Molecular Pharmacology Fast Forward. Published on February 10, 2014 as doi:10.1124/mol.113.091520
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TITLE PAGE

THE NORADRENERGIC COMPONENT IN TAPENTADOL ACTION

COUNTERACTS MOR-MEDIATED ADVERSE EFFECTS ON ADULT NEUROGENESIS

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Molecular Pharmacology Fast Forward. Published on February 10, 2014 as DOI: 10.1124/mol.113.091520
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RUNNING TITLE PAGE

Running title: *The novel analgesic tapentadol does not impair adult neurogenesis*

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Number of text pages: 34

Number of figures: 7

Number of references: 57

Number of words in Abstract: 242

Number of words in Introduction: 664

Number of words in Discussion: 1470

List of non-standard abbreviations: AR, adrenergic receptor; BrdU, bromodeoxyuridine; DG, dentate gyrus; GCL, granular cell layer; GFAP, Glial Fibrillary Acidic Protein; MAP-2, Microtubule Associated Protein-2; MORP, morphine; NeuN, Neuronal Nuclei; NTX, naltrexone; NPC, neural progenitor cells; SGZ, subgranular zone; TAP, tapentadol; EGF, Epidermal Growth Factor; bFGF, basic Fibroblast Growth Factor; Sox-2, SRY-related HMG-box gene 2; i.p., intraperitoneal; NS, neurosphere; HP, hippocampus; NE, norepinephrine; NET, NE transporter; MOR, mu opioid receptor; NRI, NE reuptake inhibitor; NPC, neural progenitor cells; NSC, neural stem cells.

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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 (NE) is a positive modulator of hippocampal neurogenesis *in vitro* and *in vivo*. Based on these observations, we investigated whether tapentadol (TAP), a novel central analgesic combining mu opioid receptor (MOR) agonism with NE reuptake inhibition (NRI), may produce less inhibition of hippocampal neurogenesis compared to morphine. When tested i*n vitro,* morphine inhibited neuronal differentiation, neurite outgrowth and survival of adult mouse hippocampal neural progenitors (NPC) and their progeny, via MOR interaction. In contrast, tapentadol was devoid of these adverse effects on cell survival and reduced neurite outgrowth and the number of newly generated neurons only at nM concentrations where the MOR component is predominant. On the contrary, at higher (μM) 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 proapototic 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, tapentadol combined mechanism of action may result in less or no reduction in adult neurogenesis compared to classical opiates.

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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). Controversial, but of considerable clinical interest, is the possibility that chronic opiate treatment in non-addicted patients may also cause cognitive impairment in the long term (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 contribute 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 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 Gorij et al., 2008). Such 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 (LTP) (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 (NSC) can self-renew or give rise to nestin-positive transiently amplifying progenitor cells which, 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 post-mitotic 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

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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; Denis-Donini et al., 2008; Deng et al., 2010; Aimone et al., 2011; 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 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). Based on 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 with learning, memory, and cognition.

Tapentadol is a novel centrally acting analgesic drug which, in both preclinical and clinical studies, has efficacy comparable to that of strong opiates in a broad spectrum of acute and chronic pain conditions, with an improved tolerability profile compared to classical opiates (Tzschentke et al., 2007, 2009; Kress, 2010; Sloan, 2010; Etropolski et al., 2011; Riemsma et al., 2011; Hartrick et al., 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 et al., 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 counter-balancing effect may result, after long-term treatment *in vivo*, in less or no dysfunction in adult neurogenesis and cognitive impairment compared to morphine.

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MATERIALS AND METHODS

Animals

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

Drugs

The source for drugs was as follows: tapentadol HCl (Grünenthal, Aachen, Germany), morphine sulphate salt pentahydrate (Sigma-Aldrich, Saint Louis, MO), naltrexone HCl (gently provided by Prof. Paola Sacerdote, University of Milan, Milan), CTOP (Tocris Bioscience, Bristol, UK), reboxetine mesylate hydrate (Sigma-Aldrich), doxazosin mesylate (Tocris Bioscience), ICI 118,551 (Tocris Bioscience), CGP 20712 A dihydrochloride (Tocris Bioscience), clonidine hydrochloride and yohimbine hydrochloride (Sigma-Aldrich), salbutamol (Prestwick Chemical, Illkirch, France).

Isolation and culture of adult hippocampal NPC

For each neurosphere preparation, three adult (4-6 mo-old) male mice were killed by cervical dislocation. The brains were extracted and hippocampi were isolated under a Zeiss dissecting microscope using fine surgical instruments and collected in ice-cold PIPES buffer pH 7.4 containing 20 mM PIPES, 25 mM glucose, 0.5 M KCl, 0.12 M NaCl (Sigma-Aldrich), 100 U/100 µg/ml Penicillin/Streptomycin solution (Life Technologies, Monza, Italy). After centrifugation (110g x 5 min), tissue was digested for 40 min at 37°C using the Papain Dissociation System (Worthington DBA, Lakewood, NJ). Cell suspension was plated onto 25-cm^2 Falcon cell-culture flask (Delchimica scientific glassware, Naples, Italy) and cultured as previously described (Valente

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et al., 2012). Primary (Passage 1, P1) neurospheres were dissociated after 7-9 d, whereas P2-P30 neurospheres every 5 D.I.V. P3-P30 neurospheres were utilized for experiments.

Neural progenitor cell differentiation

For differentiation, neurospheres were dissociated and plated onto laminin-coated (2.5 μg/cm^2, Sigma-Aldrich) Lab-Tek 8-well permanox chamber slides (Nunc, Wiesbaden, Germany) at a density of 43,750 cells/cm^2 in differentiating medium (Neurobasal-A medium containing B27 supplement, 2 mM L-glutamine and 100 U/100 μg/ml Penicillin/Streptomycin). NPC were treated in presence of indicated concentrations of drugs or vehicle for 24 h. In each experiment, 5 fields/well (corresponding to about 150-200 cells/well) were counted using the fluorescence microscope ECLIPSE E600 (NIKON, Calenzano, Italy) with a X60 objective. All experiments were run in triplicates using different cell preparations and repeated at least three times.

Immunocytochemical analysis

After fixation, neurosphere-derived differentiated cells were washed three times in PBS and permeabilized in PBS containing 0.48% (vol/vol) Triton X-100 (Sigma-Aldrich), 5 min at room temperature. The primary antibodies against MAP-2 (rabbit polyclonal, 1:600; Chemicon, Temecula, CA) and β-III tubulin (Tuj-1 clone; chicken monoclonal, 1:2,000; Aves Labs Inc., Tigard, OR) were incubated for 150 min at room temperature in an antibody solution containing 16% (vol/vol) goat serum. Secondary antibodies were as follows: Alexa Fluor 555-conjugated goat anti-rabbit antibody (1:1,400; Molecular Probes, Eugene, Oregon) and Alexa Fluor 488-conjugated goat anti-chicken antibody (1:1,400; Molecular Probes) in a solution containing 16% (vol/vol) goat serum. Nuclei were counterstained with Draq5 (1:2,000, 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 anti-fading 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.

Morphological analysis

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To evaluate neurite growth and arborization, cells were differentiated *in vitro* for 24h in presence of drugs or vehicle and 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 vs bipolar and based on the presence or absence of arborizations. In each experiment, 5 fields/well (corresponding to 60-90 Tuj-1^+ cells/well) were counted using the fluorescence microscope ECLIPSE E600 (NIKON) with a X60 objective. All experiments were run in triplicates using different cell preparations and 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, in situ terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) 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 repeated at least three times.

RNA isolation and Reverse Transcriptase-PCR analysis

Total mRNA was extracted from hippocampus and hippocampal neurospheres (1-3×10^6 cells) of adult mice by using the SV Total RNA Isolation System (Promega, Milan, Italy), according to manufacturer's instructions. The cDNA was obtained by using the ImProm-II Reverse Transcription System kit (Promega). The primer sequences used for PCR amplification were as follows: MOR, sense 5′-ATACAGGCAGGGGTCCATAG-3′ and antisense 5′-GTCCATAACACACAGTGATGATGA-3′; NE 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 primers, 0.2 mM dNTPs, 2.5 mM $MgCl_2$. PCR conditions for MOR amplification were: 95°C for 10 min; 40 cycles, 94°C for 30 s, 57°C for 30 s, 72°C for 30 s; final elongation at 72°C for 10 min. PCR conditions for NET amplification were:

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95°C for 10 min; 40 cycles, 94°C for 30 s, 61°C for 30 s, 72°C for 45 s; final elongation at 72°C for 10 min. PCR products were run onto 2% agarose gels and bands visualized by staining with ethidium bromide (Sigma-Aldrich).

***In vivo* studies**

Adult male CD1 mice (3 mo-old) 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 daily intraperitoneally (i.p) for 21 d. The last day of treatment, mice were given three doses of bromodeoxyuridine (BrdU; 50 mg/kg, i.p.), at 2 h intervals. Twenty-four h after the last BrdU dose, half of the mice (n = 5 and n = 6 of vehicle- and tapentadol-treated mice, respectively) were transcardially perfused, their brain removed and prepared for immunohistochemical analysis as previously described (Bonini et al., 2011). The remaining mice were transcardially perfused 21 d 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, Colorado); goat anti-Glial Fibrillary Acidic Protein (GFAP) (1:100; Santa Cruz Biotechnology, Santa Cruz, California); mouse anti-Neuronal Nuclei (NeuN) (1:150; Millipore, Billerica, Massachusetts) 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 mean ± s.e.m, of at least three experiments in triplicate. *In vivo* data are reported as mean ± S.D. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test or by Student's *t*-test. Statistical significance level was set for p values less than 0.05.

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RESULTS

MOR and norepinephrine transporter transcripts are expressed in undifferentiated hippocampal neural progenitors.

In our experimental setting, NPC from adult mouse hippocampus are grown in presence of EGF and bFGF-2 as floating neurospheres and phenotypically characterized by the expression of nestin and SRY-related HMG-box gene 2 (Sox-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 figure 1, RT-PCR analysis demonstrated that both MOR (Fig. 1A) and the NE transporter (NET, Fig. 1A) transcripts were expressed by undifferentiated hippocampal neurospheres (NS) as well as in adult hippocampus (HP), 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, NPC stop dividing and differentiate giving rise to neurons, astrocytes, oligodendrocytes (Cuccurazzu et al., 2013). In order to characterize the effects of drugs acting on neuronal differentiation of hippocampal NPC, we performed immunolabelling 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 [$Ki_{(MOR)}$= 2.2 nM]. As shown in figure 1B, the drug significantly decreased the percentage of MAP-2^+ neurons (ANOVA $p<0.05$) generated *in vitro* from adult hippocampal NPC, 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 [$Ki_{(MOR)}$= 1.1 nM]. To further investigate the negative effects of morphine on neuronal differentiation and/or survival of adult hippocampal NPC and their progeny, cells were differentiated *in vitro* in presence of vehicle,

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10 nM morphine, 10 nM naltrexone, or both morphine and naltrexone. Thereafter, immunolocalization with a Tuj-1 antibody was performed, since this marker allows 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, grouped as unipolar vs bipolar, and with or without neurite arborizations. As shown in figure 1C-G, morphine negatively affected neurite outgrowth of newly generated cells from adult NPC since it significantly decreased the percentage of bipolar cells (Fig. 1G; Student's *t*-test $p<0.05$) and of arborized unipolar and bipolar cells (Fig. 1G; Student's *t*-test $p<0.05$ and $p<0.01$, respectively), compared with vehicle. As expected, in parallel, the drug increased the number of cells without neurites (Fig. 1G; Student's *t*-test $p<0.01$). Additionally, naltrexone abolished the effects of morphine on neurite outgrowth (Fig. 1G; Student's *t*-test $p<0.01$). We also investigated the effect of morphine on the apoptotic and necrotic rate in NPC cultures under differentiating conditions. As shown in figure 1H, compared to 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 LDH activity in the culture medium (*data not shown*). Co-treatment with naltrexone completely prevented morphine-mediated effects on apoptotic death (Fig. 1H; Student's *t*-test $p<0.01$). Similar results were obtained in presence of CTOP (Fig. 1H). Importantly, naltrexone or CTOP alone had no effect on neuronal differentiation (Fig. 1B), neurite outgrowth (Fig. 1E,G) and apoptotic rate (Fig. 1H), suggesting that endogenous opioids were not present in culture. Overall, these data suggest that, in the *in vitro* culture model, morphine inhibits neuronal differentiation and neurite outgrowth, and increases apoptotic rate of NPC and their progeny via MOR activation.

Tapentadol negatively affects neuronal differentiation, but not survival, of adult hippocampal neural progenitors.

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The effects of tapentadol were investigated under the same experimental conditions. The drug was tested over a wide range of concentrations (1 nM-30 μM) in order to evaluate both its opioidergic and noradrenergic activity. As shown in figure 2A, tapentadol produced a U shaped curve when added to the culture medium of adult hippocampal NPC undergoing differentiation *in vitro*. Within the 30-100 nM range, consistent with MOR affinity values in mouse [$Ki_{(MOR)}$= 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 (Student's *t*-test $p<0.001$). Higher tapentadol concentrations (300 nM-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 NPC. Surprisingly, unlike morphine, at any tested concentration tapentadol did not increase significantly the apoptotic rate (Fig. 2B). Interestingly, in the concentration range of 3-30 μM tapentadol had significant antiapoptotic effects (Fig. 2B; Student's *t*-test $p<0.01$). Finally, similar to morphine, the drug had no effect on necrotic rate *in vitro* of NPC and their progeny, as measured by assessing LDH 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. 2C; Student's *t*-test $p<0.01$). Once again, higher concentrations of tapentadol (10-30 μM) exerted no effect on neurite outgrowth and arborization, compared to 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.

Thereafter we investigated the contribution of MOR agonism and NRI activity on tapentadol-mediated effects. As shown in figure 3A-D, naltrexone completely abolished the inhibitory effect of

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low tapentadol concentrations (50 nM-1 μM) on both neuronal differentiation (Fig. 3A; Student's *t*-test $p<0.001$) and neurite outgrowth (Fig. 3B-D; Student's *t*-test $p<0.01$).

In our experimental model functional β2AR are expressed. Indeed, treatment with 10-300 nM salbutamol, a selective β2AR agonist, significantly increased, in a concentration-dependent manner, the percentage of newly generated MAP-2^+ neurons, when compared to vehicle (ANOVA $p<0.05$; Fig. 4A). No effect of β2AR activation was detected at any tested salbutamol concentration on the apoptotic rate of NPC and their progeny *(data not shown)*. Based on these observations, we then investigated whether tapentadol effects on neuronal differentiation were dependent on its NRI activity and potentially mediated by β2AR. We exposed NPC cultures to 1 nM-30 μM tapentadol in presence of 100 nM ICI 118,551, a selective β2AR antagonist, or the corresponding vehicle. As shown in figure 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 to tapentadol alone (Student's *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. 4C-E), as demonstrated by a decreased percentage of bipolar cells (Fig. 4E; Student's *t*-test $p<0.01$) and of arborized bipolar cells (Fig. 4E; Student's *t*-test $p<0.05$) and, in parallel, an increased percentage of cells with no neurites or unipolar (Fig. 4E; Student's *t*-test $p<0.05$ and $p<0.001$, respectively), compared to vehicle. In addition, 100 nM ICI 118,551 counteracted the pro-survival effect of 3-30 μM tapentadol (Fig. 4F). We also evaluated the potential contribution of β1AR activation in tapentadol-mediated effects. As shown in figure 4G-H, in 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 to tapentadol alone. Moreover, CGP 20712A *per se* had no effect on neuronal differentiation and apoptotic rate when used at 10 nM (Fig. 4G-H) and over a wider range of

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concentrations (0.1 nM-100 nM, *data not shown*). Since several papers have demonstrated the involvement of α2AR subtypes in tapentadol-mediated analgesia (Bee et al., 2011; Hartrick et al., 2011; Schiene et al., 2011), we then tested the effect of yohimbine (1 nM-100 nM), a well characterized α2AR antagonist, in our culture model. As shown in figure 5A, 1-3 nM yohimbine was ineffective while 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 presence of 30 μM tapentadol, the antiapoptotic activity of the tapentadol was prevented (Fig. 5B; Student's *t*-test $p<0.05$). Conversely, no significant difference was observed in the number of MAP-2^+ neurons generated *in vitro* when NPC were treated with 30 μM tapentadol in 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-1 μM) was initially tested in our culture model. Starting at 0.1 nM, doxazosin increased, in a concentration-dependent manner, the percentage of MAP-2^+ neurons (Fig. 5D; ANOVA $p<0.05$), 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 NPC were treated with 30 μM tapentadol in absence or presence of doxazosin 0.03 nM, a concentration which *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-1 μM), a commonly utilized α2AR agonist. 10-1,000 nM clonidine significantly increased the percentage of MAP-2^+ neurons, when compared to vehicle (Fig. 5G). Conversely, at any tested concentration, clonidine did not affect the apoptotic rate of NPC and their progeny *(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

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concentrations, tapentadol counteracted the negative MOR-mediated effects on neurogenesis via β2AR activation, while it elicited antiapoptotic effects via activation of both β2AR and α2AR.

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

Morphine-mediated antineurogenic and proapoptotic effects on adult NPC 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 co-treating NPC cultures with morphine and reboxetine, a NRI antidepressant drug. As shown in figure 6A, 100 nM reboxetine, which alone promotes neuronal differentiation of adult hippocampal NPC, was able to counteract inhibition of neuronal differentiation elicited by 10 nM morphine (Student's *t*-test $p<0.01$). More interestingly, 100 nM reboxetine also counteracted the pro-apoptotic effects of morphine (Fig. 6B; Student's *t*-test $p<0.001$), 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 i.p., once/daily, with vehicle (saline) or tapentadol 20 mg/kg, for a period of 21 d. At the end of the administration period, mice were injected with the thymidine analog BrdU (50 mg/kg, i.p, three times at 2 h intervals), in order to label proliferating cells in S-phase. A group of animals ($n = 11$) was killed 24 h later, while a second group ($n = 11$)

Molecular Pharmacology Fast Forward. Published on February 10, 2014 as DOI: 10.1124/mol.113.091520
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was sacrificed 21 d after BrdU administration. The number of BrdU-labelled cells in the subgranular zone (SGZ) and in the granular cell layer (GCL) within the dentate gyrus (DG) of vehicle- and tapentadol-treated mice was quantified by a modified unbiased stereology protocol as previously described (Denis-Donini et al., 2008). As shown in figure 7A, in the 24 h group we observed no significant difference in the absolute number of $BrdU^+$ cells in the SGZ or in the GCL of mice treated with 20 mg/kg tapentadol compared to vehicle (mean number ± S.D. of $BrdU^+$ cells in the SGZ: 588 ± 87.5 and 488 ± 186.1 in vehicle- and tapentadol-treated mice, respectively; mean number ± SD of $BrdU^+$ cells in the GCL: 888 ± 297.3 and 637 ± 165.4 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 d group, we phenotypically characterized $BrdU^+$ cells by performing a triple immunolabelling with antibodies raised against BrdU, NeuN (a marker of mature neurons) and GFAP (a marker of astrocytes). The number of immunolabelled 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-labelled cells at three weeks after their labelling. As shown in figure 7B, the total number of $BrdU^+$ cells was not significantly different in mice chronically treated with 20 mg/kg tapentadol compared to vehicle-treated animals, although a trend toward increase was observed in drug-treated mice (in the SGZ: mean number ± SD 155 ± 66.1 and 202 ± 74.3 in vehicle and tapentadol-treated animals, respectively, $p = 0.29$; in the GCL: mean number ± SD 577.6 ± 254.1 and 682.7 ± 135 in vehicle and tapentadol-treated animals, respectively, $p = 0.40$). When we determined the number of labelled cells that had become neurons ($BrdU^+/NeuN^+$ cells), again no significant difference was observed between tapentadol and vehicle-treated groups in the GCL (mean number ± SD: 530 ± 258 and 621 ± 112 in vehicle and tapentadol-treated animals, respectively, $p = 0.90$), as shown in figure 7C. A very small number of proliferating cells in the hippocampus also undergoes differentiation towards

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the astroglial lineage. When we counted $BrdU^+/GFAP^+$ cells, there was not a significant difference between tapentadol- and vehicle-treated mice (*not shown*). We decided to evaluate if there was any difference between the two treatment groups in end-points correlating with neurogenesis in ventral vs dorsal hippocampus. As shown in figure 7D, in tapentadol-treated mice we observed a higher number, although not reaching statistical significance, of new neurons in the ventral hippocampus compared to vehicle-treated mice (mean number ± S.D. of $BrdU^+/NeuN^+$ cells: 262.8 ± 140 and 397.3 ± 79 in vehicle and tapentadol-treated animals; Student's *t*-test $p = 0.07$). Conversely, no such trend could be seen in the dorsal hippocampus (mean number ± S.D. of $BrdU^+/NeuN^+$ cells: 267.2 ± 128 and 224 ± 67 in vehicle and tapentadol-treated animals, respectively, Student's *t*-test $p = 0.488$) (Fig. 7D). We believe that this trend underlies a positive effect of chronic tapentadol on neurogenesis in the ventral hippocampus. Indeed, when statistical evaluation was performed intra-group, significantly more new neurons were present in the ventral compared to the dorsal hippocampus in tapentadol-treated mice (Student's *t*-test $p = 0.0021$), while no difference was present between the two subregions in vehicle-treated mice (Student's *t*-test $p = 0.96$) (Fig. 7E). Interestingly, this intra-group difference restricted to the ventral hippocampus was confirmed also for the late survival of total $BrdU^+$ cells (Student's *t*-test $p = 0.0031$) (Fig. 7F), but not for the number of undifferentiated $BrdU^+/NeuN^-/GFAP^-$ cells (Student's *t*-test $p = 0.50$) or $BrdU^+/GFAP^+$ cells (Student's *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

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al., 2009). Based on 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 which combines a MOR agonistic activity with norepinephrine reuptake inhibition in a single molecule (Tzschentke et al., 2007; Bee et al., 2011; Hartrick et al., 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 impacted on neuronal differentiation, neurite outgrowth and survival. Pharmacological blockade by naltrexone and CTOP confirmed that MOR activation was responsible for morphine deleterious effects. Interestingly, unlike morphine, tapentadol, was devoid of adverse effects on cell survival, while 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 presence of the selective β2AR antagonist ICI 118,551, micromolar tapentadol resulted in a significant reduction in the number of MAP-2^+ neurons generated by adult NPC, compared to tapentadol alone. We could also show that the β2AR agonist salbutamol is indeed proneurogenic on adult hippocampal NPC. These findings are in line with the recent observation by Masuda and colleagues (2012) that noradrenaline and salmeterol can increase proliferation of NPC derived from adult DG via activation of β2AR. Based on our experimental results with selective antagonists, herein we also suggest that β1, α1, and α2 AR do not take part in the noradrenergic component of tapentadol action that counteracts MOR deleterious negative effects. Recently Gupta and colleagues (2009) 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

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overexpressing transgenic mice and wt 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, now we report that treatment of adult hippocampal NPC with the α1AR antagonist doxazosin effectively promotes neuronal differentiation *in vitro*. On the 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, that had no effect on neuronal differentiation in 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 Ki values at α2AR are only ten times lower than those at α1AR and the drug can act as partial α2AR agonist and α1AR antagonist (Silva et al., 1996), the proneurogenic effects elicited by clonidine in our culture model may actually be both α2 and α1AR-mediated.

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

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neuronal differentiation and survival of hippocampal NPC 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 norepinephrine 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 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 could show that reboxetine, a well characterized NRI antidepressant, counteracted both antineurogenic and proapoptotic effects of morphine *in vitro* in our culture model. Based on these *in vitro* results, we then tested the effects of chronic tapentadol administration on adult hippocampal neurogenesis *in vivo*. Chronic (21 d) administration of 20 mg/kg tapentadol did not negatively affect cell proliferation and differentiation towards the neuronal lineage of newly generated cells in hippocampi of adult mice. Additionally we did not observe any effect of drug treatment on astrogliogenesis and, more importantly, on overall survival of 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). To this regard, in the present 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

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classical 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, tapentadol dual mechanism of action may result, after long-term drug treatment, in reduced dysfunction in adult neurogenesis compared to 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 et al., 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 neural progenitor cells and their progeny. Potential differences among different opioids 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 but also a non-competitive NMDA antagonist (Gorman et al., 1997). Whether this pharmacological property may explain its lack of negative effects on neurogenesis deserves further investigation since NMDA antagonists have positive effects on hippocampal neurogenesis in rodents (Nacher et al., 2001; Maekawa et al., 2009).

Our as well as other previous studies with morphine and methadone were 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 and colleagues (2012) demonstrated that chronic neuropathic pain is associated with abnormalities in hippocampal-mediated behaviour, synaptic plasticity and neurogenesis in rodents. Moreover the same authors documented reduction in hippocampal volume in chronic pain patients whose condition has a predominant neuropathic component. If such hippocampal changes are indeed related to reduced neuroplasticity, including reduced neurogenesis, future studies will need

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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. Based on our current data, an analgesic drug like tapentadol which does not interfere with generation of new neurons may represent the ideal choice in neuropathic pain states where reduced hippocampal neurogenesis may occur and 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: Grilli, Canonico, Tzschentke

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

Performed data analysis: Meneghini, Cuccurazzu, Bortolotto, Grilli

Wrote the manuscript: Grilli, Meneghini

Molecular Pharmacology Fast Forward. Published on February 10, 2014 as DOI: 10.1124/mol.113.091520
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Molecular Pharmacology Fast Forward. Published on February 10, 2014 as DOI: 10.1124/mol.113.091520
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FOOTNOTE

This work was partially supported by a research grant from Grünenthal GmbH, Germany.

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Molecular Pharmacology Fast Forward. Published on February 10, 2014 as DOI: 10.1124/mol.113.091520
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LEGENDS FOR FIGURES

Figure 1. **Deleterious effects of morphine on hippocampus-derived adult neural progenitor cells 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 neural progenitor cells (NPC) grown as neurospheres (NS RT^+). Positive control for RT-PCR experiments was cDNA from adult hippocampus (HP RT^+), while hippocampus and NS RT^- reactions (HP RT^- and NS RT^-) served as negative controls. The 50 bp DNA ladder was used as molecular weight marker (mw). (B) 24 h treatment with 0.3-10 nM morphine (MORP) reduced neuronal differentiation from adult hippocampal NPC, by decreasing the percentage of MAP-2^+ cells compared to vehicle. The antineurogenic effects of 10 nM morphine were abolished in presence of 10 nM NTX and 1 nM CTOP. Both antagonists were devoid of effects when added alone. (C-F) Representative fluorescence microscopy images of Tuj-1 immunolabelling (green) in cells derived from hippocampal NPC after 24 h treatment with vehicle (veh) (C), 10 nM morphine (D), 10 nM NTX (E), 10 nM morphine and 10 nM NTX (F). Nuclei are stained with Draq5 (red). Magnification = X400. Scale bar = 75 μm. (G) 10 nM 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 presence of 0.3-10 nM morphine. Proapoptotic effects of 10 nM morphine were totally counteracted by 10 nM NTX and 1 nM CTOP. Both antagonists were devoid of effects when added alone. Data are expressed as mean ± s.e.m. of n=3 experiments, in triplicates. *p<0.05, **p<0.01 vs vehicle-treated cells (Student's t-test).

Figure 2. **Effects of tapentadol on hippocampus-derived neural progenitor cells and their neuronal progeny.** (A) Twenty-four h treatment of NPC with a wide range of tapentadol (TAP)

Molecular Pharmacology Fast Forward. Published on February 10, 2014 as DOI: 10.1124/mol.113.091520
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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 TAP reduced neuronal differentiation from adult hippocampal NPC, by decreasing the percentage of MAP-2^+ cells compared to 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. 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 mean ± S.D. of n=3 experiments, in triplicates. *p<0.05, **p<0.01, ***p<0.001 vs vehicle-treated cells (Student's *t*-test).

Figure 3. **The deleterious effects of low tapentadol concentrations on neuronal differentiation and neurite outgrowth are MOR mediated.** (A) NTX (10 nM) completely counteracted tapentadol-induced reduction of MAP2^+ cells generated by adult hippocampal NPC. (B-C) Representative fluorescence microscopy images of Tuj-1 immunolabelling (green) in cells derived from hippocampal NPC after 24 h treatment with 300 nM tapentadol in absence (B) and in presence (C) of 10 nM NTX. Nuclei are stained with Draq5 (red). (D) The reduction in neurite outgrowth and arborization elicited by 300 nM tapentadol was counteracted in presence of 10 nM NTX. Data are expressed as mean ± S.D. of n=3 experiments, performed in triplicates. *p<0.05, **p<0.01, ***p<0.001 vs vehicle-treated cells (Student's *t*-test).

Figure 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 h with 10-300 nM salbutamol (SALB). The selective β2AR agonist significantly increased, in a concentration-dependent manner, the

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percentage of newly generated neurons. (B) Quantification of the percentage of MAP-2$^+$ cells in adult hippocampal NPC treated with 0.001-30 μM tapentadol in absence (w/o) or presence (w/) of 100 nM ICI 118,551 (ICI), a selective β2AR antagonist. ICI, which alone had no effect, significantly reduced the percentage of MAP-2$^+$ cells in presence of 10-30 μM tapentadol. (A-B) Data are expressed as mean ± S.D. of n=3 experiments, in triplicates. *p<0.05, **p<0.01, ***p<0.001 vs vehicle-treated cells; §p<0.05 vs tapentadol-treated cells (Student's *t*-test). (C-D) Representative fluorescence microscopy images of Tuj-1 immunolabelling (green) in cells derived from hippocampal NPC after 24 h treatment with 30 μM tapentadol alone (C) or in presence of 100 nM ICI 118,551 (D). Nuclei are counterstained with Draq5 (red). Magnification = X400. Scale bar = 75 μm. (E) Although 30 μM tapentadol and 100 nM ICI 118,551 had no effect alone, when added together they resulted in 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 to vehicle. Data, expressed as mean percentage ± S.D. of Tuj-1$^+$ cells over viable cells, are the result of n=3 experiments, performed in triplicates. * p<0.05, **p<0.01 vs vehicle-treated cells (Student's *t*-test). (F) The antiapoptotic effect of 3-10 mM tapentadol was counteracted by 100 nM ICI 118,551. The antagonist alone had no effect. (G-H) The effects of 3-30 μM tapentadol on neuronal differentiation (G) and apoptotic rate (F) were not significantly affected by 10 nM of CGP 20712A, a selective β1AR antagonist. The antagonist had no effect when tested alone. (F-G) Data are expressed as mean ± S.D. of n=3 experiments, in triplicates. *p<0.05, **p<0.01, ***p<0.001 vs vehicle-treated cells; §p<0.05 vs tapentadol-treated cells (Student's *t*-test).

Figure 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 h in presence of 1-100 nM yohimbine (YOH), an α2AR antagonist, or vehicle (veh). At higher concentrations (10-30-100 nM), YOH resulted in a small but significant increase in neuronal

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differentiation. (B) 3 nM YOH counteracted the proapoptotic effects of 30 μM tapentadol (TAP). (C) 3 nM YOH and 30 μM TAP had no effect on MAP-2$^+$ cells. (D) Evaluation of the percentage of MAP-2$^+$ cells in NPC cultures differentiated in presence of 0.01 nM-1 μM doxazosin (DOX), an α1AR antagonist. DOX significantly increased the percentage of newly generated neurons, with a maximal effect elicited at 100 nM. (E) An inactive concentration of DOX (0.03 nM) had no effect on neuronal differentiation in presence of tapentadol 30 μM. (F) DOX (0.03 nM) did not prevented the antiapoptotic effect of tapentadol 30 μM. (G) Percentage of MAP-2$^+$ cells in adult hippocampal NPC cultures treated with the α2AR drug clonidine (1 nM-1 μM) or vehicle. (H) The selective MOR antagonist CTOP (1-10 nM) reduced the percentage of apoptotic cells when added in presence of 100 nM TAP. *Per se*, CTOP and TAP had no effect on the apoptotic rate in culture, compared to vehicle-treated cells. (A-H) Data are expressed as mean ± S.D. of n=3 experiments, in triplicates. *p<0.05, **p<0.01, ***p<0.001 vs vehicle-treated cells (Student's *t*-test).

Figure 6. **The NRI reboxetine counteracted the deleterious effects of morphine on neuronal differentiation and survival of NPC and their progeny.** (A) Quantification of the percentage of MAP-2$^+$ cells in adult hippocampal NPC cultures treated for 24 h in presence of 10 nM morphine, 100 nM reboxetine (RBX), and in presence of both drugs, at indicated concentrations. RBX, which alone promoted neuronal differentiation of adult hippocampal NPC, was able to counteract morphine-mediated inhibition of NPC neuronal differentiation (Student's *t*-test *p<0.01). (B) Effect of RBX on morphine-mediated apoptotic death. 100 nM RBX, which alone had no effect on survival, totally counteracted the pro-apoptotic effects of 10 nM morphine. Data are expressed as mean ± S.D. of n=3 experiments, in triplicates. **p<0.01, ***p<0.001 vs vehicle-treated cells (Student's *t*-test).

Figure 7. **Chronic (21 d) treatment with tapentadol did not affect hippocampal cell proliferation or generation of new neurons in adult mice.** (A) Quantification of BrdU$^+$ cells in

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the subgranular zone (SGZ) and granular cell layer (GCL) of vehicle- and 20 mg/kg tapentadol-treated mice sacrificed 24 h after administration of the thymidine analog. Cell proliferation did not differ in the two dentate gyrus 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 (veh) and TAP, 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 TAP-treated animals. (D) Effect of TAP 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 (ventral HP), compared to vehicle. No difference was seen in the dorsal hippocampus (dorsal HP). (E) Intragroup effect of vehicle and tapentadol treatment on the number of $BrdU^+/NeuN^+$ cells in the granular cell layer of dorsal and ventral hippocampus. Unlike vehicle, which did not affect ventral and dorsal neurogenesis differently, in the tapentadol-treated group significantly more new neurons were present in the ventral compared to the dorsal hippocampus. (F) Intragroup effect of vehicle and tapentadol treatment on the number of total $BrdU^+$ cells in the granular cell layer of dorsal and ventral hippocampus. Unlike vehicle-treated mice, where cell survival was similar in the ventral and dorsal hippocampus, significantly more cells were present in the ventral compared to the dorsal hippocampus in the tapentadol-treated group. Data are expressed as mean number ± SD of n=5-6 mice/group. $^{**}p<0.01$ (Student's t-test).

Figure 1

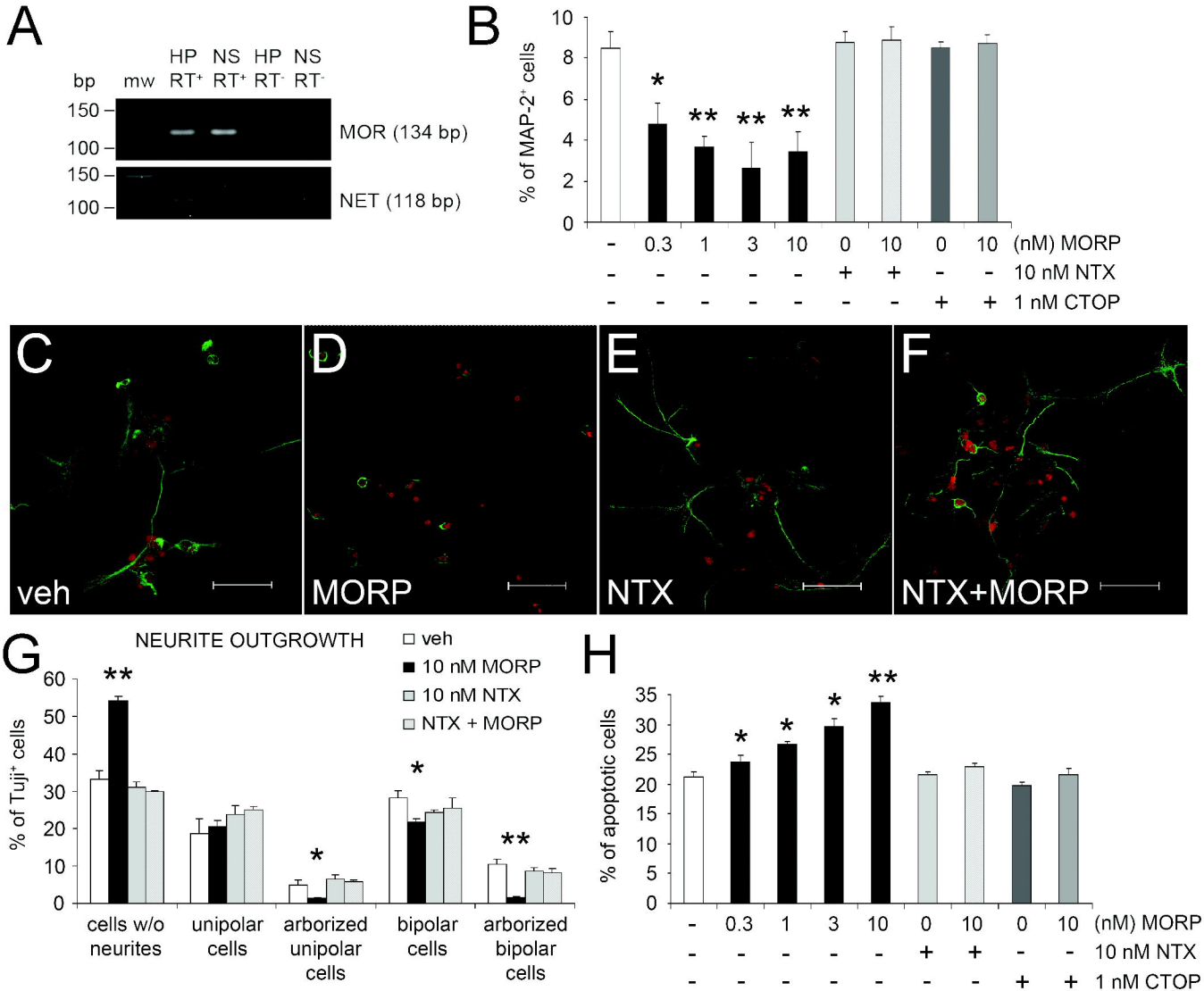

A
HP RT+
NS RT+
HP RT-
NS RT-
bp
mw
150
100
MOR (134 bp)
NET (118 bp)
B
% of MAP-2+ cells
(nM) MORP
10 nM NTX
1 nM CTOP
C
veh
D
MORP
E
NTX
F
NTX+MORP
G
NEURITE OUTGROWTH
veh
10 nM MORP
10 nM NTX
NTX + MORP
% of Tuji+ cells
cells w/o neurites
unipolar cells
arborized unipolar cells
bipolar cells
arborized bipolar cells
H
% of apoptotic cells
(nM) MORP
10 nM NTX
1 nM CTOP

Figure 2

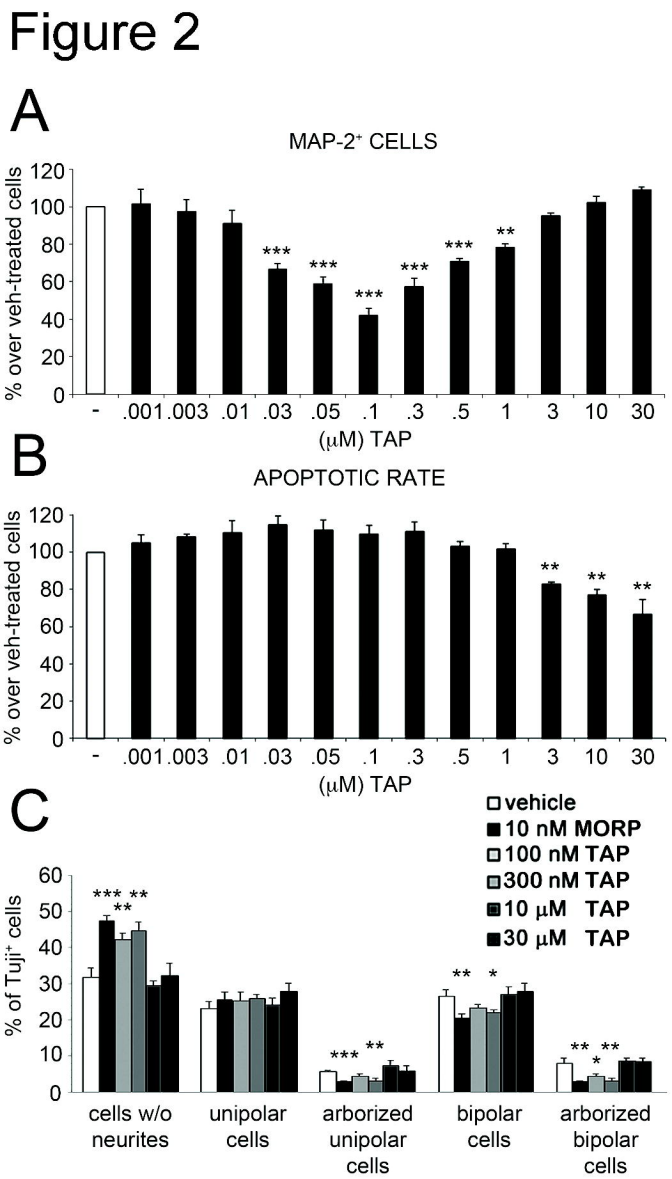

A
MAP-2+ CELLS
% over veh-treated cells
120
100
80
60
40
20
0
***
***
***
***
**
-
.001
.003
.01
.03
.05
.1
.3
.5
1
3
10
30
(μM) TAP
B
APOPTOTIC RATE
% over veh-treated cells
120
100
80
60
40
20
0
**
**
**
-
.001
.003
.01
.03
.05
.1
.3
.5
1
3
10
30
(μM) TAP
C
vehicle
10 nM MORP
100 nM TAP
300 nM TAP
10 μM TAP
30 μM TAP
% of Tuji+ cells
60
50
40
30
20
10
0
*** ** **
***
**
**
*
**
**
*
**
cells w/o neurites
unipolar cells
arborized unipolar cells
bipolar cells
arborized bipolar cells

Figure 3

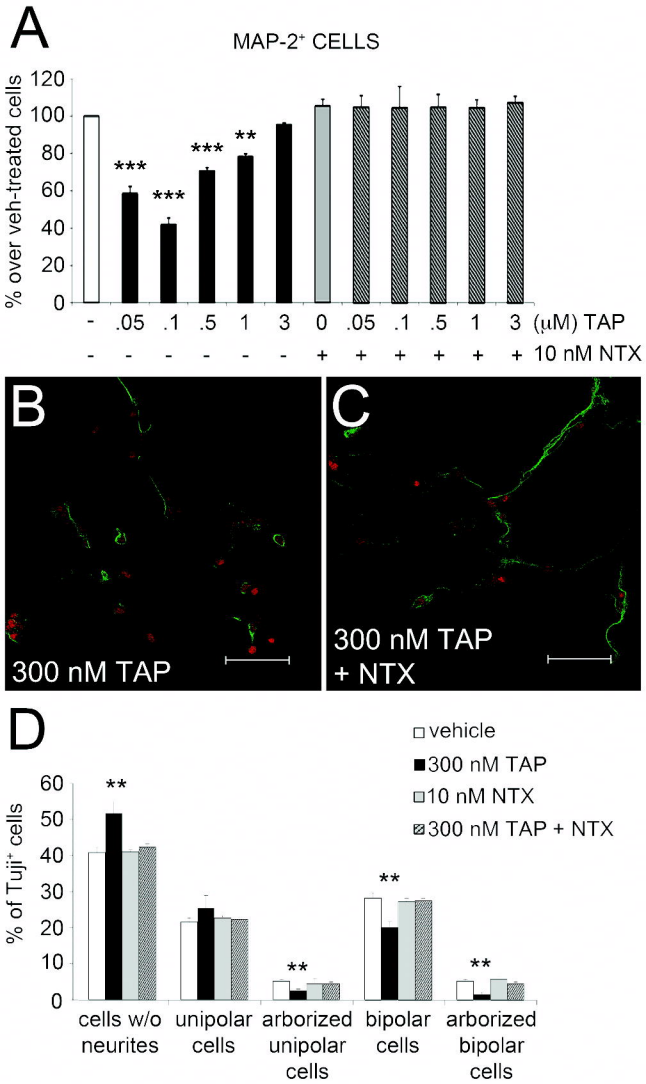

A
MAP-2+ CELLS
% over veh-treated cells
120
100
80
60
40
20
0
***
***
***
**
- .05 .1 .5 1 3 0 .05 .1 .5 1 3 (μM) TAP
- - - - - - + + + + + + 10 nM NTX
B
300 nM TAP
C
300 nM TAP
+ NTX
D
vehicle
300 nM TAP
10 nM NTX
300 nM TAP + NTX
% of Tuji+ cells
60
50
40
30
20
10
0
**
**
**
cells w/o neurites
unipolar cells
arborized unipolar cells
bipolar cells
arborized bipolar cells

Figure 4

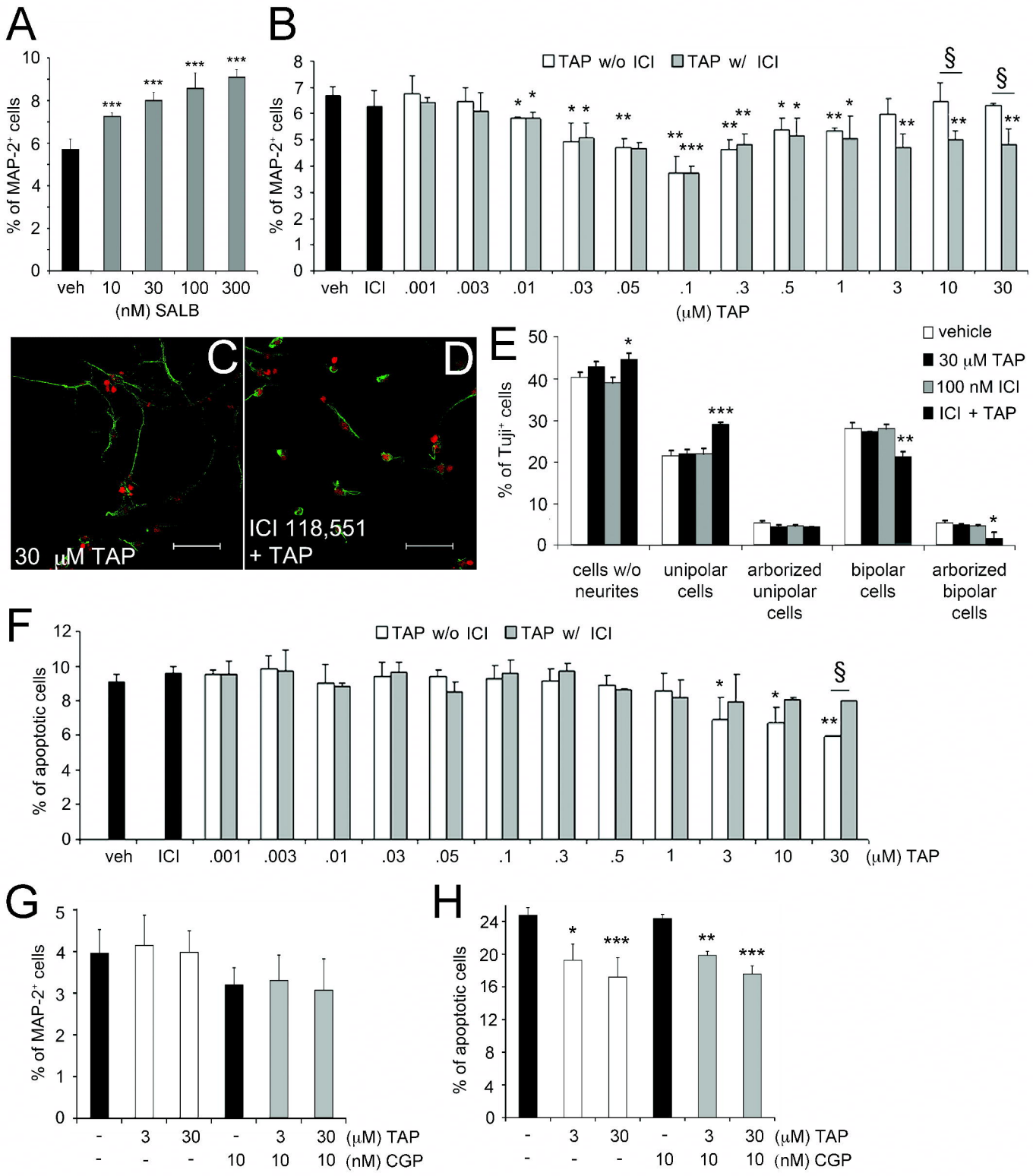

A
B
% of MAP-2+ cells
veh
10
30
100
300
(nM) SALB
TAP w/o ICI
TAP w/ ICI
ICI
.001
.003
.01
.03
.05
.1
.3
.5
1
3
(μM) TAP
C
D
30 μM TAP
ICI 118,551
+ TAP
E
% of Tuj1+ cells
vehicle
30 μM TAP
100 nM ICI
ICI + TAP
cells w/o neurites
unipolar cells
arborized unipolar cells
bipolar cells
arborized bipolar cells
F
% of apoptotic cells
G
% of MAP-2+ cells
H
% of apoptotic cells
(nM) CGP

Figure 5

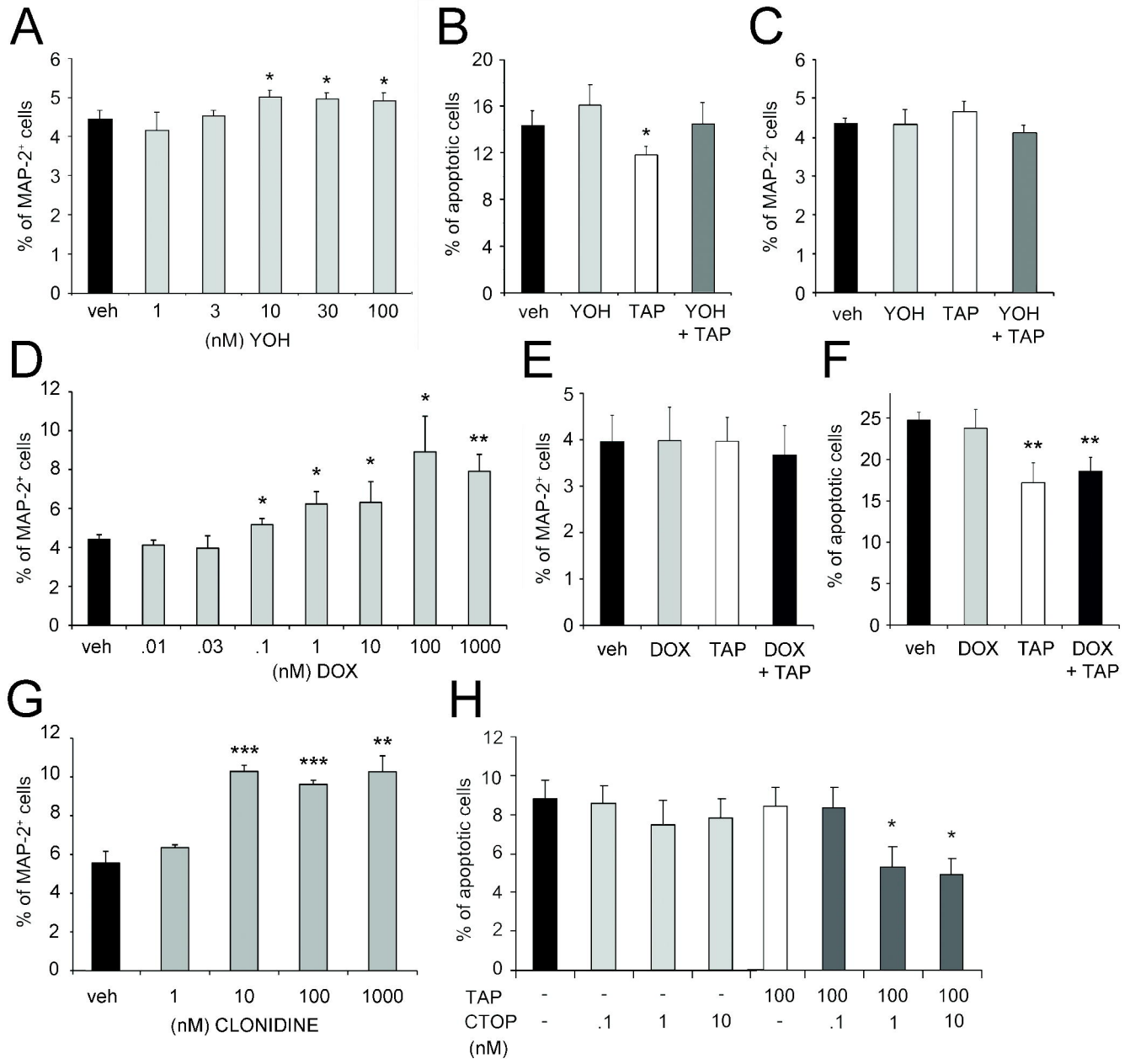

A
% of MAP-2+ cells
veh
1
3
10
30
100
(nM) YOH
B
% of apoptotic cells
veh
YOH
TAP
YOH + TAP
C
% of MAP-2+ cells
veh
YOH
TAP
YOH + TAP
D
% of MAP-2+ cells
veh
.01
.03
.1
1
10
100
1000
(nM) DOX
E
% of MAP-2+ cells
veh
DOX
TAP
DOX + TAP
F
% of apoptotic cells
veh
DOX
TAP
DOX + TAP
G
% of MAP-2+ cells
veh
1
10
100
1000
(nM) CLONIDINE
H
% of apoptotic cells
TAP
CTOP
(nM)

Figure 6

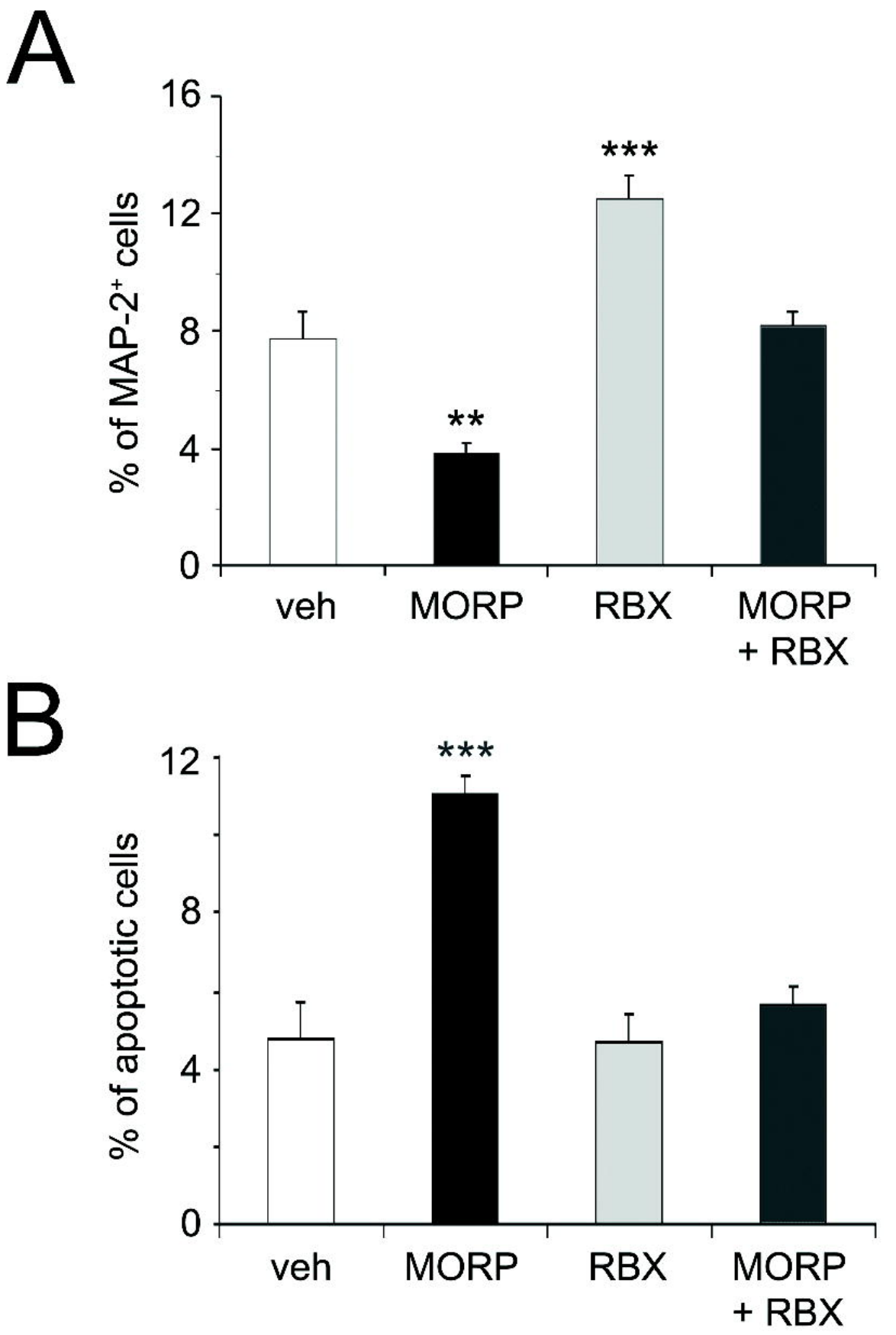
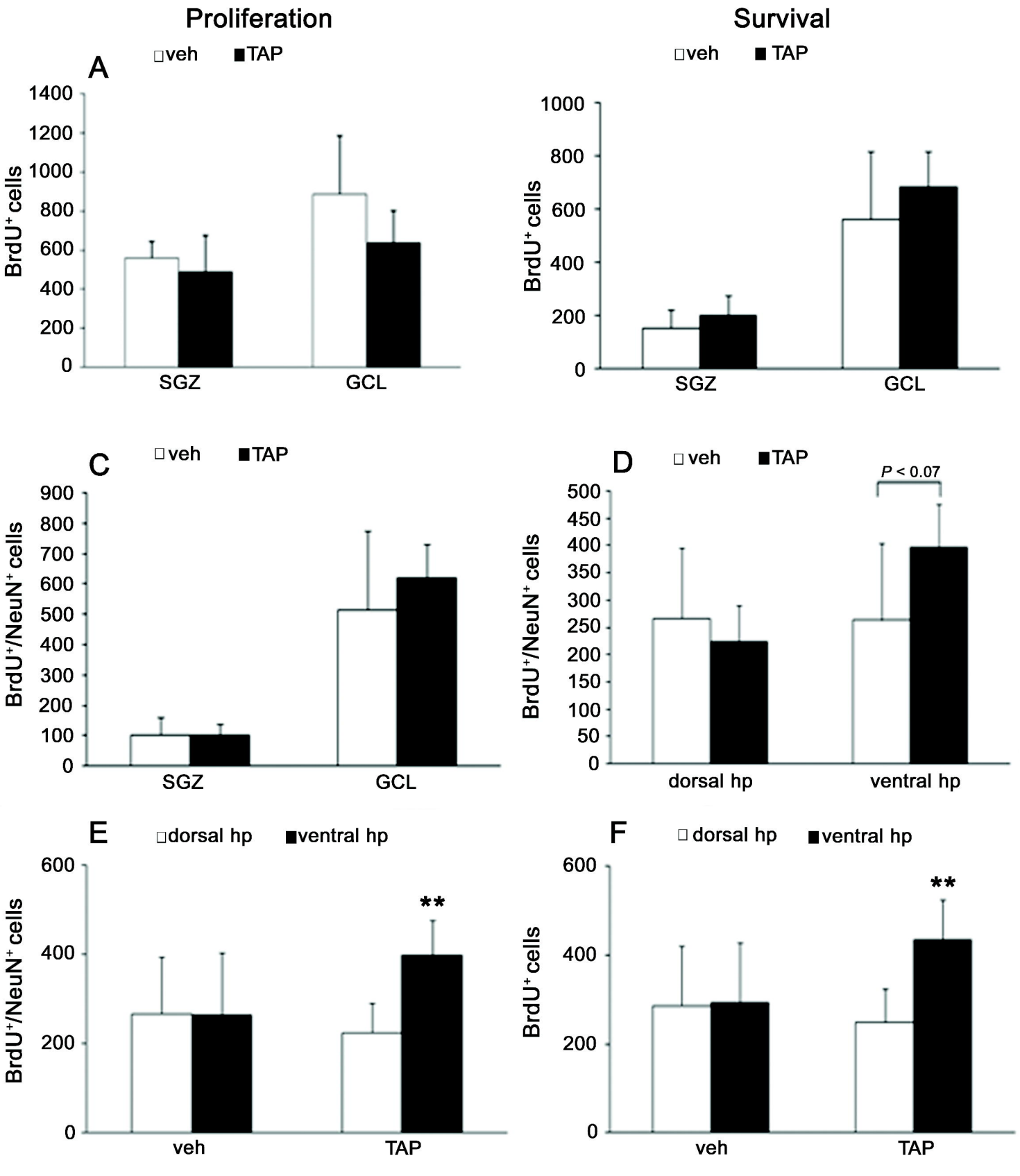

A
16
12
8
4
0
% of MAP-2+ cells
**
***
veh
MORP
RBX
MORP + RBX
B
12
8
4
0
% of apoptotic cells
***
veh
MORP
RBX
MORP + RBX

Figure 7


Proliferation
Survival
A
□veh ■TAP
BrdU⁺ cells
1400
1200
1000
800
600
400
200
0
SGZ
GCL
□veh ■TAP
BrdU⁺ cells
1000
800
600
400
200
0
SGZ
GCL
C
□veh ■TAP
BrdU⁺/NeuN⁺ cells
900
800
700
600
500
400
300
200
100
0
SGZ
GCL
D
□veh ■TAP
P < 0.07
BrdU⁺/NeuN⁺ cells
500
450
400
350
300
250
200
150
100
50
0
dorsal hp
ventral hp
E
□dorsal hp ■ventral hp
BrdU⁺/NeuN⁺ cells
600
400
200
0
**
veh
TAP
F
□dorsal hp ■ventral hp
BrdU⁺ cells
600
400
200
0
**
veh
TAP