 35.7 ± 1.5% in vehicle and PGB-treated animals, respectively; Student’s t-test: \( p < 0.01 \)) (Fig. 2B, C-F). A small but statistically not significant reduction was also observed in mice treated with 1 mpk PGB (Fig. 2B; 53 ± 9.8% and 43 ± 2% in vehicle- and PGB-treated animal, respectively; Student’s t-test: \( p = 0.159 \)). In parallel, the percentage of newly generated BrdU+/NeuN+/GFAP- neurons increased in 10 mpk PGB-treated animals compared to vehicle (37.3 ± 7.6 % and 52 ± 4.4 % in vehicle- and PGB-treated animals, respectively; Student’s t-test: \( p < 0.05 \)) (Fig. 2B, C-F). Again, a small but statistically not significant increase in the percentage of new neurons was also observed in mice treated with 1 mpk PGB (Fig. 2B; 44.7 ± 4.7 %; Student’s t-test: \( p = 0.230 \)). Finally, the percentage of BrdU+/NeuN-/GFAP+ cells was not affected by PGB treatment (9.7 ± 5.5, 12.3 ± 2.9, 12.3 ± 4.2 % in vehicle-, 1 and 10 mpk PGB-treated animals, respectively) (Fig. 2B).

Overall, in vivo data suggested that chronic administration of 10 mpk PGB resulted in enhanced neurogenesis in the DG of adult mice. Conversely, no effect of drug treatment was reported on astrogliogenesis. In parallel, the number of undifferentiated BrdU+/NeuN-/GFAP- cells was significantly reduced in the DG of mice chronically treated with 10 mpk PGB, as expected for a drug which promotes neuronal differentiation of progenitor cells. Moreover, the absolute number of surviving BrdU+ cells was not affected by drug treatment, suggesting no effect on cell survival and/or proliferation, in line with data obtained in vitro.

The \( \alpha 2\delta 1 \) Subunit of Voltage-sensitive Calcium Channels Mediates the in vitro Proneurogenic Effects of PGB and GBP. In our experimental in vitro model, PGB and GBP are proneurogenic at nanomolar concentrations, consistent with drug binding affinities for the \( \alpha 2\delta 1 \) and \( \alpha 2\delta 2 \) subunits.
of neuronal VGCC (Lotarski et al., 2011). By using a commercially available antibody raised against an extracellular epitope at the N-terminus of α2δ1, we investigated the presence of the VGCC auxiliary subunit in hippocampal neurospheres. As shown in Fig. 3A, immunoreactivity was localized at the plasma membrane in the majority of cells within adult hippocampal neurospheres. These data were confirmed by Western blot analysis of hippocampus-derived neurosphere extract and of adult hippocampus extract, used as positive control (Fig. 3B). We then evaluated whether the α2δ antagonists L-isoleucine (Fig. 3C-E) (Brown et al., 1998) and L-(+)-α-phenylglycine (Fig. 3F-H) (Mortell et al, 2006) were able to counteract PGB proneurogenic effects on hippocampal NPC. When concentrations ranging from 3 to 100 nM were tested in presence of 1 nM PGB, both α2δ antagonists caused a concentration-dependent inhibition (ANOVA: p < 0.001) of drug-induced increase of MAP-2+/nestin- (Fig. 3C,F) and MAP-2+/nestin+ (Fig. 3D,G) cell populations, with complete inhibition obtained with 30 nM L-isoleucine and 30 nM L-(+)-α-phenylglycine. In parallel, both drugs produced a concentration-dependent inhibition (ANOVA: p < 0.001) of PGB-induced decrease of MAP-2+/nestin- cells, with complete inhibition obtained at 30 nM concentration (Fig. 3E,H). Both drugs had no effect alone (not shown). Altogether these results support the hypothesis that PGB effects on neuronal differentiation of adult hippocampal NPC were α2δ-mediated.

The NF-κB Pathway is Involved in PGB-mediated Effects on Neuronal Differentiation of Adult NPC. Nuclear translocation of several transcription factors, including members of the NF-κB family, has been involved in key steps of neuronal commitment and differentiation of adult NPC in vitro and in vivo (Denis-Donini et al., 2005, 2008; Rolls et al., 2007; Koo et al., 2010; Meneghini et al., 2010). Additionally, NF-κB-mediated signalling has also been suggested to lie
downstream α2δ1 subunit activation (Park et al., 2008). Since p50/p65 heterodimers and p50 homodimers are the most abundant dimeric species in adult mammalian brain, we investigated the potential involvement of NF-κB p50 and p65 proteins in mediating PGB and GBP effects in adult neural progenitors. We treated hippocampal NPC with SN50, a cell-permeable peptide that inhibits nuclear translocation of NF-κB p50, or with SN50M, an inactive peptide used as a negative control (Lin et al., 1995). SN50 (10 µg/ml) completely counteracted 1 nM PGB-induced increase of MAP-2+/nestin^- (Fig. 4A) and MAP-2+/nestin^+ (Fig. 4B) neurons and decrease of MAP-2^-/nestin^- cells (Fig. 4C). Conversely, SN50M (10 µg/ml) did not affect PGB-mediated effects (Fig. 4A-C). We then tested JSH-23, a cell-permeable, selective blocker of NF-κB p65 nuclear translocation (IC50= 7 μM) (Shin et al., 2004). Like SN-50, 3 μM JSH-23 completely counteracted the effect of 1 nM PGB on MAP-2^+/nestin^- (Fig. 4D), MAP-2^+/nestin^+ (Fig. 4E) and MAP-2^-/nestin^- cells (Fig. 4F). Finally, 3 μM SC-514, a selective and reversible inhibitor of IkB kinase 2 (IKK2) (IC50 = 3-12 μM) (Baxter et al., 2004), abolished 1 nM PGB-mediated increase of MAP-2^+/nestin^- (Fig. 4D) and MAP-2^+/nestin^+ cells (Fig. 4E) and decrease of MAP-2^-/nestin^- cells (Fig. 4F). When applied alone, SN-50, JSH-23, SC-514 did not affect NPC differentiation (Fig. 4A-F). Moreover at the tested concentrations, SN-50, JSH-23 and SC-514 had no effect on survival of adult NPC and/or their progeny (data not shown). Representative images of MAP-2 immunolabelling (Fig. 4G-J) confirmed that the effects of 1 nM PGB (Fig. 4G) on neuronal differentiation of hippocampal NPC were completely abolished in presence of 10 µg/ml SN-50 (Fig. 4H), 3 μM JSH-23 (Fig. 4I) and 3 μM SC-514 (Fig. 4J). Overall, these data strongly suggested the involvement of NF-κB signalling in the proneurogenic effects of PGB on adult hippocampal NPC.
Chronic PGB treatment prevents depressive-like behaviour and promotes hippocampal neurogenesis in stressed mice. Finally, we decided to determine whether a chronic PGB treatment could prevent appearance of depressive-like symptoms elicited by a chronic, unpredictable restraint stress period (21 days, 3h/day immobilization). Twelve C57BL/6 mice were subjected to the stress protocol with half of them receiving vehicle (saline) or PGB (10 mpk, i.p.), once/daily, for 21 days. Control unstressed group (n = 6) was subjected to i.p. injection of saline, once/daily. The first five days of the chronic stress procedure, all experimental groups were also administered with BrdU (150 mpk i.p.). At day 21, all groups were tested for immobility in the TST and FST paradigms. One-way ANOVA analysis between experimental groups revealed a significant difference both in the TST (p < 0.01) and FST (p < 0.001). As shown in Fig. 5A-B, a Tukey’s post hoc statistical analysis confirmed that there was a significant difference between PGB-treated and saline-treated stressed mice in both the TST (Fig. 5A) and FST (Fig. 5B). As expected, saline-treated stressed mice had a significantly increased immobility compared to saline-treated unstressed mice both in the TST (116 ± 18 s and 43.2 ± 14.4 s, respectively; p < 0.01, Fig. 5A) and in the FST (91.5 ± 8.3 s and 40 ± 8.7 s, respectively; p < 0.001, Fig. 5B). Moreover, in the TST, PGB-treated stressed mice displayed an immobility time significantly lower compared to saline-treated stressed mice (43.5 ± 12 and 116 ± 18 s, respectively; p < 0.05) (Fig. 5A). Similarly, in the FST, PGB-treated stressed mice spent less time in immobility compared to saline-treated stressed mice (34.5 ± 4.2 s and 91.5 ± 8.3 s, respectively; p < 0.001) (Fig. 5B).

Thirty days after the last BrdU injection and fourteen days after completion of the stress procedure, mice were transcardially perfused and their brains immunoprocessed for BrdU/NeuN labelling and assessment of hippocampal neurogenesis, as previously described (Denis-Donini et
al, 2008). As shown in Fig. 5C, quantification of BrdU⁺/NeuN⁺ neurons in the granular cell layer revealed an increased number of cells in the PGB-treated stressed group compared to the saline-treated unstressed group (1,856 ± 78.7 and 1,413 ± 14.8; $p < 0.05$) and to the saline-treated stressed group (1,856 ± 78.7 and 1,288 ± 100.7; $p < 0.01$). Altogether these data suggest that chronic PGB treatment prevented depressive-like behavior and, in parallel, increased hippocampal neurogenesis in adult mice exposed to chronic stress. As control, we also tested the effects of 10 mpk PGB administered acutely (single i.p. injection) or chronically (i.p. injection, once daily, for 21 days) in unstressed mice. As shown in Fig. 5D, in both cases we reported no effect of the drug, compared to saline, in the TST and FST paradigms.
DISCUSSION

Deregulated hippocampal neurogenesis has been reported in neuropsychiatric conditions such as major depression and neurodegenerative disorders (Kempermann et al., 2008; Pittenger and Duman, 2008; Decarolis and Eisch, 2010; Samuel and Hen, 2011). An area of great interest is the positive influence of antidepressants on adult hippocampal neurogenesis (Duman et al., 2001; Malberg, 2004; Boldrini et al., 2009; David et al., 2009; Perera et al., 2011). Several authors have contributed to the idea that antidepressant-induced increase in hippocampal neurogenesis may be required for at least some of the drug effects in rodents, primates and, potentially, in patients (Santarelli et al., 2003; David et al., 2009; Perera et al., 2011).

Gabapentin and pregabalin are anticonvulsant, analgesic, and anxiolytic drugs whose effects have been demonstrated in preclinical models (Sills, 2006; Taylor et al., 2007). One mechanism is considered primary for their clinical efficacy, namely high affinity interaction with the α2δ1/2 subunits of voltage-gated Ca2+ channels (VGCC) (Bian et al., 2006; Gee et al., 1996) and for this reason these drugs are referred to as α2δ ligands.

Interestingly, there are reports that these drugs may be effective as add-on therapy to antidepressants in MDD (Pae, 2009), in Posttraumatic Stress Disorder (PTSD) (Pae et al., 2009) and on depressive symptoms in Generalized Anxiety Disorders (GAD) (Stein et al., 2008). No information is available on the mechanism of action of α2δ ligands in such clinical setting.

We investigated the activity of the α2δ ligands GBP and PGB in an in vitro model of adult mouse NPC. In order to identify proneurogenic drugs, we performed double immunolabelling experiments with markers of neurons (MAP-2) and undifferentiated progenitors (nestin). By these means, we could identify four cell subpopulations derived from NPC. One is the undifferentiated MAP-2/nestin+ neurosphere cell population. A second subpopulation is
composed of MAP-2+/nestin- cells and we regard them as mature neurons. We also identified a third population of MAP-2+/nestin+ cells. In vitro studies aimed at assessing neuronal differentiation of NPC usually utilize either a single marker of neuronal differentiation (βIII tubulin or MAP2) or double/triple staining for neuronal/astroglia/oligodendrocyte markers, so to evaluate differentiation of multipotent NPC toward all lineages. Under these conditions, no such double positive population could be identified. At this stage we think that double positive cells represent a population in a transition phase toward neuronal commitment, characterized by MAP-2 expression as well as by persistence of the marker of undifferentiated NPC. Indeed, although rare, MAP-2+/nestin+ cells have been detected in human fetal brain tissue (Messam et al., 2002). Finally, a fourth subpopulation is characterized by the absence of both markers. At present, we have evidence that they are Sox-2+ cells (not shown), a population of undifferentiated progenitors which can give rise to GFAP+ cells and NG2+ oligodendrocyte precursors (Brazel et al., 2005; Graham et al., 2003). In our experimental setting, PGB and GBP resulted in a concentration-dependent increase of both MAP-2+/nestin- and MAP-2+/nestin+ cells. In parallel, α2δ ligands reduced the population of double negative cells, with no effects on the MAP-2/-/nestin+ cells. No effect was elicited by PGB and GBP on survival of hippocampal NPC or their progeny (not shown). Overall, these data suggested that α2δ ligands promoted differentiation of hippocampal NPC towards the neuronal lineage, mainly at the expenses of undifferentiated Sox-2+ nestin- progenitors. Interestingly, PGB had no effect on proliferation of adult hippocampal NPC in vitro.

Activation of the NF-κB pathway participated in the proneurogenic effects elicited by α2δ ligands on adult hippocampal NPC, since both inhibition of p50 and p65 nuclear translocation and of the IκB kinase IKK2 counteracted PGB-mediated effects. Previous work showed that PGB can inhibit substance P-induced NF-κB activation in neuroblastoma, glioma and in dorsal root
ganglia cells (Park et al., 2008). On the other hand, our observation that NF-κB activation by pregabalin mediates its proneurogenic effects is in line with reports suggesting that NF-κB signalling is involved in the regulation of hippocampal neurogenesis (Rolls et al., 2007; Denis-Donini et al., 2008; Meneghini et al., 2010; Grilli and Meneghini, 2012). To provide a more complex picture, NF-κB signaling, and in particular p65 activation, has also been involved in decreased hippocampal neurogenesis in response to chronic stress and Interleukin-1 (Koo et al., 2010). The fact that both induction and inhibition of adult neurogenesis may rely on NF-κB activation is likely to reflect the complexity within the NF-κB signaling pathway. Indeed NF-κB proteins represent a family of transcription factors (p50, p65, p52, c-rel, relB) whose members can combine to form hetero- and homo-dimers of different composition, which, in turn, can be differentially activated in any given cell type and exert different, even opposite, functions, through activation of different sets of gene targets. As an example, NF-κB pathway activation is involved both in cell survival and cell death decisions in neurons (Grilli and Memo, 1997).

We also provide evidence that the α2δ1 antagonists L-isoleucine and L-(+)-α-phenylglycine inhibited PGB- and GBP-induced neuronal differentiation, suggesting that, at least in vitro, drug effects could be mediated by interaction with α2δ1. Indeed we showed that the α2δ1 subunit is expressed by adult hippocampal NPC. Interestingly, PGB and GBP mediated effects on neuronal differentiation of adult NPC were observed in the low nanomolar range, compatible with their KD values (6-7 nM) at the auxiliary α2δ1 subunit of neuronal VGCC channels (Lotarski et al., 2011). At this stage we cannot rule out the possibility that the α2δ1 subunit may mediate the proneurogenic effects of PGB and GBP independently of its modulatory function within VGCC channels. Indeed at least for another member of the α2δ subunit family, the α2δ3 protein (which does not bind PGB and GBP), its function in Drosophila has been demonstrated to be
independent of its role in biophysics and localization of Ca\(^{2+}\) channels (Kurshan et al., 2009). On the other hand, it should be underlined that, in most published reports, concentrations of \(\alpha 2\delta\) ligands that are utilized for unraveling their molecular mechanism of action are remarkably higher (in the high micromolar range) than those we utilized in our studies (Maneuf et al., 2001; Cunningham et al., 2004; Huang et al., 2006; Bauer et al., 2009; Eroglu et al., 2009).

When tested \textit{in vivo}, chronic administration of PGB resulted in enhanced neurogenesis in adult naive mice, as assessed by an increased number of BrdU\(^+\)/NeuN\(^+/\)/GFAP\(^-\) cells. Interestingly, drug treatment had no effect on astrogliogenesis but, in parallel, it decreased the number of undifferentiated BrdU\(^+\)/NeuN\(^+/\)/GFAP\(^-\) cells in the DG. Moreover, the absolute number of surviving BrdU\(^+\) cells was not affected by chronic PGB treatment, suggesting no effect on cell survival and/or proliferation, in line with data obtained \textit{in vitro}. Altogether these data support the idea that \(\alpha 2\delta\) ligands promote neurogenesis by favouring differentiation of undifferentiated progenitors toward the neuronal lineage.

Increasing evidence demonstrates that neuroplasticity, including neurogenesis, is disrupted in mood disorders and animal models of stress. It is suggested that a reduction in neurogenesis following chronic stress exposure may underlie impaired hippocampal plasticity and contribute to depression symptoms (Warner-Schmidt and Duman, 2006; Pittenger and Duman, 2008; Lucassen et al., 2010). The chronic restraint stress is frequently utilized to evaluate whether a drug could prevent depressive-like symptoms (Luo et al., 2005; Yun et al., 2010). Behavioral efficacy of an antidepressant is usually demonstrated using two common paradigms of chronic stress-induced behavioral responses, namely the forced swim test (FST) and the tail suspension test (TST) (Porsolt, 2000; Bourin et al., 2005; Cryan and Holmes, 2005). In the present study we could demonstrate that chronic PGB administration prevented the appearance of stress-induced
depressive-like behavior both in the TST and FST. It should be underlined that in mice the dose of PGB we used has been demonstrated to yield plasma concentrations (Lotarski et al., 2011) comparable with effective dosage in clinical practice (Bockbrader et al., 2010). Importantly, both acute and chronic administration of the drug had no effect on TST and FST in unstressed mice. When, at the end of the restraint procedure, mouse brains were examined, quantification of BrdU+/NeuN+ cells within the GCL revealed that, although restraint stress did not significantly reduce the number of newly generated neurons, PGB treatment increased hippocampal neurogenesis in adult mice exposed to chronic stress. Altogether, behavioural and proneurogenic PGB-mediated effects correlated in our experimental conditions.

Based on these results, we propose that the new pharmacological activity of α2δ ligands may potentially explain their efficacy as add-on therapy in MDD, as well as on depressive symptoms in PTSD and GAD (Stein et al., 2008; Pae, 2009; Pae et al., 2009). Whether the proneurogenic activity of α2δ ligands may contribute to their therapeutic profile deserves future investigation. Interestingly, α2δ ligands represent the first line treatment in chronic neuropathic pain, a disease state which has a high comorbidity with major depression. Incidentally, defective hippocampal neurogenesis has been demonstrated in animal models of neuropathic pain (Terada et al., 2008). In such clinical setting, drugs such as pregabalin, which are able to positively modulate adult neurogenesis and potentially exert antidepressant-like activity, may represent an added value compared to drugs which are “pure” analgesics.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Grilli, Valente, Bortolotto, Cuccurazzu.

Conducted experiments: Valente, Bortolotto, Cuccurazzu, Ubezio, Meneghini, Francese.

Contributed new reagents or analytical tools: none

Performed data analysis: Valente, Bortolotto, Cuccurazzu, Ubezio, Canonico, Grilli.

Wrote the manuscript: Grilli.
REFERENCES


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neurogenesis for the therapeutic action of antidepressants in adult nonhuman primates. 


FOOTNOTE

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LEGENDS FOR FIGURES

Figure 1:

Effect of $\alpha_2\delta$ ligands on neuronal differentiation and proliferation of hippocampus-derived neural progenitor cells. (A) Representative fluorescence microscopy image of a hippocampal neurosphere immunolabelled for nestin (green) and SRY-related HMG-box gene 2 (Sox-2) (red), markers of undifferentiated NPC. Magnification = X600. Scale bar = 56 μm. (B) After 24 h in absence of growth factors, hippocampal Neural Progenitor Cells (NPC) differentiated giving rise to four different cell populations identified by double Microtubule Associated Protein-2 (MAP-2) and nestin immunolabelling: MAP-2+/nestin− mature neurons, MAP-2+/nestin+, MAP-2−/nestin+ and MAP-2−/nestin− cells. Data are expressed as mean ± S.D. of $n=9$ experiments, run in triplicates. Gabapentin (GBP) and pregabalin (PGB) promote neuronal differentiation of adult hippocampal NPC. GBP (C-F) and PGB (G-J) significantly increased, in a concentration-dependent manner, the percentage of MAP-2+/nestin− (C, G) and MAP-2+/nestin+ (D, H) cells and decreased the percentage of MAP-2−/nestin− cells (F, J), with no effect on MAP-2−/nestin+ cells (E, I). Data are expressed as mean ± S.D. of $n=3$ experiments, run in triplicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs vehicle (Student’s t-test). (K-M) Representative fluorescence microscopy images of MAP-2 immunolabelling (green) in cells derived from hippocampal NPC after 24 h treatment with vehicle (K), 1 nM GBP (L) and 1 nM PGB (M). Nuclei are stained with Draq5 (blue). Magnification = X400. Scale bar = 75 μm. (N) Adult hippocampal NPC were treated with vehicle or 1 nM PGB for 6, 24, 48, 72, 96 h and proliferation rate was assessed. PGB had no effect on NPC proliferation, when compared to vehicle. Data, expressed as counts per second (CPS), represent the mean ± S.D. of experiments run in triplicates.
Figure 2:

Chronic PGB treatment promotes hippocampal neurogenesis in vivo. (A) 21 days after the last BromodeoxyUridine (BrdU) injection, the number of BrdU+ cells in the DG is similar in mice treated for twenty-one days with vehicle, 1, 10 mg/Kg body weight (mpk, i.p.) PGB. (B) Triple immunolabelling for BrdU and markers of neuronal (Neuronal Nuclei, NeuN) and glial (Glial Fibrillary Acidic Protein, GFAP) cells showed that the percentage of BrdU single positive cells is significantly reduced in mice treated with 10 mpk PGB compared to animals treated with vehicle or 1 mpk PGB. In parallel, PGB 10 mpk increased the percentage of BrdU+/NeuN+/GFAP- cells, with no effect on the percentage of BrdU+/NeuN-/GFAP+ cells. Data are expressed as mean ± S.D. *, $p < 0.05$; **, $p < 0.01$ vs vehicle (Student’s $t$-test). (C-F) Representative confocal microscopy images of BrdU (blue), NeuN (red), GFAP (green) immunolabelling in hippocampi of vehicle (C, D) and 10 mpk PGB (E, F) treated mice. BrdU single positive cells (arrows) and BrdU+/NeuN+/GFAP- cells (arrowheads) in the murine DG at X400 (C, E; scale bar = 75 µm) and X1,600 (D, F; scale bar = 18.75 µm) magnifications.

Figure 3:

The $\alpha 2\delta$ subunit mediates PGB proneurogenic effects on adult hippocampal NPC. (A) Representative confocal microscopy image of adult hippocampal NPC grown as neurosphere and immunolabelled for the $\alpha 2\delta1$ protein (green). Nuclei are stained with Draq5 (blue). The picture shows that the majority of cells within the neurosphere express $\alpha 2\delta1$. Magnification = X400. Scale bar = 75 µm. (B) Representative immunoblot showing that the subunit is expressed in extracts from undifferentiated hippocampus-derived neurospheres (HP NS) and from adult
hippocampus (HP), which was used as positive control. The protein content was normalized by α-tubulin. (C-H) The α2δ antagonists L-isoleucine (L-ISOL) (C-E) and L-(+)-α-phenylglycine (L-PHE) (F-H) prevent PGB-mediated proneurogenic effects on adult hippocampal NPC. 3-100 nM L-ISOL significantly inhibited, in a concentration-dependent manner, PGB-induced increase of MAP-2⁺/nestin⁻ (C) and MAP-2⁺/nestin⁺ cells (D) and the PGB-induced decrease of MAP-2⁻/nestin⁻ cells (E). 3-100 nM L-PHE significantly inhibited, in a concentration-dependent manner, PGB-induced increase of MAP-2⁺/nestin⁻ (F) and MAP-2⁺/nestin⁺ cells (G) and the PGB-induced decrease of MAP-2⁻/nestin⁻ cells (H). Data are expressed as mean ± S.D. of \( n = 3 \) experiments, run in triplicates. **, \( p < 0.01 \); ***, \( p < 0.001 \) vs vehicle (Student’s \( t \)-test).

**Figure 4:**

*The effects of PGB on hippocampal NPC differentiation are mediated by activation of the NF-κB signalling pathway.* In hippocampus-derived NPC, treatment with the NF-κB p50 nuclear translocation inhibitory peptide (SN-50, 10 μg/ml) inhibited the increase of MAP-2⁺/nestin⁻ (A), and MAP-2⁺/nestin⁺ (B) cells and the decrease of MAP-2⁻/nestin⁻ cells (C) induced by 1 nM PGB. Conversely, SN50M inactive peptide did not counteract PGB-mediated effects (A-C). Treatment of hippocampal NPC with the inhibitor of NF-κB p65 nuclear translocation JSH-23 (3 μM) and with the IKK2 inhibitor SC-514 (3 μM) abolished the increase of MAP-2⁺/nestin⁻ (D), and MAP-2⁺/nestin⁺ (E) cells and the decrease of MAP-2⁻/nestin⁻ cells (F) induced by 1 nM PGB. (A-F) Data are expressed as mean ± S.D. of \( n = 3 \) experiments, run in triplicates. **, \( p < 0.01 \); ***, \( p < 0.001 \) vs vehicle (Student’s \( t \)-test). (G-J) Representative confocal microscopy images of MAP-2 immunolabelling (green) on differentiated NPC after 24 h treatment with 1 nM PGB.
alone (G) or in presence of 10 μg/ml SN50 (H), 3 μM JSH-23 (I) and 3 μM SC-514 (J). Nuclei are stained with Draq5 (blue). Magnification = X400. Scale bar = 75 μm.

Figure 5:
Chronic PGB treatment prevents appearance of a depressive-like behaviour and increases hippocampal neurogenesis in stressed mice. (A-B). Behavior of restraint-stressed mice in the TST (A) and FST (B) following chronic (21 days) administration of saline or PGB (10 mpk, i.p), in comparison with saline-treated unstressed mice. Immobilization time (s) was recorded and data expressed as mean ± S.E.M. of n = 6 mice/group. Chronic PGB treatment prevented stressed-induced increase of immobilization time in TST and FST. *, p < 0.05; **, p < 0.01; ***, p < 0.001 vs saline-treated stressed mice. (C) Analysis of hippocampal neurogenesis in stressed and unstressed mice. The number of new hippocampal neurons, identified by double BrdU and NeuN staining, was significantly increased in the granular layer of PGB-treated stressed mice compared to saline-treated stressed and unstressed mice. Data are expressed as mean ± S.D. of n = 6 mice/group. *, p < 0.05, **, p < 0.01 vs PGB-treated stressed mice. (D) Neither acute nor chronic (21 days) PGB treatment affected immobility time in unstressed mice in TST and FST. Mice were injected i.p. with 10 mpk PGB or saline and time spent in immobility was measured. Data are expressed as mean ± S.E.M. of n = 6 mice/group.
Figure 5

A

BrdU$^{+}$/NeuN$^{+}$

Immobility (s)

- unstressed saline
- stressed saline
- stressed PGB

B

Immobility (s)

- unstressed saline
- stressed saline
- stressed PGB

C

BrdU$^{+}$/NeuN$^{+}$

- unstressed saline
- stressed saline
- stressed PGB

D

ACUTE

TST

FST

CHRONIC

TST

FST

Immobility (s)

- saline
- PGB