1521-0111/12/8202-271–280$25.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 82:271–280, 2012

Vol. 82, No. 2
77636/3782783

$\alpha 2\delta$ Ligands Act as Positive Modulators of Adult Hippocampal Neurogenesis and Prevent Depression-Like Behavior Induced by Chronic Restraint Stress

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Received January 11, 2012; accepted May 9, 2012

ABSTRACT

Although the role of adult hippocampal neurogenesis remains to be fully elucidated, several studies suggested that the process is involved in cognitive and emotional functions and is deregulated in various neuropsychiatric disorders, including major depression. Several psychoactive drugs, including antidepressants, can modulate adult neurogenesis. Here we show for the first time that the $\alpha 2\delta$ ligands gabapentin [1-(aminomethyl)cyclohexaneacetic acid] and pregabalin (PGB) [(*S*)-(+)-3-isobutyl-GABA or (*S*)-3-(aminomethyl)-5-methylhexanoic acid] can produce concentration-dependent increases in the numbers of newborn mature and immature neurons generated in vitro from adult hippocampal neural progenitor cells and, in parallel, a decrease in the number of undifferentiated precursor cells. These effects were confirmed in vivo, because significantly increased numbers of adult cell-generated neurons were observed in the hippocampal region of mice receiving prolonged treatment with PGB (10 mg/kg i.p. for 21 days), compared with vehicle-treated mice. We demonstrated that PGB administration prevented the appearance of depression-like behaviors induced by chronic restraint stress and, in parallel, promoted hippocampal neurogenesis in adult stressed mice. Finally, we provided data suggesting involvement of the $\alpha 2\delta 1$ subunit and the nuclear factor-κB signaling pathway in drug-mediated proneurogenic effects. The new pharmacological activities of $\alpha 2\delta$ ligands may help explain their therapeutic activity as supplemental therapy for major depression and depressive symptoms in post-traumatic stress disorder and generalized anxiety disorders. These data contribute to the identification of novel molecular pathways that may represent potential targets for pharmacological modulation in depression.

Introduction

Much experimental work has established that new neurons are generated throughout life in the hippocampus and the subventricular zone of the brains of mammals, including humans (Alvarez-Buylla et al., 2001; Kempermann et al., 2008; Kriegstein and Alvarez-Buylla, 2009; Ming and Song, 2011). Although the role of adult hippocampal neurogenesis remains to be fully elucidated, several studies suggested 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 is deregulated in various neuropsychiatric disorders (DeCarolis and Eisch, 2010; Lazarov and Marr, 2010; Samuels and Hen, 2011). On the basis of the evidence that hippocampal neurogenesis can be down-regulated under stressful conditions, including those that result in animal models of depression-like behaviors, and can be up-regulated by antidepressant drugs and treatments, the hypothesis has emerged that neurogenesis and related aspects of hippocampal plasticity may contribute to the pathophysiological processes of major depressive disorder (MDD) and its effective treatment (Malberg and Blendy, 2005; Pittenger and Duman, 2008; Hanson et al., 2011). In particular, several authors suggested that neurogenesis may be necessary for some but not all of the behavioral effects of antidepressants (Santarelli et al., 2003; David et al., 2009). Boldrini et al. (2009) demonstrated that antidepressants increased neural progenitor levels in the hippocampi of patients with depression.

This work was supported by Fondazione delle Comunità del Novarese and by Fondazione Cariplo (grants to M.G.).
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
http://dx.doi.org/10.1124/mol.112.077636.

ABBREVIATIONS: MDD, major depressive disorder; NPC, neural progenitor cell; GBP, gabapentin; PGB, pregabalin; VGCC, voltage-gated calcium channel; MAP-2, microtubule-associated protein-2; BrdU, bromodeoxyuridine; GFAP, glial fibrillary acidic protein; NeuN, neuronal nuclei; TST, tail suspension test; FST, forced swim test; DG, dentate gyrus; NF-κB, nuclear factor-κB; ANOVA, analysis of variance; JSH-23, 4-methyl-N^1-(3-phenylpropyl)-1,2-benzenediamine; SC-514, 4-amino-[2,3′-bithiophene]-5-carboxamide.

PGB [(*S*)-(+)-3-isobutyl-GABA or (*S*)-3-(aminomethyl)-5-methylhexanoic acid] and GBP [1-(aminomethyl)cyclohexaneacetic acid] are anticonvulsant, analgesic, and anxiolytic drugs whose effects have 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 interactions with the $\alpha2\delta1/2$ subunits of voltage-gated calcium channels (VGCCs) (Gee et al., 1996; Bian et al., 2006). Studies demonstrated that drug binding to the $\alpha2\delta1$ subunit is necessary for antihyperalgesic effects in a preclinical model of pain (Field et al., 2006), as well as anxiolytic effects in rodents (Lotarski et al., 2011).

Here for the first time we provide evidence that, like classic antidepressants, $\alpha2\delta$ ligands (in particular, PGB) can elicit positive modulation of adult hippocampal neurogenesis both in vitro and in vivo. Long-term PGB administration results in prevention of depression-like behavior and promotion of hippocampal neurogenesis in adult mice subjected to chronic restraint stress. On the basis of in vitro data, we propose that the proneurogenic effects of PGB are mediated through interaction with the $\alpha2\delta$ subunit and activation of the NF-κB signaling pathway, which was reported to be involved in the regulation of adult neurogenesis both in vitro and in vivo (Denis-Donini et al., 2005, 2008; Rolls et al., 2007; 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-filtered Thoren units (Thoren Caging Systems, Hazleton, PA) at the University of Piemonte Orientale animal facility, at three or four per cage, with unlimited access to water and food. Care and handling of animals were performed in accordance with the National Institutes of Health guidelines, and procedures were reviewed and approved by the local institutional animal care and use committee.

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

Isolation and Culture 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 a cell suspension was prepared as described previously (Meneghini et al., 2010). Primary (passage 1) neurospheres were dissociated after 7 to 9 days in vitro, whereas passage 2 to 16 neurospheres were dissociated every 5 days in vitro. At each passage, cells were plated in T25 flasks, at a density of 12,000 cells per cm^2, in growth medium. Passage 3 to 16 neurospheres were used for experiments.

Neural Progenitor Cell Differentiation and Proliferation. The detailed procedure for NPC differentiation was described previously (Meneghini et al., 2010). NPCs were treated in the presence of the indicated concentrations of drugs or vehicle for 24 h. For neutralization of PGB and GBP effects, NPCs were pretreated for 60 min with L-isoleucine, L-(+)-α-phenylglycine, or vehicle before the addition of $\alpha2\delta$ ligands. For NF-κB inhibition, 10 μg/ml NF-κB SN-50 peptide or NF-κB SN-50M peptide, 3 μM 4-methyl-N^1-(3-phenylpropyl)-1,2-benzenediamine (JSH-23), or 3 μM 4-amino-[2,3′-bithiophene]-5-carboxamide (SC-514) (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 with phosphate-buffered saline and were fixed with ice-cold 4% paraformaldehyde for 20 min at room temperature, for subsequent immunofluorescence analyses. In each experiment, five fields per well (corresponding to 100–150 cells per well) were counted by using an Eclipse E600 fluorescence microscope (Nikon, Calenzano, Italy) with a 60× objective. All experiments were performed in triplicate, with different cell preparations, and were repeated at least three times. Data represent mean ± S.D. values. In parallel with differentiation analyses, necrotic and apoptotic rates in culture were evaluated in each experiment, as described previously (Meneghini et al., 2010). For evaluation of cell proliferation, dissociated NPCs were plated onto LuminNUNC F96 MicroWell plates (NUNC GmbH & Co. KG, Wiesbaden, Germany), at a density of 10,000 cells per well, in growth medium [Neurobasal-A medium containing B27 supplement, 2 mM L-glutamine, 20 ng/ml epidermal growth factor (Sigma-Aldrich, Milan, Italy) 100 units/ml penicillin, and 100 μg/ml streptomycin; Invitrogen, Carlsbad, CA], and were grown in the presence of 1 nM PGB or vehicle for 6 to 96 h. Proliferation rates were determined by using the CellTiter-Glo luminescent cell viability assay (Promega, Milan, Italy), according to the manufacturer's instructions. All experiments were performed in triplicate, and data (expressed as counts per second) represent mean ± S.D. values.

Immunocytochemical Analyses. Adult mouse neurospheres were harvested onto superfrost microscope slides (Menzel-Glaser, Braunschweig, Germany) through cytospin centrifugation (235*g* for 5 min; Thermo Fisher Scientific, Waltham, MA) and were fixed with ice-cold methanol (Sigma-Aldrich) for 10 min at −20°C, for subsequent immunofluorescence analyses. The following primary antibodies were used: mouse anti-$Ca_v\alpha2\delta1$ antibody (1:500; Alomone Labs, Jerusalem, Israel), chicken anti-nestin polyclonal antibody (1:4000; Neuromics, Edina, MN), and rabbit anti-Sox-2 antibody (1:500; Millipore Bioscience Research Reagents, Temecula, CA). After fixation, neurosphere-derived, differentiated cells were incubated with antibodies against microtubule-associated protein-2 (MAP-2) (rabbit polyclonal antibody, 1:600; Millipore Bioscience Research Reagents) or nestin (mouse monoclonal antibody, 1:1200; Abcam Inc., Cambridge, MA; or chicken monoclonal antibody, 1:4000; Neuromics). Secondary antibodies were as follows: Alexa Fluor 488-conjugated goat anti-mouse antibody (1:1600; Invitrogen), Alexa Fluor 555-conjugated goat anti-rabbit antibody (1:1400; Invitrogen), and Alexa Fluor 488-conjugated goat anti-chicken antibody (1:1400; Invitrogen). Nuclei were counterstained with DRAQ5 dye (1:2000; Enzo Life Sciences, Inc., Farmingdale, NY) diluted in phosphate-buffered saline. Slides were prepared with coverslips by using fluorescent mounting medium (Dako Denmark A/S, Glostrup, Denmark) as an antifading agent. Adobe Photoshop CS (Adobe Systems Inc., San Jose, CA) 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 Analyses. For protein isolation, neurosphere and tissue extracts were prepared as described previously (Meneghini et al., 2010). Immunoblotting was performed overnight with the primary antibody anti-$Ca_v\alpha2\delta1$ (1:1500; Alomone Labs), in an antibody solution containing 3% (w/v) bovine serum albumin in Tris-buffered saline with 0.1% Tween 20. After washing, blots were incubated for 60 min at room temperature with peroxidase-conjugated goat anti-mouse antibody (1:10,000; Bio-Rad Laboratories, Hercules, CA), and the immunocomplexes were observed by using the Supersignal West Pico chemiluminescent substrate (Thermo Fisher Scientific). Densitometric analyses were performed with the Quantity One software system (Bio-Rad Laboratories), and each band signal was normalized to the α-tubulin signal (mouse monoclonal antibody, 1:2000; Sigma-Aldrich) in each lane.

In Vivo Neurogenesis Studies. Adult (3–4-month-old), male, CD1 mice were distributed randomly into vehicle or PGB treatment groups ($n = 6$ per group). PGB (1 or 10 mg/kg of body weight) and corresponding vehicle (saline solution) were administered intraperi-

toneally once daily for 21 days. For the first 5 days of treatment, mice were also given a daily dose of bromodeoxyuridine (BrdU) (150 mg/kg of body weight i.p.; Sigma-Aldrich). Twenty-one days after the last BrdU administration, mice were perfused transcardially. Brain tissue was prepared for subsequent analyses as described previously (Denis-Donini et al., 2008). The following primary antibodies were used: rat monoclonal anti-BrdU antibody (1:200; Novus Biologicals Inc., Littleton, CO), goat anti-glial fibrillary acidic protein (GFAP) antibody (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and mouse anti-neuronal nuclei (NeuN) antibody (1:150; Millipore Corp., Billerica, MA). For quantification and phenotypic characterization of newborn cells, a modified, unbiased, stereological protocol was used, as described previously (Denis-Donini et al., 2008).

Unpredictable Chronic Restraint Stress and PGB Treatment. Adult (3-month-old), male, C57BL/6J mice were subjected to restraint ($n = 12$) by being individually placed in well ventilated polypropylene tubes (internal diameter, 3 cm; length, 11.5 cm). The test duration was 3 h each day for 21 days; the administration time was changed every day, to be unpredictable. During immobilization stress, mice did not have access to food or water. After restraint stress administration, mice were returned to their home cage environment. The unstressed control mice ($n = 6$) were left undisturbed in their home cages for the entire duration of the stress experimental procedure. One observer, who was not aware of the stress procedure or drug administration, performed the behavioral tests. In the PGB long-term administration experiments, behavioral tests were performed 24 h after the last drug (or vehicle) injection. In the PGB short-term administration experiments, tests were performed 2 h after the drug (or vehicle) injection, a time reported to correspond to peak behavioral effects (Lotarski et al., 2011). For the first 5 days of the experimental procedure, animals were given BrdU (150 mg/kg i.p., once daily). Thirty days after the last BrdU injection and 14 days after the last restraint session, mice were perfused transcardially and their brains were immunoprocessed for double BrdU/NeuN labeling, as described previously (Denis-Donini et al., 2008).

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

Forced Swim Test. Mice were individually placed in a transparent glass cylinder (diameter, 13 cm; height, 24 cm) filled with 11.5 cm of warm water (22–23°C). Immobility was defined as making only 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 in their home cages.

Statistical Analyses. Data were reported as mean ± S.D. or S.E.M. values from at least three experiments and were analyzed with one-way analysis of variance (ANOVA) followed by Tukey's post hoc test or with Student's t test. Statistical significance was set for p values of <0.05.

Results

GBP and PGB Have Effects on the Differentiation of Adult Mouse Neural Progenitor Cells. In our experimental setting, adult murine NPCs were grown in the presence of epidermal growth factor and basic fibroblast growth factor, as floating neurospheres. Under these conditions, cells were phenotypically characterized by the expression of well recognized markers of undifferentiated neural progenitors, such as nestin and Sox-2 (Fig. 1A), and by the absence of markers of committed neurons, glial cells, or oligondrocytes (Meneghini et al., 2010). After removal of growth factors from the medium, NPCs stopped dividing and differentiated. Through double-immunolabeling with antibodies against nestin and MAP-2, we were able to evaluate the stages of neuronal differentiation of NPCs in vitro. After 24 h, hippocampus-derived NPCs gave rise to a subpopulation of MAP-2$^+$/nestin$^-$ cells (3.9 ± 0.6% of the total), which we regard as newly generated neurons, MAP-2$^+$/nestin$^+$ cells (32.7 ± 2.0% of the total), which we consider a population of cells in a phase of transition toward neuronal commitment, a small population of MAP-2$^-$/nestin$^+$ undifferentiated cells (6.8 ± 0.7% of the total), which are indistinguishable from neurosphere-constituting progenitors, and a large population of MAP-2$^-$/nestin$^-$ cells (56.6 ± 2.4% of the total) (Fig. 1B). Double-negative cells expressed Sox-2, a marker of undifferentiated progenitors (Brazel et al., 2005), and mainly generated GFAP$^+$ cells and NG2$^+$ oligodendrocytic precursors (data not shown). Under these experimental conditions, we evaluated the effects of PGB and GBP (in concentration ranges consistent with their binding affinities at the α2δ subunit) on neuronal differentiation of adult mouse hippocampal NPCs. Both drugs exhibited a remarkable ability to promote neuronal differentiation of adult NPCs. In particular, when hippocampal NPCs were incubated with GBP, we observed a concentration-dependent increase in the proportion of MAP-2$^+$/nestin$^-$ mature neurons (ANOVA, $p < 0.001$), in comparison with vehicle, with a maximal increase elicited by 1 nM GBP (154.6 ± 15.1% increase, compared with vehicle-treated cells; Student's t test, $p < 0.001$) (Fig. 1C). A smaller but significant increase in the proportion of MAP-2$^+$/nestin$^+$ immature neurons was produced by GBP at all tested concentrations (Fig. 1D). Compared with vehicle, the drug significantly decreased the proportion of MAP-2$^-$/nestin$^-$ cells, in a concentration-dependent manner (ANOVA, $p < 0.001$) (Fig. 1F), with maximal inhibition elicited by 1 nM GBP (44.7 ± 6.4% decrease, compared with vehicle-treated cells; Student's t test, $p < 0.01$). The drug produced no effect on the MAP-2$^-$/nestin$^+$ cell population (Fig. 1E).

We evaluated PGB effects on hippocampal NPC neuronal differentiation. Like GBP, PGB (0.1–10 nM) produced dramatic, concentration-dependent increases in the proportions of MAP-2$^+$/nestin$^-$ cells (Fig. 1G) and MAP-2$^+$/nestin$^+$ cells (Fig. 1H) and a decrease in the number of MAP-2$^-$/nestin$^-$ cells (Fig. 1J), compared with vehicle (ANOVA, $p < 0.001$). In particular, maximal effects were elicited by 1 nM PGB (168.6 ± 15.9, 72.5 ± 4.3, and −53.7 ± 2.9% changes, compared with vehicle-treated cells, for MAP-2$^+$/nestin$^-$, MAP-2$^+$/nestin$^+$, and MAP-2$^-$/nestin$^-$ cell populations, respectively; Student's t test, $p < 0.001$) (Fig. 1, G, H, and J). As demonstrated for GBP, PGB produced no effect on the MAP-2$^-$/nestin$^+$ cell population (Fig. 1I). MAP-2 immunolabeling experiments revealed that cells treated with 1 nM GBP (Fig. 1L) and 1 nM PGB (Fig. 1M) increased neuritic arborizations, compared with vehicle-treated cells (Fig. 1K). We also demonstrated that, within the tested concentration ranges, neither GBP nor PGB affected survival rates for NPCs and/or their progeny under our experimental conditions (data not shown). Overall, these data suggested that GBP and PGB had the ability to promote neuronal differentiation of adult hippocampal NPCs in vitro. To investigate whether α2δ ligands could 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,

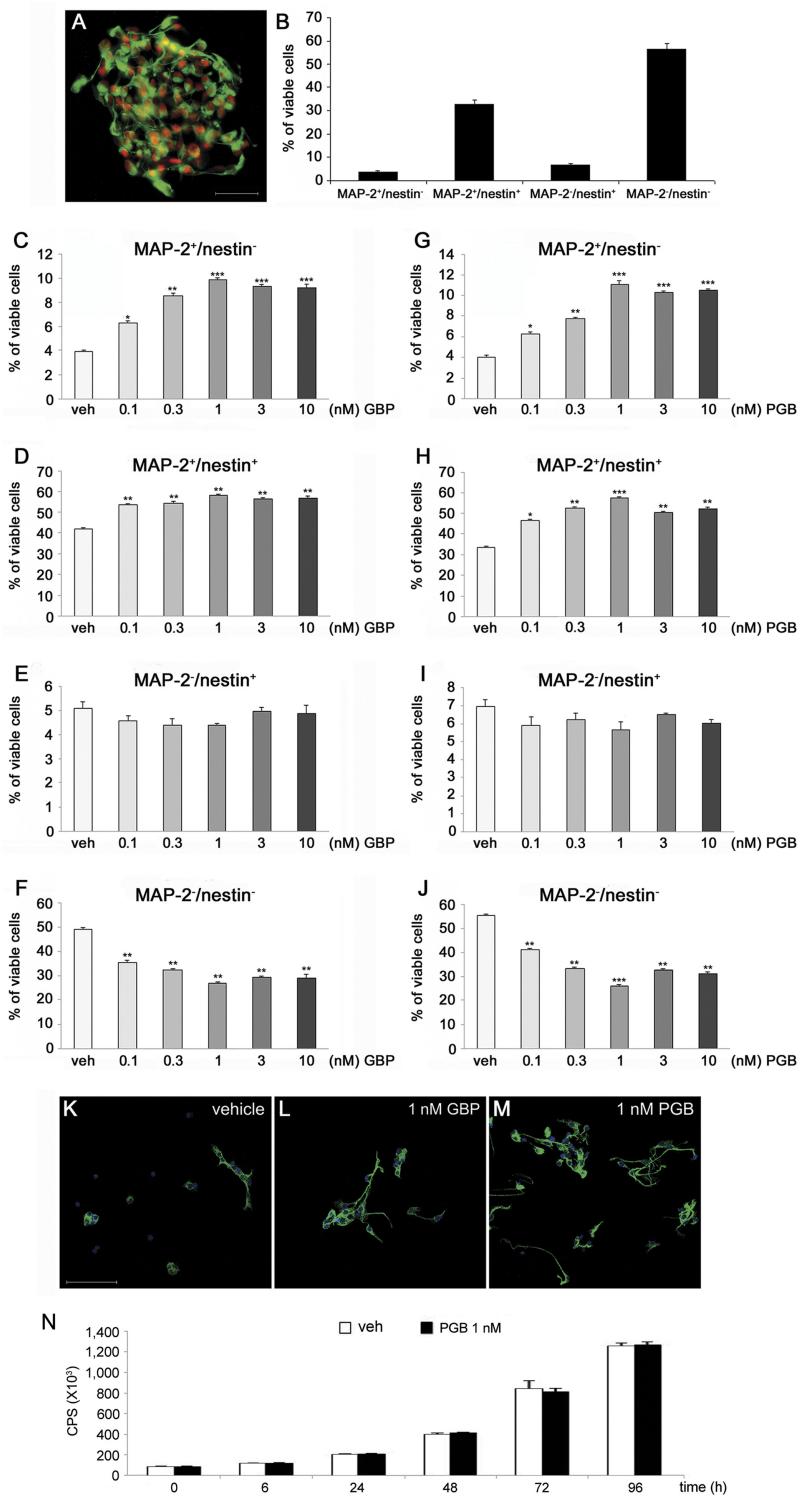

Fig. 1. $\alpha 2\delta$ ligands exhibit effects on neuronal differentiation, and not on proliferation, of hippocampus-derived neural progenitor cells. A, representative fluorescence microscopic image of a hippocampal neurosphere immunolabeled for nestin (green) and Sox-2 (red), markers of undifferentiated NPCs. Magnification, 600×. Scale bar, 56 μm. B to J, quantitation of cell populations after vehicle (veh) or ligand treatment. B, after 24 h in the absence of growth factors, hippocampal NPCs differentiated, giving rise to four cell populations identified through MAP-2/nestin double-immunolabeling, namely, MAP-2$^+$/nestin$^-$ mature neurons and MAP-2$^+$/nestin$^+$, MAP-2$^-$/nestin$^+$, and MAP-2$^-$/nestin$^-$ cells. Data are expressed as mean ± S.D. of nine experiments, performed in triplicate. C to J, GBP (C–F) and PGB (G–J) promoted neuronal differentiation of adult hippocampal NPCs. GBP and PGB significantly increased, in a concentration-dependent manner, the proportions of MAP-2$^+$/nestin$^-$ (C and G) and MAP-2$^+$/nestin$^+$ (D and H) cells and decreased the proportion of MAP-2$^-$/nestin$^-$ cells (F and J), with no effect on MAP-2$^-$/nestin$^+$ cells (E and I). Data are expressed as mean ± S.D. of three experiments, performed in triplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, versus vehicle (Student's t test). K to M, representative fluorescence microscopic images of MAP-2 immunolabeling (green) in cells derived from hippocampal NPCs after 24-h treatment with vehicle (K), 1 nM GBP (L), or 1 nM PGB (M). Nuclei were stained with DRAQ5 dye (blue). Magnification, 400×. Scale bar, 75 μm. N, proliferation rates for adult hippocampal NPCs treated with vehicle or 1 nM PGB for 6, 24, 48, 72, or 96 h. PGB had no effect on NPC proliferation, compared with vehicle. Data, expressed as counts per second (CPS), represent the mean ± S.D. of experiments performed in triplicate.

which suggested that the drug did not affect cell proliferation of adult hippocampal neural progenitors.

PGB Promotes Adult Hippocampal Neurogenesis In Vivo. The in vitro results prompted us to investigate whether $\alpha 2\delta$ ligands had any effect on adult hippocampal neurogenesis in vivo. Adult male mice (n = 18) received intraperitoneal injections of vehicle (saline solution) or 1 or 10 mg/kg PGB once daily for 21 days. During the first 5 days of treatment, mice were also treated with the thymidine analog BrdU (150 mg/kg of body weight i.p), to label cells in S-phase. Twenty-one days after the last BrdU injection, mice were killed and their brain tissue was analyzed for the presence and phenotype of BrdU$^+$ cells in the hippocampus. The numbers of BrdU-labeled cells in the subgranular zone and in the granular cell layer within the dentate gyrus (DG) of vehicle- and drug-treated mice were quantified with a modified, unbiased, stereological protocol, as described previously (Denis-Donini et al., 2008). As shown in Fig. 2A, we observed no significant difference in the absolute numbers of surviving BrdU$^+$ cells in the DG of mice treated with 1 or 10 mg/kg

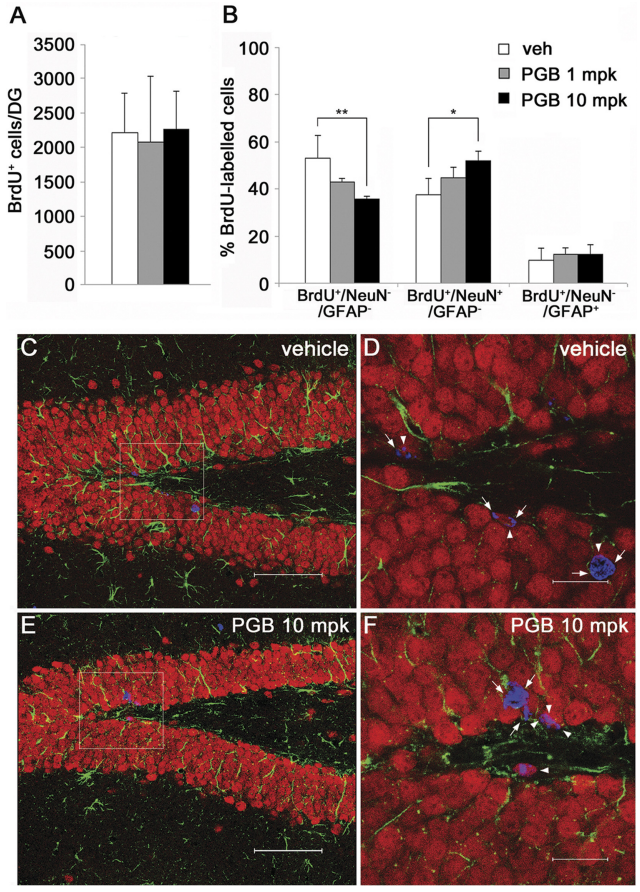

Fig. 2. PGB treatment promotes hippocampal neurogenesis in vivo. A, numbers of $BrdU^+$ cells in the DG 21 days after the last BrdU injection. Values were similar for mice treated for 21 days with vehicle (veh) or 1 or 10 mg/kg (mpk) PGB (administered intraperitoneally). B, triple-immunolabeling for BrdU and markers of neuronal (NeuN) and glial (GFAP) cells. Results showed that the proportion of single-positive $BrdU^+$ cells was significantly reduced in mice treated with 10 mg/kg PGB, compared with animals treated with vehicle or 1 mg/kg PGB. In parallel, PGB at 10 mg/kg of body weight increased the proportion of $BrdU^+/NeuN^+/GFAP^-$ cells, with no effect on the proportion of $BrdU^+/NeuN^-/GFAP^+$ cells. Data are expressed as mean ± S.D. *, $p < 0.05$; **, $p < 0.01$, versus vehicle (Student's t test). C to F, representative confocal microscopic images of BrdU (blue), NeuN (red), and GFAP (green) immunolabeling in hippocampi of vehicle-treated (C and D) and 10 mg/kg PGB-treated (E and F) mice. Single-positive $BrdU^+$ cells (arrows) and $BrdU^+/NeuN^+/GFAP^-$ cells (arrowheads) in the murine DG are indicated at 400× magnification (C and E; scale bar, 75 μm) and 1600× magnification (D and F; scale bar, 18.75 μm).

PGB, compared with vehicle (2218 ± 586, 2082 ± 964, and 2266 ± 562 $BrdU^+$ cells in the DG of vehicle-, 1 mg/kg PGB-, and 10 mg/kg 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 100 $BrdU^+$ cells were randomly selected from brain sections throughout the entire hippocampal DG extension in vehicle- and drug-treated animals. The proportions of $BrdU^+/NeuN^-/GFAP^-$, $BrdU^+/NeuN^+/GFAP^-$, and $BrdU^+/NeuN^-/GFAP^+$ cells were calculated. Compared with vehicle-treated animals, the number of $BrdU^+/NeuN^-/GFAP^-$ cells was significantly reduced in the DG of mice receiving chronic treatment with 10 mg/kg PGB (53 ± 9.8% and 35.7 ± 1.5% in vehicle- and PGB-treated animals, respectively; Student's t test, $p < 0.01$) (Fig. 2, B–F). A small and statistically insignificant reduction was observed in mice treated with 1 mg/kg PGB (53 ± 9.8% and 43 ± 2% in vehicle- and PGB-treated animals, respectively; Student's t test, $p = 0.159$) (Fig. 2B). In parallel, the proportion of newly generated $BrdU^+/NeuN^+/GFAP^-$ neurons increased in 10 mg/kg PGB-treated animals, compared with vehicle-treated animals (37.3 ± 7.6% and 52 ± 4.4% in vehicle- and PGB-treated animals, respectively; Student's t test, $p < 0.05$) (Fig. 2, B–F). A small and statistically insignificant increase in the proportion of new neurons was also observed in mice treated with 1 mg/kg PGB (44.7 ± 4.7%; Student's t test, $p = 0.230$) (Fig. 2B). The proportion of $BrdU^+/NeuN^-/GFAP^+$ cells was not affected by PGB treatment (9.7 ± 5.5, 12.3 ± 2.9, and 12.3 ± 4.2% in vehicle-, 1 mg/kg PGB-, and 10 mg/kg PGB-treated animals, respectively) (Fig. 2B).

Overall, in vivo data suggested that chronic administration of 10 mg/kg PGB resulted in enhanced neurogenesis in the DG of adult mice. No effect of drug treatment on astrogliogenesis was observed. In parallel, the number of undifferentiated $BrdU^+/NeuN^-/GFAP^-$ cells was significantly reduced in the DG of mice receiving chronic treatment with 10 mg/kg PGB, as expected for a drug that promotes neuronal differentiation of progenitor cells. The absolute number of surviving $BrdU^+$ cells was not affected by drug treatment, which suggests that there were no effects on cell survival and/or proliferation, in line with data obtained in vitro.

The α2δ1 Subunit of Voltage-Sensitive Calcium Channels Mediates In Vitro Proneurogenic Effects of PGB and GBP. In our experimental in vitro model, PGB and GBP were proneurogenic at nanomolar concentrations, consistent with drug binding affinities for the α2δ1 and α2δ2 subunits of neuronal VGCCs (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 auxillary 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 through Western blot analysis of hippocampus-derived neurosphere extract and adult hippocampus extract (used as a positive control sample) (Fig. 3B). We then evaluated whether the α2δ antagonists L-isoleucine (Fig. 3, C–E) (Brown et al., 1998) and L-(+)-α-phenylglycine (Fig. 3, F–H) (Mortell et al., 2006) were able to counteract PGB proneurogenic effects on hippocampal NPCs. When concentrations ranging from 3 to 100 nM were tested in the presence of 1 nM PGB, both α2δ antagonists caused concentration-dependent inhibition (ANOVA, $p < 0.001$) of drug-induced increases in $MAP\text{-}2^+/nestin^-$ (Fig. 3, C and F) and $MAP\text{-}2^+/nestin^+$ (Fig. 3, D and G) cell populations, with complete inhibition being obtained with 30 nM L-isoleucine and 30 nM L-(+)-α-phenylglycine. Both drugs produced concentration-dependent inhibition (ANOVA, $p < 0.001$) of PGB-induced decreases in the $MAP\text{-}2^-/nestin^-$ cell population, with complete inhibition being obtained with 30 nM concentration (Fig. 3, E and H). The two drugs had no effect alone (data not shown). These results support the hypothesis that PGB effects on neuronal differentiation of adult hippocampal NPCs were α2δ-mediated.

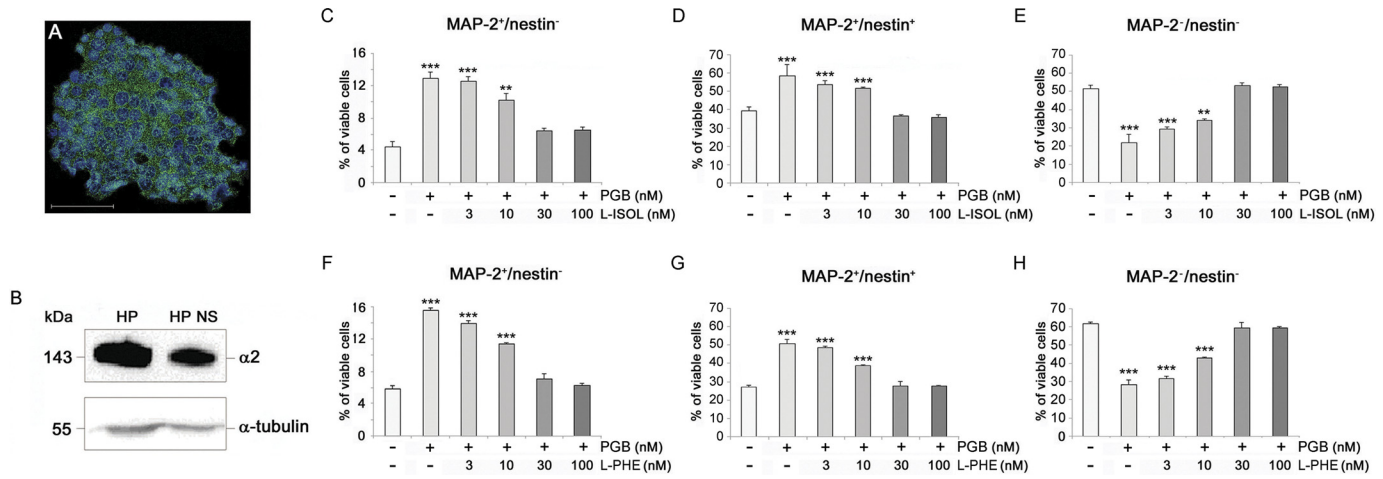

Fig. 3. The $\alpha 2\delta$ subunit mediates PGB proneurogenic effects on adult hippocampal NPCs. A, representative confocal microscopic image of adult hippocampal NPCs grown as neurospheres and immunolabeled for the $\alpha 2\delta 1$ protein (green). Nuclei were stained with DRAQ5 dye (blue). The image indicates that the majority of cells within the neurosphere expressed $\alpha 2\delta 1$. Magnification, 400×. 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 a positive control sample. The protein content was normalized with respect to α-tubulin levels. C to H, quantitation of cell populations after antagonist and ligand treatments. The $\alpha 2\delta$ antagonists L-isoleucine (L-ISOL) (C–E) and L-(+)-α-phenylglycine (L-PHE) (F–H) prevented PGB-mediated proneurogenic effects on adult hippocampal NPCs. L- Isoleucine at 3 to 100 nM significantly inhibited, in a concentration-dependent manner, the PGB-induced increases in the MAP-2$^+$/nestin$^-$ (C) and MAP-2$^+$/nestin$^+$ (D) cell populations and decrease in the MAP-2$^-$/nestin$^-$ cell population (E). L-(+)-α-Phenylglycine at 3 to 100 nM significantly inhibited, in a concentration-dependent manner, the PGB-induced increases in the MAP-2$^+$/nestin$^-$ (F) and MAP-2$^+$/nestin$^+$ (G) cell populations and decrease in the MAP-2$^-$/nestin$^-$ cell population (H). Data are expressed as mean ± S.D. of three experiments, performed in triplicate. **, $p < 0.01$; ***, $p < 0.001$, versus vehicle (Student's t test).

The NF-κB Pathway Is Involved in PGB-Mediated Effects on Neuronal Differentiation of Adult NPCs. Nuclear translocation of several transcription factors, including members of the NF-κB family, is involved in key steps in the neuronal commitment and differentiation of adult NPCs in vitro and in vivo (Denis-Donini et al., 2005, 2008; Rolls et al., 2007; Koo et al., 2010; Meneghini et al., 2010). NF-κB-mediated signaling was suggested to occur downstream of $\alpha 2\delta 1$ subunit activation (Park et al., 2008). Because 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 NPCs 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 increases in MAP-2$^+$/nestin$^-$ (Fig. 4A) and MAP-2$^+$/nestin$^+$ (Fig. 4B) neurons and decrease in MAP-2$^-$/nestin$^-$ cells (Fig. 4C). SN50M (10 μg/ml) did not affect PGB-mediated effects (Fig. 4, A–C). We then tested JSH-23, a cell-permeable, selective blocker of NF-κB p65 nuclear translocation (IC_{50} = 7 μM) (Shin et al., 2004). Like SN-50, 3 μM JSH-23 completely counteracted the effects of 1 nM PGB on MAP-2$^+$/nestin$^-$ (Fig. 4D), MAP-2$^+$/nestin$^+$ (Fig. 4E), and MAP-2$^-$/nestin$^-$ (Fig. 4F) cells. Finally, 3 μM SC-514, a selective reversible inhibitor of IκB kinase 2 (IC_{50} = 3–12 μM) (Baxter et al., 2004), abolished 1 nM PGB-mediated increases in MAP-2$^+$/nestin$^-$ (Fig. 4D) and MAP-2$^+$/nestin$^+$ (Fig. 4E) cells and decrease in MAP-2$^-$/nestin$^-$ cells (Fig. 4F). When applied alone, SN-50, JSH-23, and SC-514 did not affect NPC differentiation (Fig. 4, A–F). At the tested concentrations, SN-50, JSH-23, and SC-514 had no effects on the survival of adult NPCs and/or their progeny (data not shown). Representative images of MAP-2 immunolabeling confirmed that the effects of 1 nM PGB on neuronal differentiation of hippocampal NPCs (Fig. 4G) were completely abolished in the 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 signaling in the proneurogenic effects of PGB on adult hippocampal NPCs.

Chronic PGB Treatment Prevents Depressive Behavior and Promotes Hippocampal Neurogenesis in Stressed Mice. Finally, we decided to determine whether long-term PGB treatment could prevent the appearance of depression-like symptoms elicited by long-term, unpredictable, restraint stress (immobilization for 3 h/day for 21 days). Twelve C57BL/6 mice were subjected to the stress protocol, with half of them receiving vehicle (saline solution) or PGB (10 mg/kg of body weight i.p.) once daily for 21 days. Control unstressed mice (n = 6) were subjected to intraperitonal injections of saline solution once daily. For the first 5 days of the chronic stress procedure, all experimental groups were also treated with BrdU (150 mg/kg of body weight i.p.). On day 21, all groups were tested for immobility with the TST and FST methods. One-way ANOVA between experimental groups revealed significant differences in both the TST ($p < 0.01$) and FST ($p < 0.001$) results. As shown in Fig. 5, A and B, Tukey's post hoc statistical analysis confirmed significant differences between PGB-treated and saline-treated stressed mice for both the TST (Fig. 5A) and the FST (Fig. 5B). As expected, saline-treated stressed mice had significantly increased immobility, compared with saline-treated unstressed mice, both in the TST (116 ± 18 and 43.2 ± 14.4 s, respectively; $p < 0.01$) (Fig. 5A) and in the FST (91.5 ± 8.3 and 40 ± 8.7 s, respectively; $p < 0.001$) (Fig. 5B). In the TST, PGB-treated stressed mice displayed a significantly shorter immobility time, compared with saline-treated stressed mice (43.5 ± 12 and 116 ± 18 s, respectively; $p < 0.05$) (Fig. 5A). In the FST, PGB-treated stressed mice spent less time in

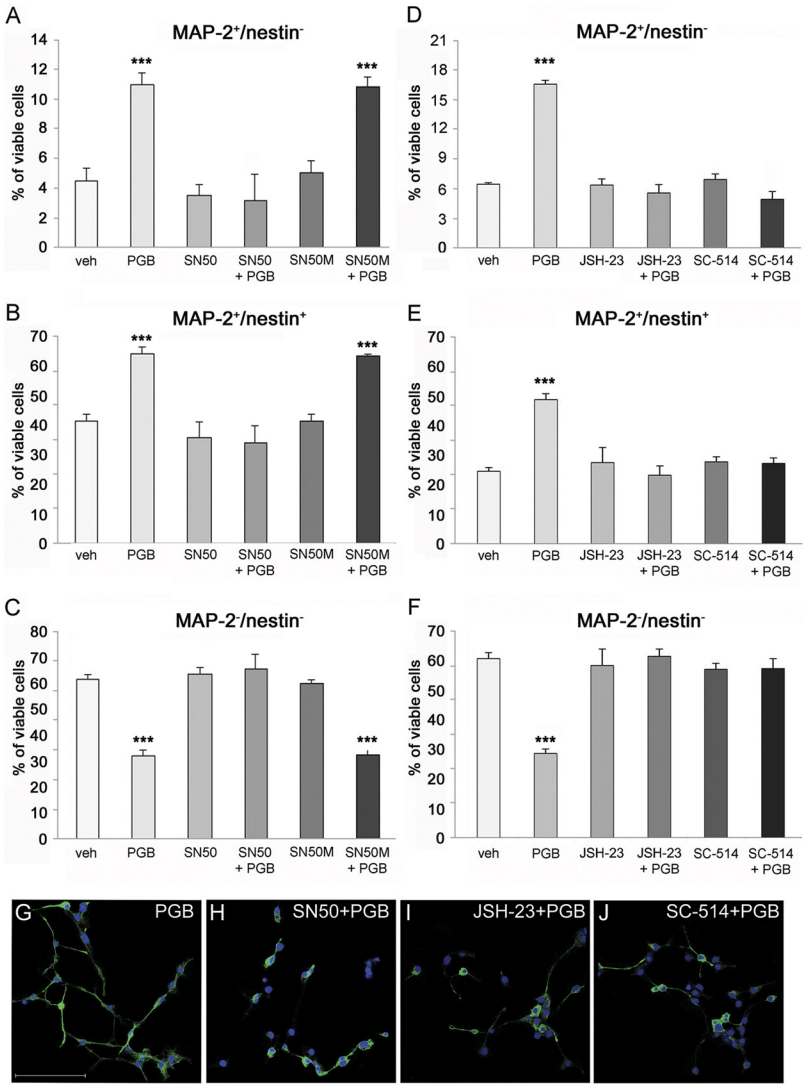

Fig. 4. PGB effects on hippocampal NPC differentiation are mediated through activation of the NF-κB signaling pathway. A to F, quantitation of cell populations after vehicle (veh), ligand, and/or peptide treatment. In hippocampus-derived NPCs, treatment with a NF-κB p50 nuclear translocation-inhibitory peptide (SN-50, at 10 μg/ml) inhibited the increases in the MAP-2$^+$/nestin$^-$ (A) and MAP-2$^+$/nestin$^+$ (B) cell populations and the decrease in the MAP-2$^-$/nestin$^-$ cell population (C) induced by 1 nM PGB. The inactive peptide SN50M did not counteract PGB-mediated effects (A–C). Treatment of hippocampal NPCs with an inhibitor of NF-κB p65 nuclear translocation (JSH-23, at 3 μM) or an IκB kinase 2 inhibitor (SC-514, at 3 μM) abolished the increases in the MAP-2$^+$/nestin$^-$ (D) and MAP-2$^+$/nestin$^+$ (E) cell populations and the decrease in the MAP-2$^-$/nestin$^-$ cell population (F) induced by 1 nM PGB. Data are expressed as mean ± S.D. of three experiments, performed in triplicate. ***, $p < 0.001$, versus vehicle (Student's t test). G to J, representative confocal microscopic images of MAP-2 immunolabeling (green) of differentiated NPCs after 24-h treatment with 1 nM PGB alone (G) or in presence of 10 μg/ml SN50 (H), 3 μM JSH-23 (I), or 3 μM SC-514 (J). Nuclei were stained with DRAQ5 dye (blue). Magnification, 400×. Scale bar, 75 μm.

immobility, compared with saline-treated stressed mice (34.5 ± 4.2 and 91.5 ± 8.3 s, respectively; $p < 0.001$) (Fig. 5B).

Thirty days after the last BrdU injection and 14 days after completion of the stress procedure, mice were transcardially perfused and their brains were immunoprocessed for BrdU/NeuN labeling and assessment of hippocampal neurogenesis, as described previously (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 (1856 ± 78.7 cells per granular cell layer), compared with the saline-treated unstressed group (1413 ± 14.8 cells per granular cell layer; $p < 0.05$) and the saline-treated stressed group (1288 ± 100.7 cells per granular cell layer; $p < 0.01$). These data suggested that chronic PGB treatment prevented depression-like behavior and, in parallel, increased hippocampal neurogenesis in adult mice exposed to long-term stress. As control experiments, we also tested the effects of short-term (single intraperitoneal injection) and long-term (intraperitoneal injection once daily for 21 days) treatment with 10 mg/kg PGB in unstressed mice. In both cases, we noted no effect of the drug, compared with saline solution, with the TST and FST methods (Fig. 5D).

Discussion

Deregulated hippocampal neurogenesis in neuropsychiatric conditions such as major depression and neurodegenerative disorders has been reported (Kempermann et al., 2008; Pittenger and Duman, 2008; DeCarolis and Eisch, 2010; Samuels 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 contributed to the idea that antidepressant-induced increases in hippocampal neurogenesis may be required for at least some drug effects in rodents, primates, and possibly patients (Santarelli et al., 2003; David et al., 2009; Perera et al., 2011).

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

Interestingly, there are reports that these drugs may be effective as supplemental therapy with antidepressants for

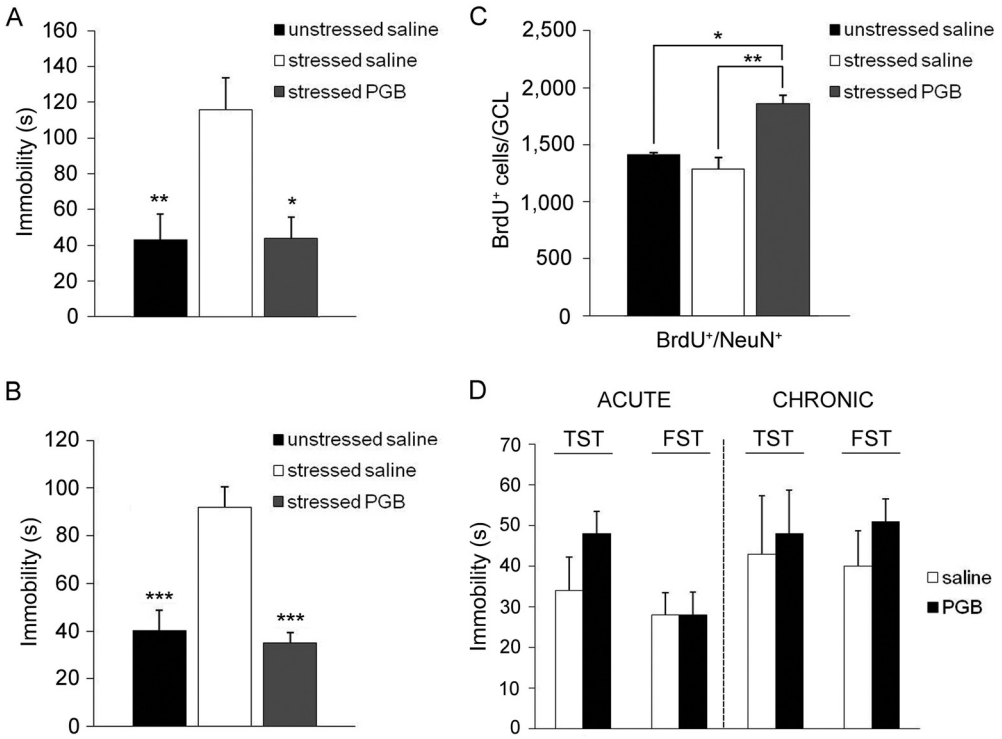

Fig. 5. Chronic PGB treatment prevents the appearance of depression-like behavior and increases hippocampal neurogenesis in stressed mice. A and B, behavior of restraint-stressed mice in the TST (A) and FST (B) after long-term (21-day) administration of saline solution or PGB (10 mg/kg i.p.), in comparison with saline-treated, unstressed mice. Immobilization times were recorded, and data are expressed as mean ± S.E.M. of results for six mice per group. Chronic PGB treatment prevented stress-induced increases in immobilization times in the TST and FST. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, versus saline-treated stressed mice. C, analysis of hippocampal neurogenesis in stressed and unstressed mice. The number of new hippocampal neurons, identified on the basis of double staining for BrdU and NeuN, was significantly increased in the granular cell layer (GCL) of PGB-treated, stressed mice, compared with saline-treated, stressed or unstressed mice. Data are expressed as mean ± S.D. of findings for six mice per group. *, $p < 0.05$; **, $p < 0.01$, versus PGB-treated, stressed mice. D, effects of PGB treatment on immobility times for unstressed mice. Neither acute nor chronic (21-day) PGB treatment affected immobility times for unstressed mice in the TST and FST. Mice were given intraperitoneal injections of 10 mg/kg PGB or saline solution, and the time spent in immobility was measured. Data are expressed as mean ± S.E.M. of findings for six mice per group.

MDD (Pae, 2009), post-traumatic stress disorder (Pae et al., 2009), and depression-like symptoms in generalized anxiety disorders (Stein et al., 2008). No information is available on the mechanism of action of $\alpha 2\delta$ ligands in such clinical settings.

We investigated the activity of the $\alpha 2\delta$ ligands GBP and PGB in an in vitro model of adult mouse NPCs. To identify proneurogenic drugs, we performed double-immunolabeling experiments with markers of neurons (MAP-2) and undifferentiated progenitors (nestin). Through these means, we could identify four cell subpopulations derived from NPCs. One population was the undifferentiated MAP-2^-/nestin$^+$ neurosphere cell population. A second subpopulation was composed of MAP-2^+/nestin$^-$ cells, which we regard as mature neurons. The third population was composed of MAP-2^+/nestin$^+$ cells. In vitro studies aimed at assessing neuronal differentiation of NPCs usually use either a single marker of neuronal differentiation (βIII tubulin or MAP-2) or double/triple staining for markers of neurons, astroglia, and/or oligondrocytes, to evaluate the differentiation of multipotent NPCs toward all lineages. Under these conditions, no double-positive population could be identified. At this point, we think that double-positive cells represent a population in a phase of transition toward neuronal commitment, characterized by MAP-2 expression and persistence of the marker of undifferentiated NPCs. Although rare, MAP-2^+/nestin$^+$ cells were detected in human fetal brain tissue (Messam et al., 2002). A fourth subpopulation was characterized by the absence of both markers. We have evidence that those cells are Sox-2^+ cells (data not shown), a population of undifferentiated progenitors that can give rise to GFAP$^+$ cells and NG2^+ oligodendrocyte precursors (Graham et al., 2003; Brazel et al., 2005). In our experimental setting, PGB and GBP resulted in concentration-dependent increases in both MAP-2^+/nestin$^-$ and MAP-2^+/nestin$^+$ cell populations. In parallel, $\alpha 2\delta$ ligands reduced the population of double-negative cells, with no effects on MAP-2^-/nestin$^+$ cells. No effect on the survival of hippocampal NPCs or their progeny was elicited by PGB or GBP (data not shown). Overall, these data suggested that $\alpha 2\delta$ ligands promoted differentiation of hippocampal NPCs toward the neuronal lineage, mainly at the expense of undifferentiated Sox-2^+ and nestin$^-$ progenitors. PGB had no effect on proliferation of adult hippocampal NPCs in vitro.

Activation of the NF-κB pathway was involved in the proneurogenic effects elicited by $\alpha 2\delta$ ligands in adult hippocampal NPCs, because inhibition of both p50 and p65 nuclear translocation and IκB kinase 2 counteracted PGB-mediated effects. Previous work showed that PGB could inhibit substance P-induced NF-κB activation in neuroblastoma, glioma, and dorsal root ganglia cells (Park et al., 2008). However, our observation that NF-κB activation by pregabalin mediates its proneurogenic effects is in line with reports suggesting that NF-κB signaling 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). NF-κB signaling (in particular, p65 activation) also was shown to be 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 likely reflects the complexity within the NF-κB signaling pathway. NF-κB proteins represent a family of transcription factors (p50, p65, p52, c-Rel, and RelB) whose members can combine to form heterodimers and homodimers of different compositions, which can be differentially activated in any given cell type and can exert different (even opposite) functions through activation of different sets of gene targets. As an example, NF-κB pathway activation is involved in both 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, which suggests that, at least in vitro, drug effects may be mediated through interactions with α2δ1. We showed that the α2δ1 subunit is expressed by adult hippocampal NPCs. Interestingly, PGB- and GBP-mediated effects on neuronal differentiation of adult NPCs were observed in the low nanomolar range, which is consistent with their K_D values (6–7 nM) for the auxiliary α2δ1 subunit of neuronal VGCCs (Lotarski et al., 2011). At this time, we cannot rule out the possibility that the α2δ1 subunit mediates the proneurogenic effects of PGB and GBP independent of its modulatory function within VGCCs. For another member of the α2δ subunit family, the α2δ3 protein (which does not bind PGB and GBP), its function in *Drosophila melanogaster* was demonstrated to be independent of its role in biophysical processes and localization of Ca^{2+} channels (Kurshan et al., 2009). It should be emphasized, however, that the concentrations of α2δ ligands used in most reported studies to elucidate the ligands' molecular mechanisms of action were much higher (in the high micromolar range) than those we used 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 in vivo, long-term administration of PGB resulted in enhanced neurogenesis in adult naive mice, as indicated by increased numbers of $BrdU^+/NeuN^+/GFAP^-$ cells. Drug treatment had no effect on astrogliogenesis but decreased the number of undifferentiated $BrdU^+/NeuN^-/GFAP^-$ cells in the DG. The absolute numbers of surviving $BrdU^+$ cells were not affected by long-term PGB treatment, which suggests that there was no effect on cell survival and/or proliferation, in line with data obtained in vitro. Together, these data support the idea that α2δ ligands promote neurogenesis by favoring 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 was suggested that a reduction in neurogenesis after chronic stress exposure might underlie impaired hippocampal plasticity and might contribute to symptoms of depression (Warner-Schmidt and Duman, 2006; Pittenger and Duman, 2008; Lucassen et al., 2010). The chronic restraint stress test is frequently used to evaluate whether drugs can prevent depression-like symptoms (Luo et al., 2005; Yun et al., 2010). Behavioral efficacy of antidepressants is usually demonstrated by 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 demonstrated that long-term PGB administration prevented the appearance of stress-induced depression-like behavior in both the TST and the FST. It should be noted that the dose of PGB we used in mice was demonstrated (Lotarski et al., 2011) to yield plasma concentrations comparable to those achieved with effective dosages in clinical practice (Bockbrader et al., 2010). Acute or chronic administration of the drug had no effect on TST and FST results for unstressed mice. When mouse brains were examined at the end of the restraint procedure, quantification of $BrdU^+/NeuN^+$ cells within the granular cell layer 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. Behavioral and proneurogenic PGB-mediated effects were correlated under our experimental conditions.

On the basis of these results, we propose that the new pharmacological activity of α2δ ligands may explain their efficacy as supplemental therapy for MDD, as well as depression-like symptoms in post-traumatic stress disorder and generalized anxiety disorder (Stein et al., 2008; Pae, 2009; Pae et al., 2009). Whether the proneurogenic activity of α2δ ligands may contribute to their therapeutic profiles deserves future investigation. Interestingly, α2δ ligands represent first-line treatment for chronic neuropathic pain, a disease state with a high rate of comorbidity with major depression. Defective hippocampal neurogenesis was demonstrated in animal models of neuropathic pain (Terada et al., 2008). In such clinical settings, drugs such as pregabalin, which are able to modulate adult neurogenesis positively and possibly to exert antidepressant activity, may yield additional benefits, compared with drugs that are "pure" analgesics.

Authorship Contributions

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

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

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

Wrote the manuscript: Grilli.

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