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The Noradrenergic Component in Tapentadol Action Counteracts μ-Opioid Receptor–Mediated Adverse Effects on Adult Neurogenesis

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ABSTRACT

Opiates were the first drugs shown to negatively impact neurogenesis in the adult mammalian hippocampus. Literature data also suggest that norepinephrine is a positive modulator of hippocampal neurogenesis in vitro and in vivo. On the basis of these observations, we investigated whether tapentadol, a novel central analgesic combining μ-opioid receptor (MOR) agonism with norepinephrine reuptake inhibition (NRI), may produce less inhibition of hippocampal neurogenesis compared with morphine. When tested in vitro, morphine inhibited neuronal differentiation, neurite outgrowth, and survival of adult mouse hippocampal neural progenitors and their progeny, via MOR interaction. By contrast, tapentadol was devoid of these adverse effects on cell survival and reduced neurite outgrowth and the number of newly generated neurons only at nanomolar concentrations where the MOR component is predominant. On the contrary, at higher (micromolar) concentrations, tapentadol elicited proneurogenic and antiapoptotic effects via activation of β2 and α2 adrenergic receptors, respectively. Altogether, these data suggest that the noradrenergic component in tapentadol has the potential to counteract the adverse MOR-mediated effects on hippocampal neurogenesis. As a proof of concept, we showed that reboxetine, an NRI antidepressant, counteracted both antineurogenic and apoptotic effects of morphine in vitro. In line with these observations, chronic tapentadol treatment did not negatively affect hippocampal neurogenesis in vivo. In light of the increasing long-term use of opiates in chronic pain, in principle, the tapentadol combined mechanism of action may result in less or no reduction in adult neurogenesis compared with classic opiates.

Introduction

Clinical research has shown that opiate addicts display deficits in memory tasks, attention, verbal fluency, and general cognitive performance, relative to controls (Cipolli and Galliani, 1987; Guerra et al., 1987; Gruber et al., 2007). The possibility that chronic opiate treatment in nonaddicted patients may also cause cognitive impairment in the long term is controversial, but is of considerable clinical interest (Kendall et al., 2010; Kurita et al., 2011; Højsted et al., 2012). Although the central depressant effects of opiates complicate the interpretation of drug effects on cognition, evidence from basic research contributes to the idea that chronically administered opiates may interfere with cognition independently of performance effects. In rodents, chronic morphine treatment resulted in impaired acquisition of radial maze and Y-maze choice escape tasks, but did not alter performance of the task if it was learned before drug exposure (Spain and Newsom, 1991). Moreover, in animal models, dependence on morphine did not impair learning ability in the reference memory version of the water Morris maze, but partially impaired memory retention for the previously learned spatial information (Miladi Gorji et al., 2008). These findings suggest that long-term opiate use may produce maladaptive changes in brain structures involved in learning and memory, such as the hippocampus. Underlying mechanisms for a disruption of long-term memory retention in morphine-dependent animals have been suggested to rely on impairment in hippocampal long-term potentiation (Pu et al., 2002), but other mechanisms may play a role and deserve exploration. An intriguing property of the adult hippocampal dentate gyrus (DG) is its ability to generate new neurons throughout life, a process referred to as adult neurogenesis. In particular, new cells are born in the subgranular zone (SGZ) of the DG, where resident neural stem cells can self-renew or give rise to nestin-positive transiently amplifying progenitor cells

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ABBREVIATIONS: ANOVA, analysis of variance; AR, adrenergic receptor; BrdU, bromodeoxyuridine; CGP 20712A dihydrochloride, 1-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol dihydrochloride; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH^2; DG, dentate gyrus; GCL, granular cell layer; GFAP, glial fibrillary acidic protein; ICI 118,551, 3-(isopropylamino)-1-[(7-methyl-4-indanyl)oxy] butan-2-ol; MAP-2, microtubule-associated protein-2; MOR, μ-opioid receptor; NE, norepinephrine; NET, norepinephrine transporter; NeuN, neuronal nuclei; NPC, neural progenitor cell; NRI, norepinephrine reuptake inhibition; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT, reverse transcription; SGZ, subgranular zone.

that, in turn, can generate neuroblasts capable of terminal neuronal differentiation (Kempermann et al., 2003, 2004; Seri et al., 2004; Bonaguidi et al., 2012). Finally, their postmitotic neuronal progeny can integrate into the adult hippocampal circuitry or it can be deleted by apoptosis (Deng et al., 2010). Each step of adult neurogenesis is regulated and it has been postulated to affect hippocampal function in the uninjured brain (Zhao et al., 2008). Interestingly, it has been proposed that new neurons born in the adult hippocampus may contribute to a variety of hippocampal-related functions, including learning and memory (Shors et al., 2001; Aimone et al., 2006, 2011; Denis-Donini et al., 2008; Deng et al., 2010; Couillard-Despres et al., 2011; Sahay et al., 2011).

In rodents, hippocampal adult neurogenesis is profoundly affected by chronically administered opiates. Adult in vivo exposure to μ-opioid receptor (MOR) agonists decreases SGZ progenitor proliferation, maturation, and survival of new neurons (Eisch et al., 2000; Mandyam et al., 2004; Kahn et al., 2005; Eisch and Harburg, 2006; Arguello et al., 2009). On the basis of these observations, it can be hypothesized that alteration in hippocampal neurogenesis may represent one mechanism by which opiates may exert long-lasting effects on the neural circuitry involved in learning, memory, and cognition.

Both preclinical and clinical studies show that tapentadol is a novel centrally acting analgesic drug that has efficacy comparable with that of strong opiates in a broad spectrum of acute and chronic pain conditions, with an improved tolerability profile compared with classic opiates (Tzschentke et al., 2007, 2009; Kress, 2010; Sloan, 2010; Etropolski et al., 2011; Riemsma et al., 2011; Hartrick and Rodríguez Hernandez, 2012; Pergolizzi et al., 2012). Tapentadol combines MOR agonistic activity with norepinephrine reuptake inhibition (NRI) in a single molecule (Tzschentke et al., 2007; Bee et al., 2011; Hartrick and Rozek, 2011; Schröder et al., 2011).

Since literature data suggest that norepinephrine (NE) exerts positive modulation on adult hippocampal neurogenesis (Kulkarni et al., 2002; Jha et al., 2006), we explored whether the noradrenergic activity of tapentadol may counteract the negative MOR-mediated action on neurogenesis. In principle, this counterbalancing effect may result, after long-term treatment in vivo, in less or no dysfunction in adult neurogenesis and cognitive impairment compared with morphine.

Materials and Methods

Animals. Adult male CD1 mice (aged 4–6 months) were purchased from Charles River Laboratories (Calco, Italy). All animals were maintained in high-efficiency particulate air–filtered Thoren units (Thoren Caging Systems, Inc., Hazleton, PA) at the University of Eastern Piedmont animal facility, were kept at three to four per cage, and had unlimited access to water and food. Care and handling of animals were performed in accordance with the National Institutes of Health guidelines and also reviewed and were approved by the local institutional animal care and use committee.

Drugs. The sources for drugs were as follows: tapentadol HCl (Grünenthal, Aachen, Germany), morphine sulfate salt pentahydrate (Sigma-Aldrich, St. Louis, MO), naltrexone HCl (provided by Professor Paola Sacerdote, University of Milan, Milan, Italy), D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH^2 (CTOP) (Tocris Bioscience, Bristol, UK), reboxetine mesylate hydrate (Sigma-Aldrich), doxazosin mesylate (Tocris Bioscience), ICI 118,551 [3-(isopropylamino)-1-[(7-methyl-4-indanyl)oxy] butan-2-ol; Tocris Bioscience], CGP 20712A dihydrochloride [1-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol dihydrochloride; Tocris Bioscience], clonidine hydrochloride and yohimbine hydrochloride (Sigma-Aldrich), and salbutamol (Prestwick Chemical, Illkirch, France).

Isolation and Culture of Adult Hippocampal Neural Progenitor Cells. For each neurosphere preparation, three adult male mice (aged 4–6 months) were euthanized by cervical dislocation. The brains were extracted and hippocampi were isolated under a Zeiss (Carl Zeiss, Oberkochen, Germany) dissecting microscope using fine surgical instruments and collected in ice-cold 1,4-piperazinediethanesulfonic acid buffer, pH 7.4, containing 20 mM 1,4-piperazinediethanesulfonic acid, 25 mM glucose, 0.5 M KCl, 0.12 M NaCl (Sigma-Aldrich), and 100 U/100 μg/ml penicillin/streptomycin solution (Life Technologies, Monza, Italy). After centrifugation (110g × 5 minutes), tissue was digested for 40 minutes at 37°C using the Papain Dissociation System (Worthington DBA, Lakewood, NJ). Cell suspension was plated onto a 25-cm^2 Falcon cell-culture flask (Delchimica Scientific Glassware, Naples, Italy) and cultured as previously described (Valente et al., 2012). Primary (passage 1, P1) neurospheres were dissociated after 7–9 days, whereas P2–P30 neurospheres every 5 days in vitro. P3–P30 neurospheres were used for experiments.

Neural Progenitor Cell Differentiation. For differentiation, neurospheres were dissociated and plated onto laminin-coated (2.5 μg/cm^2; Sigma-Aldrich) Laboratory-Tek eight-well Permanox Chamber Slides (Nunc, Wiesbaden, Germany) at a density of 43,750 cells/cm^2 in differentiating medium (Neurobasal-A medium [Life Technologies] containing B27 supplement, 2 mM L-glutamine, and 100 U/100 μg/ml penicillin/streptomycin). Neural progenitor cells (NPCs) were treated in the presence of indicated concentrations of drugs or vehicle for 24 hours. In each experiment, five fields per well (corresponding to about 150–200 cells per well) were counted using the Eclipse E600 fluorescence microscope (Nikon, Calenzano, Italy) with a ×60 objective. All experiments were run in triplicate using different cell preparations and were repeated at least three times.

Immunocytochemical Analysis. After fixation, neurosphere-derived differentiated cells were washed three times in phosphate-buffered saline (PBS) and permeabilized in PBS containing 0.48% (v/v) Triton X-100 (Sigma-Aldrich), for 5 minutes at room temperature. The primary antibodies against microtubule-associated protein-2 (MAP-2; rabbit polyclonal, 1:600; Chemicon, Temecula, CA) and β-III tubulin (Tuj-1 clone, chicken monoclonal, 1:2000; Aves Laboratories Inc., Tigard, OR) were incubated for 150 minutes at room temperature in an antibody solution containing 16% (v/v) goat serum. Secondary antibodies were as follows: Alexa Fluor 555–conjugated goat anti-rabbit antibody (1:1400; Molecular Probes, Eugene, OR) and Alexa Fluor 488–conjugated goat anti-chicken antibody (1:1400; Molecular Probes) in a solution containing 16% (v/v) goat serum. Nuclei were counterstained with Draq5 (1:2000; Alexis Biochemicals, San Diego, CA) or Hoechst (0.8 ng/ml; Sigma-Aldrich), diluted in PBS. Slides were coverslipped with fluorescent mounting medium (DakoCytomation, Glostrup, Denmark) as an antifading agent. Adobe Photoshop CS (Adobe Systems Incorporated, San Jose, CA) was used for digital processing of the images. Only light intensity, brightness, and contrast adjustments were applied to improve information.

Morphologic Analysis. To evaluate neurite growth and arborization, cells were differentiated in vitro for 24 hours in the presence of drugs or vehicle; thereafter, an immunolocalization with an antibody against Tuj-1 was performed. Tuj-1^+ newly generated neurons and neuroblasts were then grouped based on the absence or presence of neurites. In the latter case, Tuj-1^+ cells were also grouped as unipolar versus bipolar and based on the presence or absence of arborizations. In each experiment, five fields per well (corresponding to 60–90 Tuj-1^+ cells per well) were counted using the Eclipse E600 fluorescence microscope (Nikon) with a ×60 objective. All experiments were run in triplicate using different cell preparations and were repeated at least three times.

Assessment of Cell Viability. Necrosis evaluation in NPC cultures was performed as previously described (Meneghini et al., 2010). For apoptosis quantification, the in situ terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling assay was performed by the In Situ Cell Death detection kit (Roche

Diagnostic, Monza, Italy), in accordance with the manufacturer's instructions. All experiments were run in triplicate using different cell preparations and were repeated at least three times.

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction Analysis. Total mRNA was extracted from hippocampus and hippocampal neurospheres (1–3 $\times$ 10^6 cells) of adult mice by using the SV Total RNA Isolation System (Promega, Milan, Italy), according to the manufacturer's instructions. The cDNA was obtained by using the ImProm-II Reverse Transcription System kit (Promega). The primer sequences used for polymerase chain reaction (PCR) amplification were as follows: MOR, sense 5′-ATACAGGCAGGGGTCCATAG-3′ and antisense 5′-GTCCATAACACACAGTGATGATGA-3′; and norepinephrine transporter (NET), sense 5′-CTTCTGGCGCGAATGAAT-3′ and antisense 5′-CATTGCGTTCCTTCACCACT-3′. PCR reactions were carried out by using GoTaq Flexi DNA polymerase (Promega) in a final volume of 25 μl containing 20 ng cDNA, 0.4 mM of each primer, 0.2 mM dNTPs, and 2.5 mM $MgCl_2$. PCR conditions for MOR amplification were as follows: 95°C for 10 minutes; 40 cycles, 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds; and final elongation at 72°C for 10 minutes. PCR conditions for NET amplification were as follows: 95°C for 10 minutes; 40 cycles, 94°C for 30 seconds, 61°C for 30 seconds, and 72°C for 45 seconds; and final elongation at 72°C for 10 minutes. PCR products were run onto 2% agarose gels and bands were visualized by staining with ethidium bromide (Sigma-Aldrich).

In Vivo Studies. Adult male CD1 mice (aged 3 months) were randomly distributed into vehicle (n = 10) and tapentadol treatment groups (n = 12). Tapentadol (20 mg/kg body weight) and corresponding vehicle (saline) were administered intraperitoneally daily for 21 days. On the last day of treatment, mice were given three doses of bromodeoxyuridine (BrdU; 50 mg/kg i.p.), at 2-hour intervals. Twenty-four hours after the last BrdU dose, half of the mice (n = 5 and 6 of vehicle- and tapentadol-treated mice, respectively) were transcardially perfused, and their brains were removed and prepared for immunohistochemical analysis as previously described (Bonini et al., 2011). The remaining mice were transcardially perfused 21 days after the last BrdU administration. Coronal brain sections (40-μm thick) were cut and immunoprocessed for rat monoclonal anti-BrdU (1:200; Novus Biologicals, Littleton, CO), goat anti–glial fibrillary acidic protein (GFAP; 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), and mouse anti–neuronal nuclei (NeuN; 1:150; Millipore, Billerica, MA) as previously described (Denis-Donini et al., 2008). For quantification and phenotypic characterization of proliferating and newborn cells, a modified unbiased stereology protocol was used, as previously reported (Meneghini et al., 2013).

Statistical Analysis. In all in vitro experiments, data are reported as the mean ± S.E.M. of at least three experiments in triplicate. In vivo data are reported as the mean ± S.D. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's post hoc test or t test. The statistical significance level was set for P values <0.05.

Results

MOR and NET Transcripts Are Expressed in Undifferentiated Hippocampal Neural Progenitors. In our experimental setting, NPCs from the adult mouse hippocampus are grown in the presence of epidermal growth factor and basic fibroblast growth factor-2 as floating neurospheres and are phenotypically characterized by the expression of nestin and SRY-related HMG-box gene 2, markers of undifferentiated neural progenitors, and by the absence of markers of mature and immature neurons such as MAP-2, Tuj-1, and doublecortin (Valente et al., 2012). As shown in Fig. 1, reverse transcription (RT)-PCR analysis demonstrated that both MOR (Fig. 1A) and the NET (Fig. 1A) transcripts were expressed by undifferentiated hippocampal neurospheres as well as in adult hippocampus, used as a positive control. Hippocampus and neurosphere RT^- reactions (HP RT^- and NS RT^-) served as negative controls.

Morphine Negatively Affects Neuronal Differentiation and Survival of Hippocampal Neural Progenitors Acting via MOR Activation. Upon removal of growth factors from the medium, NPCs stop dividing and differentiate giving rise to neurons, astrocytes, and oligodendrocytes (Cuccurazzu et al., 2013). To characterize the effects of drugs acting on neuronal differentiation of hippocampal NPCs, we performed immunolabeling experiments with antibodies against the neuronal marker MAP-2. Under differentiating conditions, we evaluated the effect of NPC exposure to morphine concentrations (0.3–10 nM), which are consistent with drug affinity values for mouse MOR [$K_{i(MOR)}$ = 2.2 nM]. As shown in Fig. 1B, the drug significantly decreased the percentage of MAP-2^+ neurons (ANOVA, $P < 0.05$) generated in vitro from adult hippocampal NPCs, in a concentration-dependent manner. Moreover, the deleterious effects of morphine on neuronal differentiation were abolished by naltrexone (10 nM) or by CTOP (1 nM), a highly selective MOR antagonist [$K_{i(MOR)}$ = 1.1 nM]. To further investigate the negative effects of morphine on neuronal differentiation and/or survival of adult hippocampal NPCs and their progeny, cells were differentiated in vitro in the presence of vehicle, 10 nM morphine, 10 nM naltrexone, or both morphine and naltrexone. Thereafter, immunolocalization with a Tuj-1 antibody was performed, since this marker allows us to effectively immunodecorate all neurites of newly generated neurons and neuroblasts. Tuj-1^+ cells were then grouped based on the absence or presence of neurites, and in the latter case, were grouped as unipolar versus bipolar, and with or without neurite arborizations. As shown in Fig. 1, C–G, morphine negatively affected neurite outgrowth of newly generated cells from adult NPCs since it significantly decreased the percentage of bipolar cells (t test, $P < 0.05$; Fig. 1G) and of arborized unipolar and bipolar cells (t test, $P < 0.05$ and $P < 0.01$, respectively; Fig. 1G) compared with the vehicle. As expected, in parallel, the drug increased the number of cells without neurites (t test, $P < 0.01$; Fig. 1G). In addition, naltrexone abolished the effects of morphine on neurite outgrowth (t test, $P < 0.01$; Fig. 1G). We also investigated the effect of morphine on the apoptotic and necrotic rate in NPC cultures under differentiating conditions. As shown in Fig. 1H, compared with vehicle, morphine increased the percentage of apoptotic nuclei over the total number of cells in a concentration-dependent manner (ANOVA, $P < 0.01$). Conversely, morphine had no effect on necrosis, as measured by assessing lactate dehydrogenase activity in the culture medium (data not shown). Cotreatment with naltrexone completely prevented morphine-mediated effects on apoptotic death (t test, $P < 0.01$; Fig. 1H). Similar results were obtained in the presence of CTOP (Fig. 1H). Importantly, naltrexone or CTOP alone had no effect on neuronal differentiation (Fig. 1B), neurite outgrowth (Fig. 1, E and G), and apoptotic rate (Fig. 1H), suggesting that endogenous opioids were not present in the culture. Overall, these data suggest that, in the in vitro culture model, morphine inhibits neuronal differentiation and neurite outgrowth and increases the apoptotic rate of NPCs and their progeny via MOR activation.

Tapentadol Negatively Affects Neuronal Differentiation, but Not Survival, of Adult Hippocampal Neural Progenitors. The effects of tapentadol were investigated under the same experimental conditions. The drug was tested over a wide range of concentrations (1 nM to 30 μM) to evaluate

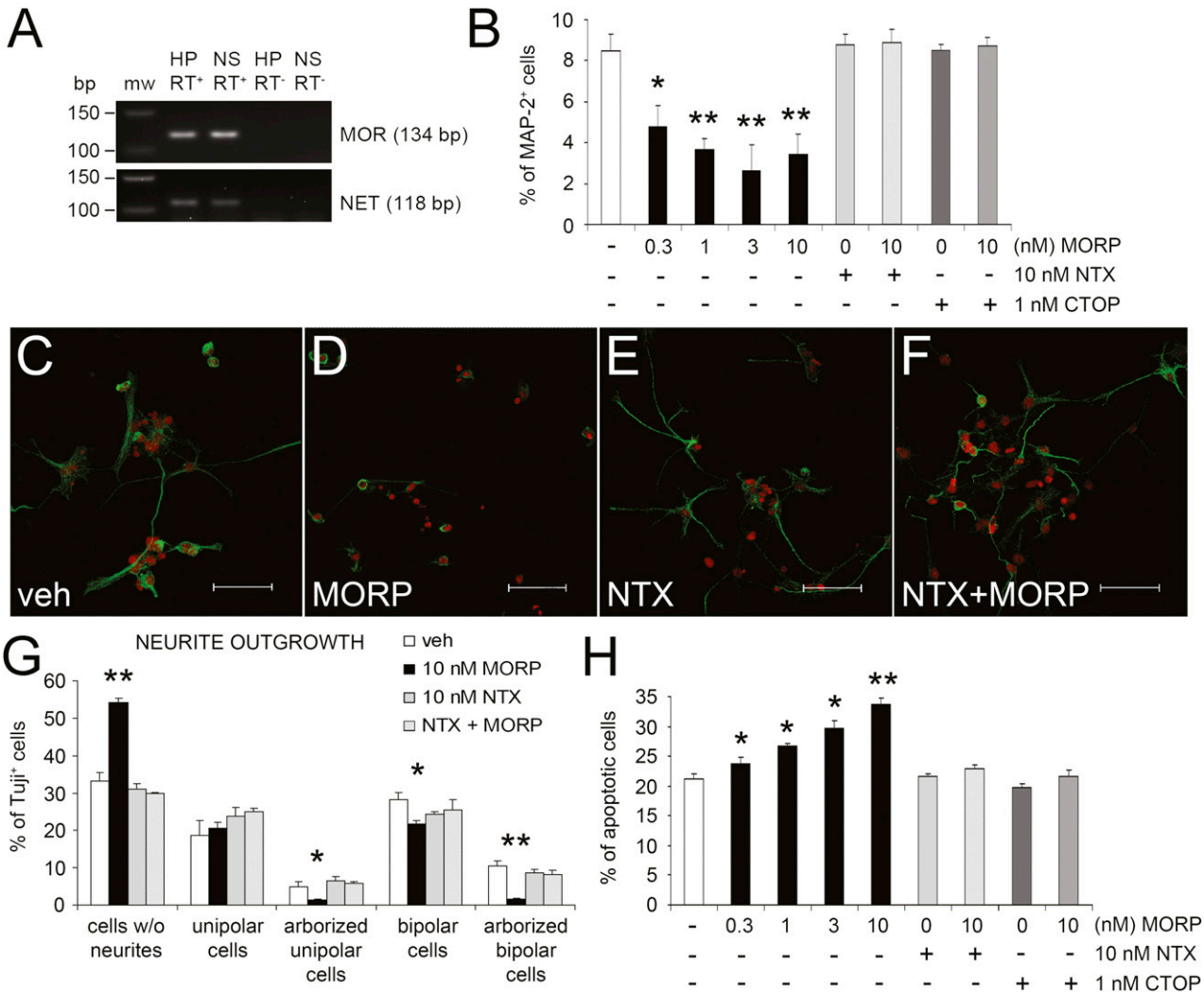

Fig. 1. Deleterious effects of morphine on hippocampus-derived adult NPCs and their neuronal progeny. (A) RT-PCR analysis confirmed that both MOR and NET transcripts (134- and 118-bp amplicons, respectively) are expressed in hippocampal NPCs grown as neurospheres (NS RT$^+$). The positive control for RT-PCR experiments was cDNA from adult hippocampus (HP RT$^+$), whereas hippocampus and NS RT$^-$ reactions (HP RT$^-$ and NS RT$^-$) served as negative controls. The 50-bp DNA ladder was used as a molecular weight marker. (B) Twenty-four–hour treatment with 0.3–10 nM morphine reduced neuronal differentiation from adult hippocampal NPCs by decreasing the percentage of MAP-2$^+$ cells compared with vehicle. The antineurogenic effects of 10 nM morphine were abolished in the presence of 10 nM naltrexone or 1 nM CTOP. Both antagonists were devoid of effects when added alone. (C–F) Representative fluorescence microscopy images of Tuj-1 immunolabeling (green) in cells derived from hippocampal NPCs after 24-hour treatment with vehicle (C), 10 nM morphine (D), 10 nM naltrexone (E), and 10 nM morphine and 10 nM naltrexone (F). Nuclei are stained with Draq5 (red). (G) Ten nanomoles of morphine treatment decreased neurite outgrowth and arborization in newborn Tuj-1$^+$ neuroblasts, as demonstrated by a significant decrease in the percentage of bipolar cells, arborized unipolar and bipolar cells, and, in parallel, by an increase in the number of cells without neurites. Naltrexone, inactive alone, abolished the effects of morphine on neurite outgrowth. (H) The percentage of apoptotic cells in NPC cultures increased in a concentration-dependent manner in the presence of 0.3–10 nM morphine. Proapoptotic effects of 10 nM morphine were totally counteracted by 10 nM naltrexone and 1 nM CTOP. Both antagonists were devoid of effects when added alone. Data are expressed as the mean ± S.E.M. of three experiments in triplicate. *$P < 0.05$; **$P < 0.01$ versus vehicle-treated cells (t test). HP, hippocampus; MORP, morphine; mw, molecular weight; NS, neurosphere; NTX, naltrexone; veh, vehicle. Bar, 75 μm in C–F. Original magnification, 400× in C–F.

both its opioidergic and noradrenergic activity. As shown in Fig. 2A, tapentadol produced a U-shaped curve when added to the culture medium of adult hippocampal NPCs undergoing differentiation in vitro. Within the 30–100 nM range, consistent with MOR affinity values in mouse [$K_{i(MOR)} = 96$ nM], the drug produced a concentration-dependent decrease in the percentage of newly generated MAP-2$^+$ neurons (ANOVA, $P < 0.01$), with a maximal effect observed at 100 nM (t test, $P < 0.001$). Higher tapentadol concentrations (300 nM to 1 μM) still decreased neuronal differentiation but drug effects progressively diminished in a concentration-dependent manner (ANOVA $P < 0.01$). Moreover, tapentadol concentrations of 3–30 μM had no effect on neuronal differentiation of adult mouse hippocampal NPCs. Surprisingly, unlike morphine, tapentadol did not significantly increase the apoptotic rate at any tested concentration (Fig. 2B). Interestingly, tapentadol had significant antiapoptotic effects in the concentration range of 3–30 μM (t test, $P < 0.01$; Fig. 2B). Finally, similar to morphine, the drug had no effect on the necrotic rate in vitro of NPCs and their progeny, as measured by assessing lactate dehydrogenase activity in the culture medium (data not shown). When tapentadol was tested for its influence on neurite outgrowth in NPC cultures under differentiating conditions, drug concentrations corresponding to 100 and 300 nM showed inhibitory effects, similarly to 10 nM morphine

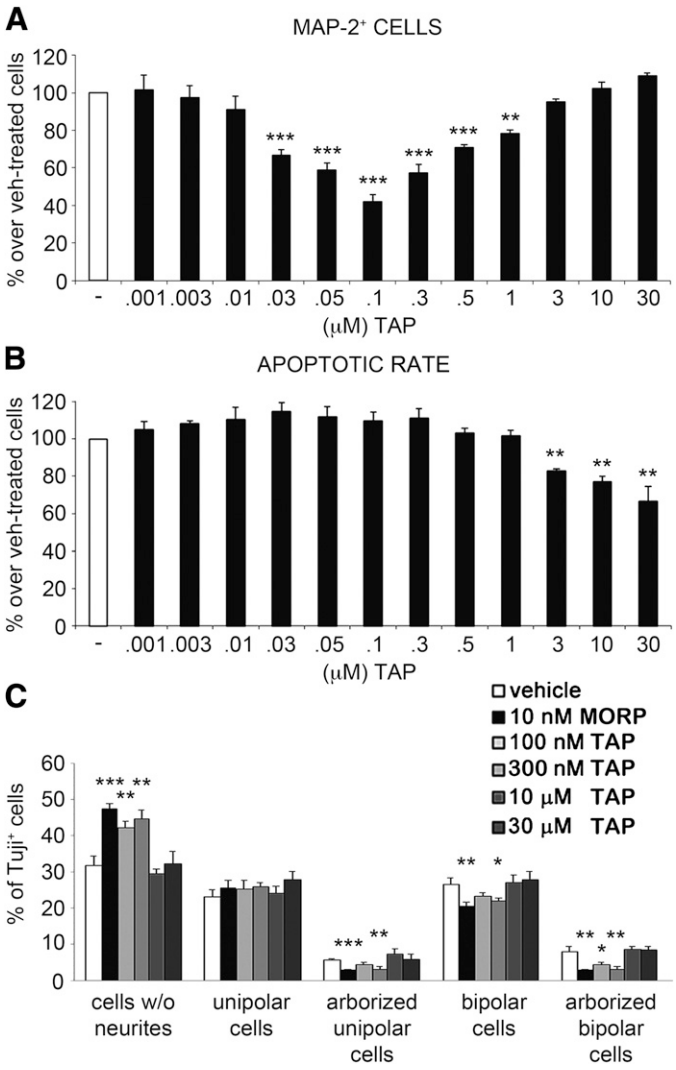

Fig. 2. Effects of tapentadol on hippocampus-derived NPCs and their neuronal progeny. (A) Twenty-four–hour treatment of NPCs with a wide range of tapentadol concentrations (0.001–30 μM) resulted in a characteristic U-shaped curve on the number of newly born MAP-2$^+$ cells. Specifically, 0.03–0.5 μM tapentadol reduced neuronal differentiation from adult hippocampal NPCs by decreasing the percentage of MAP-2$^+$ cells compared with vehicle. (B) Under a wide range of concentrations (0.001–1 μM), tapentadol had no effect on the apoptotic rate in NPC cultures. Higher drug concentrations (3–30 μM) exerted an antiapoptotic effect. (C) Effects of low and high concentrations of tapentadol on neuritic outgrowth of NPC cultures under differentiating conditions. The results showed that 100–300 nM tapentadol, similarly to 10 nM morphine, decreased both neurite outgrowth and arborization in Tuj-1$^+$ neuroblasts, as demonstrated by a significant decrease in the percentage of bipolar cells, arborized unipolar and bipolar cells, and by an increase in the number of cells without neurites. Conversely, 10–30 μM tapentadol had no effect on neurite outgrowth and arborization in Tuj-1$^+$ cells. Data are expressed as the mean ± S.E.M. of three experiments in triplicate. *$P <$ 0.05; **$P < 0.01$; ***$P < 0.001$ versus vehicle-treated cells (t test). MORP, morphine; TAP, tapentadol; veh, vehicle.

(t test, $P < 0.01$; Fig. 2C). Once again, higher concentrations of tapentadol (10–30 μM) exerted no effect on neurite outgrowth and arborization compared with vehicle-treated cells (Fig. 2C).

The Concentration-Dependent Effects of Tapentadol on NPC Neuronal Differentiation and Survival Are Mediated by MOR and by α2/β2 Adrenergic Receptors. We next investigated the contribution of MOR agonism and NRI activity on tapentadol-mediated effects. As shown in Fig. 3, naltrexone completely abolished the inhibitory effect of low tapentadol concentrations (50 nM to 1 μM) on both neuronal differentiation (t test, $P < 0.001$; Fig. 3A) and neurite outgrowth (t test, $P < 0.01$; Fig. 3, B–D).

Functional β2 adrenergic receptors (ARs) are expressed in our experimental model. Indeed, treatment with 10–300 nM salbutamol, a selective β2AR agonist, significantly increased the percentage of newly generated MAP-2$^+$ neurons in a concentration-dependent manner compared with vehicle (ANOVA, $P < 0.05$; Fig. 4A). No effect of β2AR activation on the apoptotic rate of NPCs and their progeny was detected at any tested salbutamol concentration (data not shown). On the basis of these observations, we then investigated whether the effects of tapentadol on neuronal differentiation were dependent on its NRI activity and potentially mediated by β2AR. We exposed NPC cultures to 1 nM to 30 μM tapentadol in the presence of 100 nM ICI 118,551, a selective β2AR antagonist, or the corresponding vehicle. As shown in Fig. 4B, ICI 118,551 had no effect on tapentadol-mediated effects up to a drug concentration of 1 μM. Within tapentadol concentration range of 3–30 μM, which has no apparent effect on neuronal differentiation in vitro, pretreatment with ICI 118,551 significantly reduced the percentage of MAP-2$^+$ newly generated neurons, when compared with tapentadol alone (t test, $P <$ 0.05). Similarly, although both 100 nM ICI 118,551 and 30 μM tapentadol had no effect alone, when added together they resulted in a significant reduction of neurite outgrowth in Tuj-1$^+$ cells (Fig. 4, C–E), as demonstrated by a decreased percentage of bipolar cells (t test, $P < 0.01$; Fig. 4E) and arborized bipolar cells (t test, $P < 0.05$; Fig. 4E) and, in parallel, an increased percentage of cells with no neurites or unipolar (t test, $P < 0.05$ and $P < 0.001$, respectively; Fig. 4E) compared with vehicle. In addition, 100 nM ICI 118,551 counteracted the prosurvival effect of 3–30 μM tapentadol (Fig. 4F). We also evaluated the potential contribution of β1AR activation in tapentadol-mediated effects. As shown in Fig. 4, G and H, in the presence of tapentadol 3–30 μM, treatment with 10 nM CGP 20712A, a selective β1AR antagonist, did not produce significantly different numbers of MAP-2$^+$ neurons (Fig. 4G) and apoptotic cells (Fig. 4H), compared with tapentadol alone. Moreover, CGP 20712A per se had no effect on neuronal differentiation and apoptotic rate when used at 10 nM (Fig. 4, G and H) and over a wider range of concentrations (0.1–100 nM; data not shown). Since several articles have demonstrated the involvement of α2AR subtypes in tapentadol-mediated analgesia (Bee et al., 2011; Hartrick and Rozek, 2011; Schiene et al., 2011), we then tested the effect of yohimbine (1–100 nM), a well characterized α2AR antagonist, in our culture model. As shown in Fig. 5A, 1–3 nM yohimbine was ineffective, whereas higher drug concentrations (10–100 nM) produced a small, but statistically significant, increase in the percentage of MAP-2$^+$ neurons ($P < 0.05$), without affecting NPC survival (data not shown). When an inactive concentration of yohimbine (3 nM) was added to the culture medium in the presence of 30 μM tapentadol, the antiapoptotic activity of the tapentadol was prevented (t test, $P < 0.05$; Fig. 5B). Conversely, no significant difference was observed in the number of MAP-2$^+$ neurons generated in vitro when NPCs were treated with 30 μM tapentadol in the absence or presence of yohimbine (Fig. 5C). We also evaluated the potential involvement of α1AR in tapentadol-mediated activity. The effect of the selective α1AR antagonist doxazosin (0.01 nM to 1 μM) was initially tested in

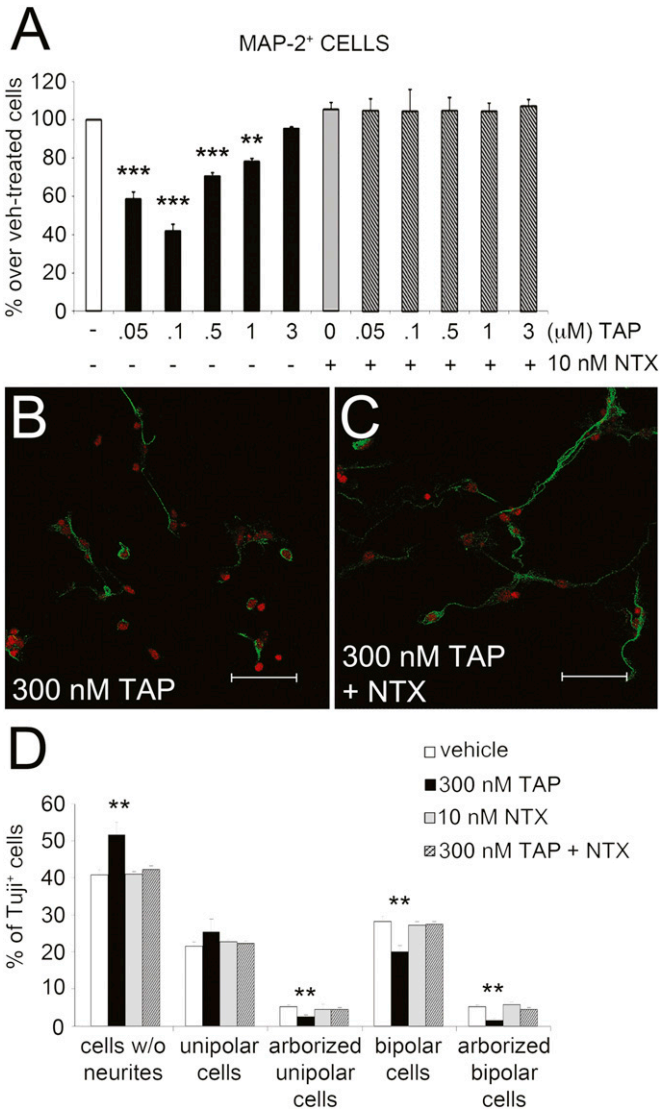

Fig. 3. The deleterious effects of low tapentadol concentrations on neuronal differentiation and neurite outgrowth are MOR mediated. (A) Naltrexone (10 nM) completely counteracted tapentadol-induced reduction of MAP2$^+$ cells generated by adult hippocampal NPCs. (B and C) Representative fluorescence microscopy images of Tuj-1 immunolabeling (green) in cells derived from hippocampal NPCs after 24-hour treatment with 300 nM tapentadol in the absence (B) and presence (C) of 10 nM naltrexone. Nuclei are stained with Draq5 (red). (D) The reduction in neurite outgrowth and arborization elicited by 300 nM tapentadol was counteracted in the presence of 10 nM naltrexone. Data are expressed as the mean ± S.E.M. of three experiments performed in triplicate. $^{**}P < 0.01$; $^{***}P < 0.001$ versus vehicle-treated cells (t test). NTX, naltrexone; TAP, tapentadol; veh, vehicle.

our culture model. Starting at 0.1 nM, doxazosin increased, in a concentration-dependent manner, the percentage of MAP-2$^+$ neurons (ANOVA, $P < 0.05$; Fig. 5D), with its maximal effect reached at 100 nM, suggesting the presence of an α1AR-mediated noradrenergic tone in our culture system. No significant difference was observed in the number of MAP-2$^+$ neurons generated in vitro when NPCs were treated with 30 μM tapentadol in the absence or presence of doxazosin 0.03 nM, a concentration that per se has no effect on neurogenesis (Fig. 5E). Moreover, unlike yohimbine, 0.03 nM doxazosin could not counteract the antiapoptotic effect of 30 μM tapentadol (Fig. 5F). To complete our pharmacological characterization, we then tested clonidine (1 nM to 1 μM), a commonly used α2AR agonist. Our results showed that 10–1000 nM clonidine significantly increased the percentage of MAP-2$^+$ neurons compared with vehicle (Fig. 5G). Conversely, clonidine did not affect the apoptotic rate of NPCs and their progeny at any tested concentration (data not shown). The proneurogenic effects elicited by clonidine could be potentially explained by the fact that the drug can act as a partial agonist at α2AR and as an antagonist at α1AR (Silva et al., 1996). Altogether, our extensive pharmacological characterization suggested that at micromolar concentrations, tapentadol counteracted the negative MOR-mediated effects on neurogenesis via β2AR activation, whereas it elicited antiapoptotic effects via activation of both β2AR and α2AR.

Since nanomolar (MOR interacting) concentrations of tapentadol had no effect on apoptosis (unlike morphine), we decided to test the effect of CTOP in the presence of tapentadol. Although, as expected, 100 nM tapentadol alone had no effect on apoptosis, MOR blockade by CTOP (1–10 nM) in the presence of tapentadol resulted in a reduced apoptotic rate compared with drug alone (Fig. 5H). These data suggest that, in vitro, the MOR-dependent proapoptotic effects of tapentadol are also counteracted by antiapoptotic drug–mediated effects.

Morphine-Mediated Antineurogenic and Proapoptotic Effects on Adult NPCs and Their Progeny Can Be Counteracted by a Noradrenaline Reuptake Inhibitor. Overall, our data suggested that the noradrenergic component in tapentadol has the potential to counteract most of the deleterious MOR-mediated effects on hippocampal neurogenesis. As a proof of concept, we decided to mimic the dual mechanism of action of tapentadol, by cotreating NPC cultures with morphine and reboxetine, an NRI antidepressant drug. As shown in Fig. 6A, 100 nM reboxetine, which alone promotes neuronal differentiation of adult hippocampal NPCs, was able to counteract inhibition of neuronal differentiation elicited by 10 nM morphine (t test, $P < 0.01$). More interestingly, 100 nM reboxetine also counteracted the proapoptotic effects of morphine (t test, $P < 0.001$; Fig. 6B), even if at that concentration the drug alone has no effect on cell survival.

Chronic Tapentadol Treatment Does Not Negatively Affect Hippocampal Neurogenesis In Vivo. Adult male mice ($n = 22$) were injected intraperitoneally once daily with vehicle (saline) or 20 mg/kg tapentadol for a period of 21 days. At the end of the administration period, mice were injected with the thymidine analog BrdU (50 mg/kg i.p., three times at 2-hour intervals) to label proliferating cells in the S-phase. A group of animals ($n = 11$) was euthanized 24 hours later, whereas a second group ($n = 11$) was euthanized 21 days after BrdU administration. The number of BrdU-labeled cells in the SGZ and in the granular cell layer (GCL) within the DG of vehicle- and tapentadol-treated mice was quantified by a modified unbiased stereology protocol as previously described (Denis-Donini et al., 2008). As shown in Fig. 7A, we observed no significant difference in the absolute number of BrdU$^+$ cells in the SGZ or GCL of mice treated with 20 mg/kg tapentadol in the 24-hour group compared with vehicle (mean 588 ± 87.5 and 488 ± 186.1 BrdU$^+$ cells in the SGZ in vehicle- and tapentadol-treated mice, respectively; mean 888 ± 297.3 and 637 ± 165.4 BrdU$^+$ cells in the GCL in vehicle- and tapentadol-treated mice, respectively). These data confirm that, unlike morphine (Eisch et al., 2000; Arguello et al., 2009), chronic tapentadol treatment has no negative effects on the proliferative rate of adult hippocampal neural progenitors. In the 21-day group, we

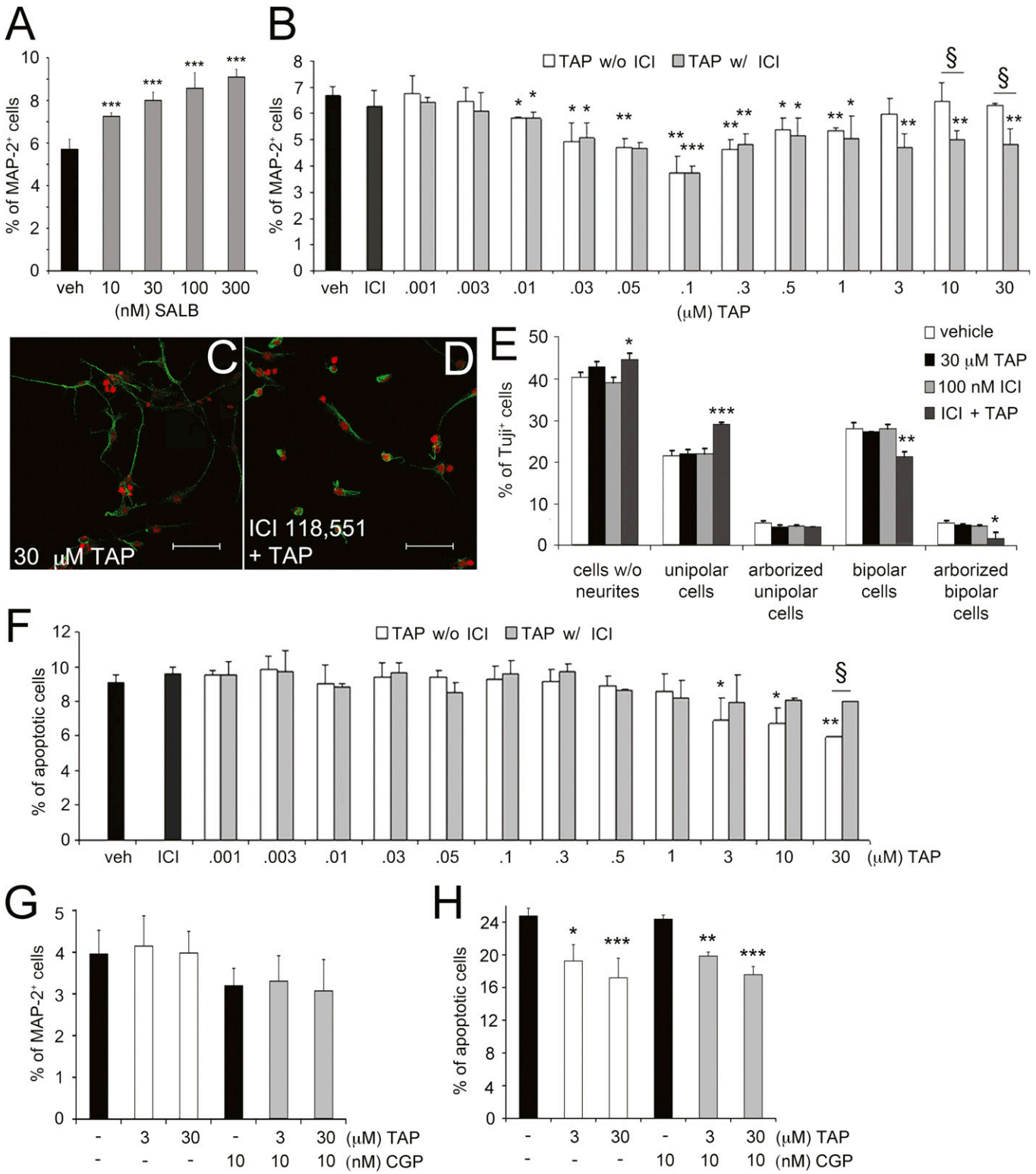

Fig. 4. Activation of β2AR mediates tapentadol-dependent effects on neuronal differentiation, neurite outgrowth, and apoptotic rate. (A) Evaluation of the percentage of MAP-2$^+$ cells in adult hippocampal NPC cultures treated for 24 hours with 10–300 nM salbutamol. The selective β2AR agonist significantly increased the percentage of newly generated neurons in a concentration-dependent manner. (B) Quantification of the percentage of MAP-2$^+$ cells in adult hippocampal NPCs treated with 0.001–30 μM tapentadol in the absence or presence of 100 nM ICI 118,551, a selective β2AR antagonist. ICI 118,551, which alone had no effect, significantly reduced the percentage of MAP-2$^+$ cells in the presence of 10–30 μM tapentadol. (A and B) Data are expressed as the mean ± S.D. of three experiments in triplicate. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus vehicle-treated cells; $^{\S}P < 0.05$ versus tapentadol-treated cells (t test). (C and D) Representative fluorescence microscopy images of Tuj-1 immunolabeling (green) in cells derived from hippocampal NPCs after 24-hour treatment with 30 μM tapentadol alone (C) or in the presence of 100 nM ICI 118,551 (D). Nuclei are counterstained with Draq5 (red). (E) Although 30 μM tapentadol and 100 nM ICI118,551 had no effect alone, when added together they resulted in a significant reduction of neurite outgrowth in Tuj-1$^+$ cells, as demonstrated by a decreased percentage of bipolar and arborized bipolar cells and a parallel increased percentage of cells with no neurites or unipolar cells compared with vehicle. Data are expressed as the mean percentage ± S.E.M. of Tuj-1$^+$ cells over

phenotypically characterized $BrdU^+$ cells by performing a triple immunolabeling with antibodies raised against BrdU, NeuN (a marker of mature neurons), and GFAP (a marker of astrocytes). The number of immunolabeled cells was quantified in the GCL of mice hippocampi, the region where newly generated cells can migrate and, if they survive, acquire their neuronal or astroglial phenotype. Moreover, the total number of $BrdU^+$ cells was determined to evaluate differences in the survival rate of BrdU-labeled cells at 3 weeks after their labeling. As shown in Fig. 7B, the total number of $BrdU^+$ cells was not significantly different in mice chronically treated with 20 mg/kg tapentadol compared with vehicle-treated animals, although a trend toward increase was observed in drug-treated mice (mean 155 ± 66.1 and 202 ± 74.3 in the SGZ in vehicle- and tapentadol-treated animals, respectively, $P = 0.29$; mean 577.6 ± 254.1 and 682.7 ± 135 in the GCL in vehicle and tapentadol-treated animals, respectively, $P = 0.40$). When we determined the number of labeled cells that had become neurons ($BrdU^+/NeuN^+$ cells), again no significant difference was observed between tapentadol- and vehicle-treated groups in the GCL (mean 530 ± 258 and 621 ± 112 in vehicle and tapentadol-treated animals, respectively, $P = 0.90$), as shown in Fig. 7C. A very small number of proliferating cells in the hippocampus also undergo differentiation toward the astroglial lineage. When we counted $BrdU^+/GFAP^+$ cells, there was not a significant difference between tapentadol- and vehicle-treated mice (data not shown). We decided to evaluate whether there was any difference between the two treatment groups in end points correlating with neurogenesis in ventral versus dorsal hippocampus. As shown in Fig. 7D, we observed a higher number, although not reaching statistical significance, of new neurons in the ventral hippocampus in tapentadol-treated mice compared with vehicle-treated mice (mean 262.8 ± 140 and 397.3 ± 79 $BrdU^+/NeuN^+$ cells in vehicle- and tapentadol-treated animals; t test, $P = 0.07$). Conversely, no such trend could be seen in the dorsal hippocampus (mean 267.2 ± 128 and 224 ± 67 $BrdU^+/NeuN^+$ cells in vehicle- and tapentadol-treated animals, respectively, $P = 0.488$; Fig. 7D). We believe that this trend underlies a positive effect of chronic tapentadol on neurogenesis in the ventral hippocampus. Indeed, when intragroup statistical evaluation was performed, significantly more new neurons were present in the ventral compared with the dorsal hippocampus in tapentadol-treated mice (t test, $P = 0.0021$), whereas no difference was present between the two subregions in vehicle-treated mice (t test, $P = 0.96$; Fig. 7E). Interestingly, this intragroup difference restricted to the ventral hippocampus was also confirmed for the late survival of total $BrdU^+$ cells (t test, $P = 0.0031$; Fig. 7F), but not for the number of undifferentiated $BrdU^+/NeuN^-/GFAP^-$ cells (t test, $P = 0.50$) or $BrdU^+/GFAP^+$ cells (t test, $P = 0.492$) in tapentadol-treated mice (data not shown).

Discussion

Findings correlating long-term use of opiates and cognitive dysfunction raise the possibility that chronic opiate use may produce maladaptive plasticity in brain structures involved in learning and memory, such as the hippocampus. In rodents, in vivo exposure to MOR agonists decreases SGZ proliferation by inhibition of progenitor proliferation, maturation, and survival of new neurons (Eisch et al., 2000; Mandyam et al., 2004; Kahn et al., 2005; Eisch and Harburg, 2006; Arguello et al., 2009). On the basis of these observations, it can be hypothesized that alterations in hippocampal neurogenesis may represent one mechanism by which opiates exert long-lasting effects on the neural circuitry involved with cognition.

Tapentadol is a novel centrally acting analgesic drug that combines MOR agonistic activity with NRI in a single molecule (Tzschentke et al., 2007; Bee et al., 2011; Hartrick and Rozek, 2011; Schröder et al., 2011). When we compared the effects of tapentadol and morphine on adult hippocampal NPC cultures, distinct differences between the two molecules could be observed. Morphine adversely affected neuronal differentiation, neurite outgrowth, and survival. Pharmacological blockade by naltrexone and CTOP confirmed that MOR activation was responsible for the deleterious effects of morphine. Interestingly, unlike morphine, tapentadol was devoid of adverse effects on cell survival, although it reduced neurite outgrowth and the number of newly generated neurons only within a concentration range known to interact with MOR and not at higher concentrations that affect NE reuptake. Extensive pharmacological characterization of the AR subtypes involved in such effects demonstrated that tapentadol counteracts MOR-mediated antineurogenic effects mainly via β2AR receptors. Indeed, in the presence of the selective β2AR antagonist ICI 118,551, micromolar tapentadol resulted in a significant reduction in the number of $MAP\text{-}2^+$ neurons generated by adult NPCs compared with tapentadol alone. We also showed that the β2AR agonist salbutamol is indeed proneurogenic on adult hippocampal NPCs. These findings are in line with the recent observation by Masuda et al. (2012) that noradrenaline and salmeterol can increase proliferation of NPCs derived from adult DG via activation of β2AR. On the basis of our experimental results with selective antagonists, herein we also suggest that β1ARs, α1ARs, and α2ARs do not take part in the noradrenergic component of tapentadol action that counteracts the deleterious negative effects of MOR. Gupta et al. (2009) recently suggested that α1AR, expressed by neural progenitors, could regulate adult neurogenesis both in vivo and in vitro in a complex manner. In particular, they showed increased incorporation of BrdU in neurogenic areas of α1AR overexpressing transgenic mice and wild-type mice treated with the α1 selective agonist cirazoline, suggesting a positive effect of that receptor subtype on proliferation. Moreover, they reported that α1AR stimulation reduced neuronal differentiation in adult neurosphere cultures. In line with these observations, we now report that treatment of adult hippocampal NPCs with the α1AR antagonist doxazosin effectively promotes neuronal differentiation in vitro. On the

viable cells, and are the result of three experiments performed in triplicate. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus vehicle-treated cells (t test). (F) The antiapoptotic effect of 3–10 μM tapentadol was counteracted by 100 nM ICI 118,551. The antagonist alone had no effect. (G and H) The effects of 3–30 μM tapentadol on neuronal differentiation (G) and apoptotic rate (H) were not significantly affected by 10 nM of CGP 20712A, a selective β1AR antagonist. The antagonist had no effect when tested alone. (F and H) Data are expressed as mean ± S.D. of three experiments in triplicate. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus vehicle-treated cells; $^{\S}P < 0.05$ versus tapentadol-treated cells (t test). Bar, 75 μm in C and D. Original magnification, 400× in C and D. CGP, CGP 20712A; ICI, ICI 118,551; SALB, salbutamol; TAP, tapentadol; w/, presence; w/o, absence.

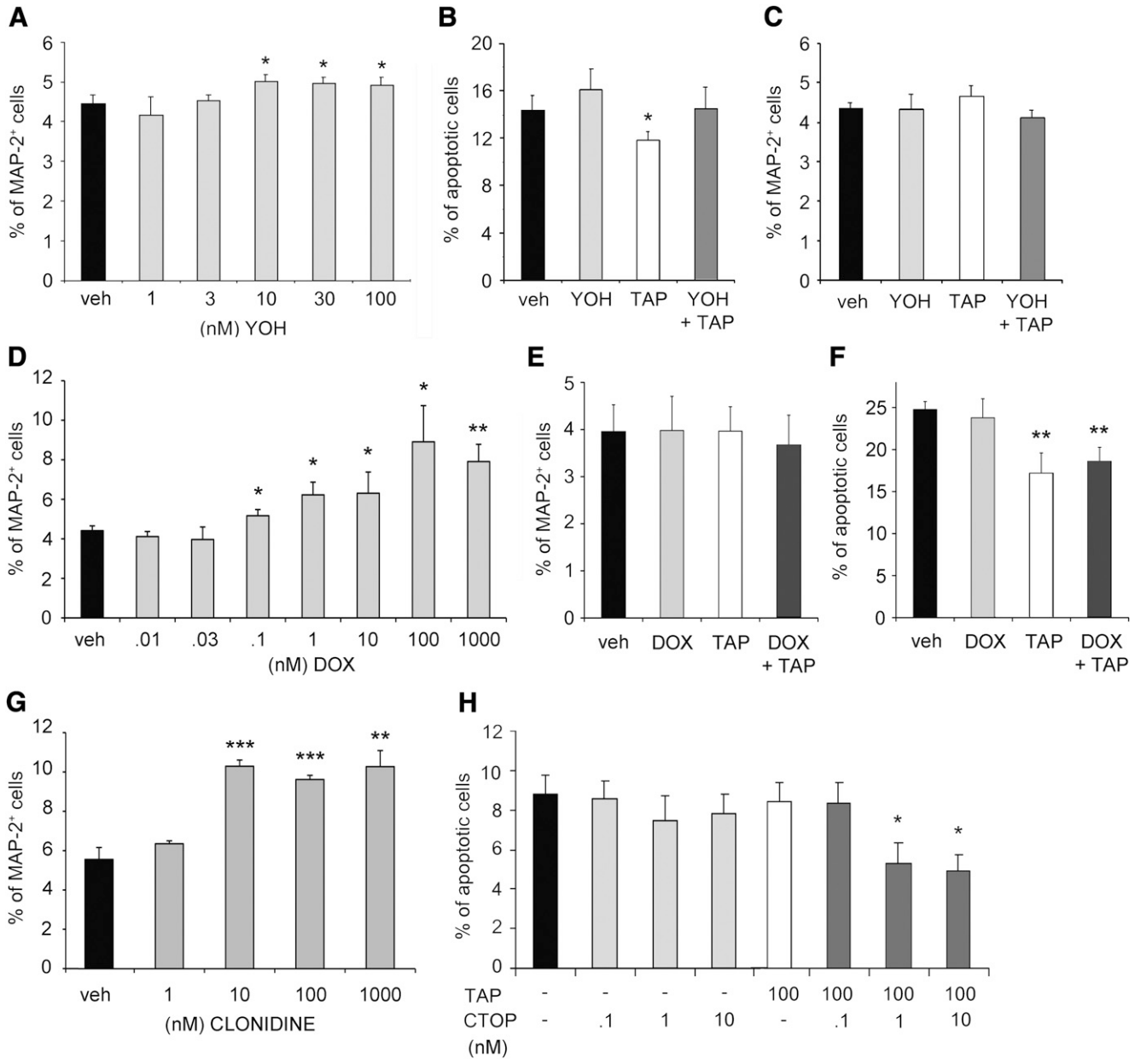

Fig. 5. Blockade of α2AR counteracts the antiapoptotic effects elicited by tapentadol. (A) Quantification of the percentage of MAP-2$^+$ cells in adult hippocampal NPC cultures treated for 24 hours in the presence of 1–100 nM yohimbine, an α2AR antagonist, or vehicle. At higher concentrations (10, 30, or 100 nM), yohimbine resulted in a small but significant increase in neuronal differentiation. (B) Three nanomoles of yohimbine counteracted the proapoptotic effects of 30 μM tapentadol. (C) Three nanomoles of yohimbine and 30 μM tapentadol had no effect on MAP-2$^+$ cells. (D) Evaluation of the percentage of MAP-2$^+$ cells in NPC cultures differentiated in the presence of 0.01 nM to 1 μM doxazosin, an α1AR antagonist. Doxazosin significantly increased the percentage of newly generated neurons, with a maximal effect elicited at 100 nM. (E) An inactive concentration of doxazosin (0.03 nM) had no effect on neuronal differentiation in the presence of 30 μM tapentadol. (F) Doxazosin (0.03 nM) did not prevent the antiapoptotic effect of 30 μM tapentadol. (G) The percentage of MAP-2$^+$ cells in adult hippocampal NPC cultures treated with the α2AR drug clonidine (1 nM to 1 μM) or vehicle. (H) The selective MOR antagonist CTOP (1–10 nM) reduced the percentage of apoptotic cells when added in the presence of 100 nM tapentadol. Per se, CTOP and tapentadol had no effect on the apoptotic rate in culture compared with vehicle-treated cells. Data are expressed as the mean ± S.D. of three experiments in triplicate. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus vehicle-treated cells (t test). DOX, doxazosin; TAP, tapentadol; veh, vehicle; YOH, yohimbine.

other hand, an inactive concentration of doxazosin had no effect on neuronal differentiation in presence of tapentadol. Similar results were obtained with the α2AR antagonist yohimbine, which had no effect on neuronal differentiation in the presence of micromolar concentrations of tapentadol. The available literature data suggest that activation of α2AR decreases adult hippocampal neurogenesis (Yanpallewar et al., 2010). These conclusions are mainly based on the observation that α2AR agonists clonidine and guanabenz decrease proliferation and not differentiation or survival of neural progenitors in vivo (Yanpallewar et al., 2010). Furthermore, in vivo α2AR blockade by yohimbine accelerated the neurogenic effects of chronic imipramine administration. In partial agreement with these data in our experimental settings, yohimbine, per se, could produce a small but significant increase in neuronal differentiation. To our surprise, when we tested clonidine in vitro, the drug promoted neuronal differentiation of adult hippocampal neural progenitors. Since clonidine K_i values at α2AR are only

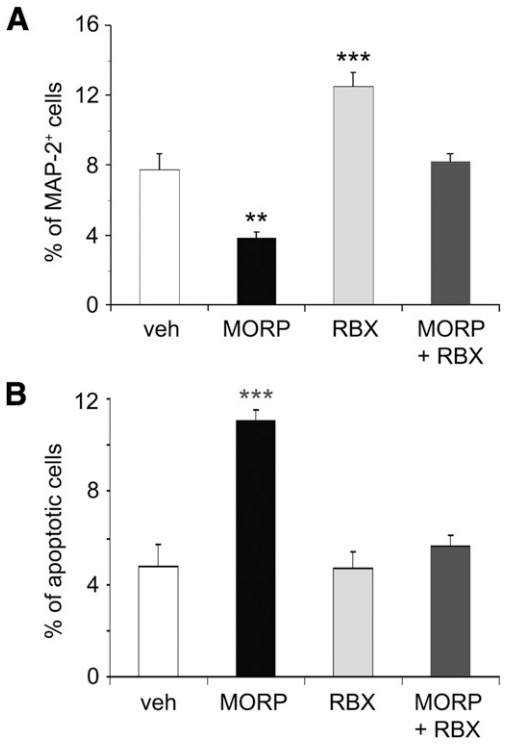

Fig. 6. The NRI reboxetine counteracted the deleterious effects of morphine on neuronal differentiation and survival of NPCs and their progeny. (A) Quantification of the percentage of MAP-2$^+$ cells in adult hippocampal NPC cultures treated for 24 hours in presence of 10 nM morphine, 100 nM reboxetine, and in the presence of both drugs, at the indicated concentrations. Reboxetine, which alone promoted neuronal differentiation of adult hippocampal NPCs, was able to counteract morphine-mediated inhibition of NPC neuronal differentiation. (B) The effect of reboxetine on morphine-mediated apoptotic death. One-hundred nanomoles of reboxetine, which alone had no effect on survival, totally counteracted the proapoptotic effects of 10 nM morphine. Data are expressed as the mean ± S.E.M. of three experiments in triplicate. $^{**}P < 0.01$; $^{***}P < 0.001$ versus vehicle-treated cells (t test). MORP, morphine; RBX, reboxetine; veh, vehicle.

10 times lower than those at α1AR and the drug can act as a partial α2AR agonist and an α1AR antagonist (Silva et al., 1996), the proneurogenic effects elicited by clonidine in our culture model may actually be mediated by both α2- and α1AR.

In our culture model, only yohimbine and ICI 118,551, but not CGP 20712A or doxazosin, could prevent the antiapoptotic effect of tapentadol, suggesting the involvement of β2- and α2AR subtypes in such activity. Interestingly, the strong analgesic effects of the MOR/NRI drug are also α2AR-mediated in animal models of pain (Tzschentke et al., 2007; Schröder et al., 2011).

Since there is no evidence of direct interaction of tapentadol with β2- and α2AR (Tzschentke et al., 2007), at the present stage of knowledge we propose that the effects of the MOR/NRI drug on neuronal differentiation and survival of hippocampal NPCs may represent an indirect consequence of drug-mediated blockade of noradrenaline reuptake.

Overall, these data are in agreement with literature reports demonstrating that the neurotransmitter NE exerts positive modulation of hippocampal neurogenesis. Indeed, experimentally induced depletion of both brain NE and 5-HT levels, unlike depletion of 5-HT levels only, reduced both proliferation and survival of adult-generated neurons in hippocampus (Jha et al., 2006). Moreover, hippocampal neurogenesis is dramatically reduced in NE-deficient mice (Kulkarni et al., 2002).

To support the idea that the noradrenergic component in tapentadol has the potential to counteract the adverse MOR-mediated effects on hippocampal neurogenesis, we showed that reboxetine, a well characterized NRI antidepressant, counteracted both antineurogenic and proapoptotic effects of morphine in vitro in our culture model. On the basis of these in vitro results, we then tested the effects of chronic tapentadol administration on adult hippocampal neurogenesis in vivo. Chronic (21-day) administration of 20 mg/kg tapentadol did not negatively affect cell proliferation and differentiation toward the neuronal lineage of newly generated cells in hippocampi of adult mice. In addition, we did not observe any effect of drug treatment on astrogliogenesis and, more importantly, on overall survival of new adult-generated cells. The drug dose of 20 mg/kg was chosen based on several considerations. The dose is 2–10 times higher than ED_{50} values for drug analgesic activity in rodent inflammatory and neuropathic pain models (Tzschentke et al., 2007; Schiene et al., 2011). Moreover, in vivo intracerebral microdialysis has demonstrated that at 10 mg/kg i.p., tapentadol produces an increase in extracellular levels of NE (+450% of baseline) in the ventral hippocampus of rodents (Tzschentke et al., 2007). In this study, positive effects of tapentadol treatment on end points correlating with adult neurogenesis (neuronal differentiation of adult progenitor cells and overall survival of progenitors and their progeny) were specifically restricted to the ventral hippocampus, a subregion where classic antidepressants, well established positive modulators of hippocampal neurogenesis, exert their proneurogenic effects (Banasr et al., 2006; Tanti et al., 2012).

Altogether, our data support the idea that the noradrenergic component in tapentadol has the potential to counteract the adverse MOR-mediated effects on hippocampal neurogenesis in vitro and in vivo. In principle, the tapentadol dual mechanism of action may result, after long-term drug treatment, in reduced dysfunction in adult neurogenesis compared with morphine. We believe that this property of tapentadol may be of relevance, since both mechanisms of action contribute to its analgesic efficacy (Tzschentke et al., 2007; Hartrick and Rozek, 2011; Schröder et al., 2011).

Our preclinical data highlight the need for a better understanding of the cellular and molecular effects of opioids on adult NPCs and their progeny. Potential differences among different opiates deserve to be carefully investigated. A recent in vivo study in the rat found that chronically administered methadone did not alter several quantified parameters relevant to adult hippocampal neurogenesis, including the number of Ki67-, doublecortin-, or BrdU-immunoreactive cells (Sankararaman et al., 2012). Methadone is an atypical opiate, since it is a MOR agonist as well as a noncompetitive *N*-methyl-D-aspartate antagonist (Gorman et al., 1997). Whether this pharmacological property may explain its lack of negative effects on neurogenesis deserves further investigation since *N*-methyl-D-aspartate antagonists have positive effects on hippocampal neurogenesis in rodents (Nacher et al., 2001; Maekawa et al., 2009).

This study, as well as other previous studies with morphine and methadone, was performed in naïve mice. Interestingly, several groups demonstrated disruption of hippocampal neuroplasticity in rodent models of chronic neuropathic pain (Terada et al., 2008; Mutso et al., 2012). In particular, Mutso et al. (2012) demonstrated that chronic neuropathic pain is

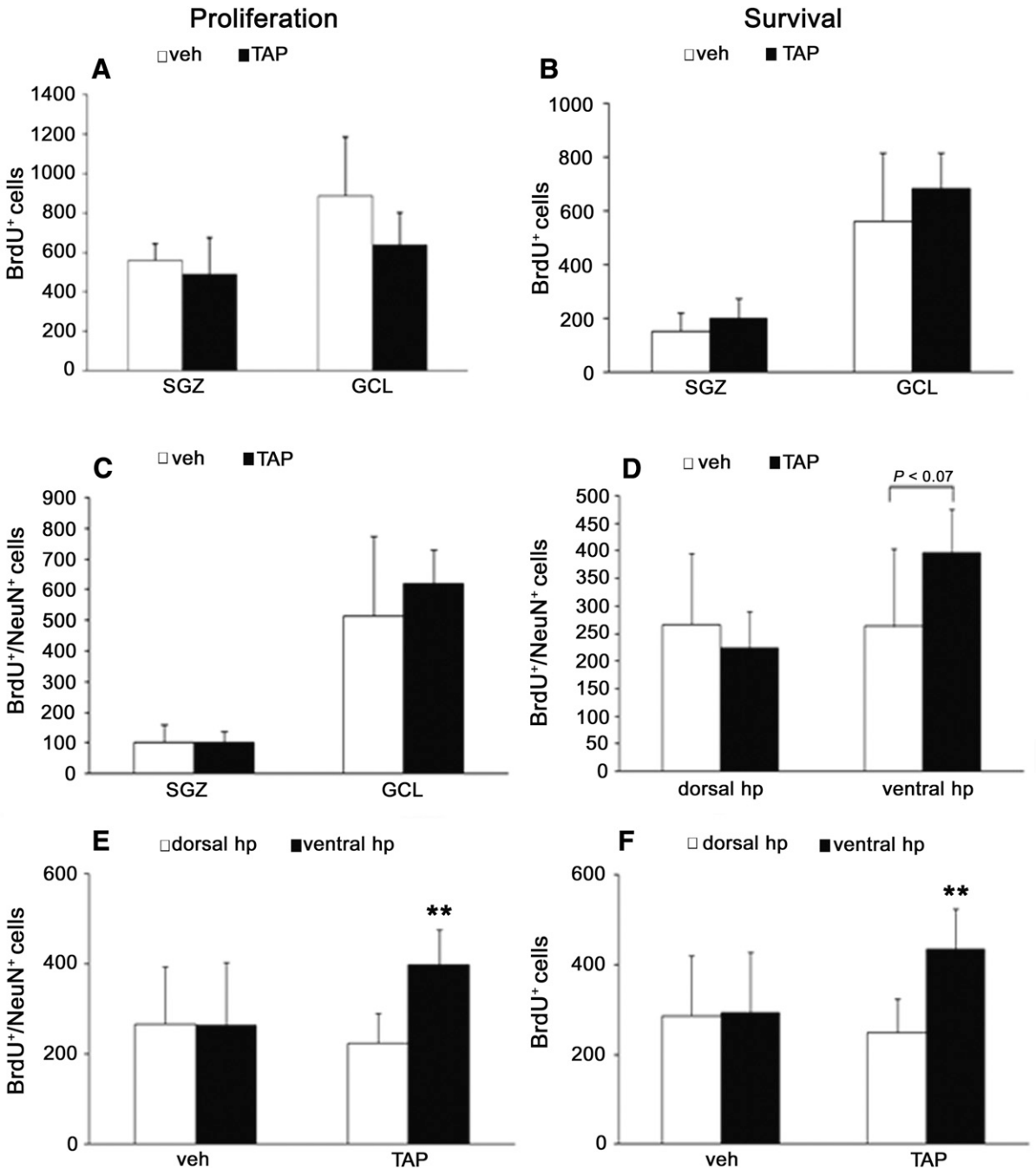

Fig. 7. Chronic (21-day) treatment with tapentadol did not affect hippocampal cell proliferation or generation of new neurons in adult mice. (A) Quantification of $BrdU^+$ cells in the SGZ and GCL of vehicle-treated and 20 mg/kg tapentadol-treated mice euthanized 24 hours after administration of the thymidine analog. Cell proliferation did not differ in the two DG subregions. (B) Three weeks after the last BrdU injection, the total number of $BrdU^+$ cells in the SGZ and GCL was similar in mice treated with vehicle and tapentadol, confirming no difference in cell survival of newly generated cells. (C) The percentage of newly born neurons, as identified by counting the number of $BrdU^+/NeuN^+$ cells, was not different in the SGZ and GCL of vehicle- and tapentadol-treated animals. (D) Effect of tapentadol and vehicle treatment on the number of newly generated neurons in the GCL of dorsal and ventral hippocampi. Although not statistically significant ($P = 0.07$), tapentadol had a more pronounced effect on ventral hippocampus compared with vehicle. No difference was seen in the dorsal hippocampus. (E) Intragroup effect of vehicle and tapentadol treatment on the number of $BrdU^+/NeuN^+$ cells in the GCL of the dorsal and ventral hippocampus. Unlike vehicle, which did not affect ventral and dorsal neurogenesis differently, significantly more new neurons were present in the ventral compared with the dorsal hippocampus in the tapentadol-treated group. (F) Intragroup effect of vehicle and tapentadol treatment on the number of total $BrdU^+$ cells in the GCL of dorsal and ventral hippocampus. Unlike vehicle-treated mice, in which cell survival was similar in the ventral and dorsal hippocampus, significantly more cells were present in the ventral compared with the dorsal hippocampus in the tapentadol-treated group. Data are expressed as the mean ± S.D. five to six mice per group. $^{**}P < 0.01$ (t test). hp, hippocampus; TAP, tapentadol; veh, vehicle.

associated with abnormalities in hippocampal-mediated behavior, synaptic plasticity, and neurogenesis in rodents. Moreover, the same authors documented the reduction in hippocampal volume in patients with chronic pain whose condition has a predominant neuropathic component. If such hippocampal changes are indeed related to reduced neuroplasticity, including reduced neurogenesis, future studies will need to address the possibility that they may potentially contribute,

at least in part, to emotional disturbances, including depressed mood, which are frequently present in patients suffering from chronic pain. On the basis of our current data, an analgesic drug like tapentadol that does not interfere with the generation of new neurons may represent the ideal choice in neuropathic pain states in which reduced hippocampal neurogenesis may occur and may in turn diminish the risk not only for cognitive impairment but also for development of mood disorders associated with chronic pain.

Authorship Contributions

Participated in research design: Tzschentke, Canonico, Grilli.

Conducted experiments: Meneghini, Cuccurazzu, Bortolotto, Ramazzotti, Ubezio.

Performed data analysis: Meneghini, Cuccurazzu, Bortolotto, Grilli.

Wrote or contributed to the writing of the manuscript: Meneghini, Grilli.

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