Proneurogenic Effects of Trazodone in Murine and Human Neural Progenitor Cells

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ABSTRACT: Several antidepressants increase adult hippocampal neurogenesis (ahNG) in rodents, primates, and, potentially, humans. This effect may at least partially account for their therapeutic activity. The availability of antidepressants whose mechanism of action involves different neurotransmitter receptors represents an opportunity for increasing our knowledge on their distinctive peculiarities and for dissecting the contribution of receptor subtypes in ahNG modulation. The aim of this study was to evaluate, *in vitro*, the effects of the antidepressant trazodone (TZD) on ahNG by using primary cultures of murine adult hippocampal neural progenitor cells (ahNPCs) and human induced pluripotent stem cell (iPSC)-derived NPCs. We demonstrated that TZD enhances neuronal differentiation of murine as well as human NPCs. TZD is a multimodal antidepressant, which binds with high affinity to 5-HT_{2a}, α_1, and 5-HT_{1a} and with lower affinity to 5-HT_{2c}, α_2 and 5-HTT. We demonstrated that TZD proneurogenic effects were mediated by 5-HT_{2a} antagonism both in murine and in human NPCs and by 5-HT_{2c} antagonism in murine cells. Moreover NF-κB p50 nuclear translocation appeared to be required for TZD-mediated proneurogenic effects. Interestingly, TZD had no proneurogenic effects in 5-HT depleted ahNPCs. The TDZ bell-shaped dose−response curve suggested additional effects. However, in our model 5-HT_{1a} and α_1/α_2 receptors had no role in neurogenesis. Overall, our data also demonstrated that serotoninergic neurotransmission may exert both positive and negative effects on neuronal differentiation of ahNPCs *in vitro*.

KEYWORDS: antidepressant, neural progenitor cells, trazodone, neurogenesis, serotonin, 5-HT_2
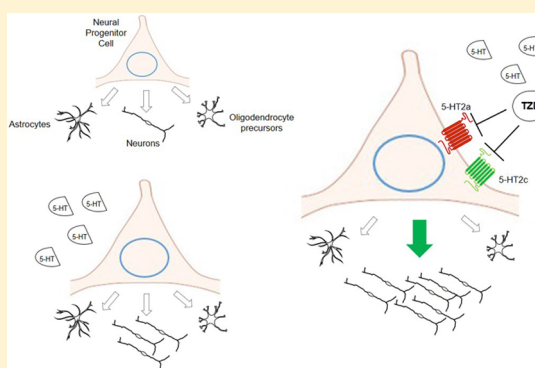
INTRODUCTION

Lifelong generation of new neurons takes place in the subgranular zone (SGZ) of the dentate gyrus (DG) from a pool of adult hippocampal neural progenitor cells (ahNPCs).[1] Newly generated neurons can integrate into preexisting neuronal networks and potentially contribute to specific functions such as hippocampal-dependent learning and memory, mood regulation, and stress response.[2] Extensive research efforts have contributed to the idea that adult hippocampal neurogenesis (ahNG) represents an important form of neural plasticity, which is deregulated in several neuropsychiatric disorders, including major depressive disorder (MDD), and whose reduction is correlated with cognitive impairment and mood alterations.[3−6]

Adult hippocampal NG is susceptible to pharmacological modulation.[7−11] Several antidepressant drugs including trycyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), and serotonin−norepinephrine reuptake inhibitors (SNRIs), counteract stress-reduced ahNG in preclinical models of depressive-like behavior.[12,13] Increased number of hippo-

campal neural progenitors was also reported in post-mortem studies in MDD patients treated with antidepressants.[14−16] Although still debated, ahNG may be required for some of the behavioral effects of antidepressants, at least in rodent models.[17,18]

Trazodone (TDZ) is a triazolopyridine derivative that in clinical studies has demonstrated an antidepressant activity comparable to TCAs, SSRIs, and SNRIs.[19] TZD is structurally unrelated to other antidepressant classes and is characterized by a unique profile.[20] In the past, the drug has been classified as a serotonin receptor antagonist and reuptake inhibitor (SARI), able to provide simultaneous inhibition of the serotonin transporter (SERT), partial agonism of serotonin 5-HT_{1a} receptors, and antagonism of 5-HT_{2a} and 5-HT_{2c} receptors. With such a profile, the drug potentially avoids tolerability issues that are often associated with 5-HT_{2a} and 5-HT_{2c}

receptor stimulation (i.e., insomnia, anxiety, and sexual dysfunction).[21] In addition to those properties, TDZ also exerts antagonistic activity against α_1- and α_2-adrenergic receptors (ARs), with minimal anticholinergic effects.[21] In such respect, by taking advantage of a recently introduced concept, TZD can be recognized as the first ever multimodal antidepressant.[22]

In line with the accepted hypothesis that antidepressants may exert their therapeutic activity at least in part via positive effects on neurogenesis, modulation of ahNG has become part of the discovery activities of recently approved antidepressant drugs.[23,24] In addition, promotion of adult hippocampal neurogenesis is currently explored as a strategy for anti-depressant drug screening.[25−27] Despite intense research efforts in this field, our current knowledge on the relative contribution of different receptors in the modulation of ahNG and in mediating the effects of antidepressant drugs is limited. Extensive literature data suggest a potential and complex contribution of receptors engaged by TZD, such as 5-HT$_{1a}$, 5-HT$_{2a}$ and 5-HT$_{2c}$, α_1, and α_2, in the modulation of adult neurogenesis.[28−30] How the unique pharmacological profile of TZD may impact adult neurogenesis is therefore of interest and could potentially increase our understanding of ahNG modulation by neurotransmitters.

■ RESULTS AND DISCUSSION

Serotonin Promotes Neuronal Differentiation of Adult Hippocampal NPCs. For our studies, we used a well characterized *in vitro* model of murine ahNPCs. In this experimental setting, when ahNPCs from the adult mouse hippocampus are grown in the presence of growth factors, they are phenotypically characterized by expression of nestin and SRY-related HMG-box gene 2 (SOX2), bonafide markers of undifferentiated neural progenitors, and by the absence of markers of mature and immature neurons such as MAP-2, Tuj-1, and doublecortin.[27] Upon removal of growth factors and exposure to a serum-free defined medium, ahNPCs stop dividing and differentiate onto laminin-coated chamber slides. By double immunolabeling for markers of neurons (MAP-2) and undifferentiated ahNPCs (nestin), the appearance of new neurons that are MAP-2$^+$ and nestin$^−$ and newly generated neuroblasts (cells positive for both MAP-2 and nestin) can be evaluated and quantified as previously described.[26] Under these experimental conditions, within 24 h, ahNPCs give rise to about 2% and 30% of neurons and neuroblasts, respectively (Figure 1a,b). We initially exposed ahNPCs to increasing concentrations of the endogenous neurotransmitter 5-HT (0.0001−100 μM). As depicted in Figure 1, 5-HT is able to significantly increase the percentage of newly generated neurons and neuroblasts compared to vehicle-treated cells (p < 0.001, ANOVA, Figure 1a,b). Altogether these data confirm that in our *in vitro* model ahNPC differentiation is positively modulated by 5-HT levels, in line with previous reports.[31]

TZD Promotes Neuronal but Not Glial Differentiation of ahNPCs in Vitro. Subsequently, ahNPCs were differentiated in the presence of a wide range of TZD concentrations (0.0003−1 μM) for 24 h. Such concentration range was chosen with the purpose of studying the potential involvement of all receptor subtypes bound with high and low affinity by TZD.[21] Compared to vehicle-treated cells, a significant increase in the percentage of MAP-2$^+$ and nestin$^−$ cells was observed in the presence of 0.01−0.3 μM TZD (0.1 μM TZD, p < 0.001 vs vehicle, ANOVA, Figure 2a), suggesting that the drug is able to
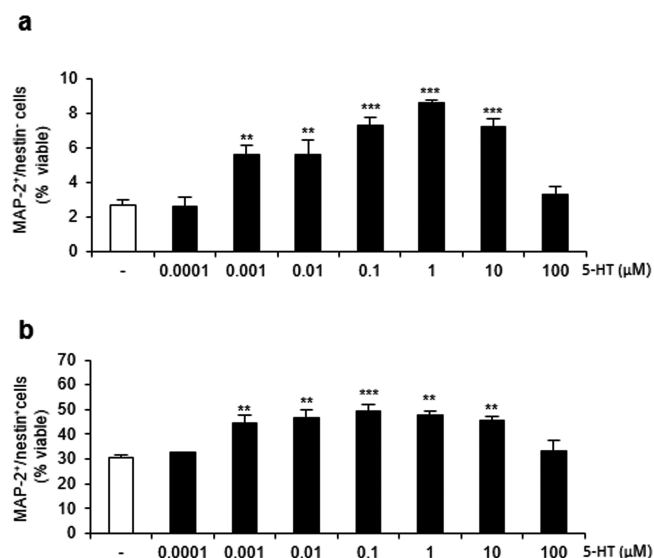
Figure 1. 5-HT promotes neuronal differentiation of murine ahNPCs *in vitro*. Adult hippocampal NPCs were treated with 5-HT (0.0001−100 μM) or vehicle, for 24 h. Effects on the percentage of MAP-2$^+$/nestin$^−$ (a) and MAP-2$^+$/nestin$^+$ (b) cells generated from ahNPCs were evaluated. Data are expressed as mean ± SD: **p < 0.01, ***p < 0.001 vs vehicle-treated cells (one-way ANOVA, Tukey's *post hoc*).

promote neuronal differentiation of murine ahNPCs in a concentration-dependent manner. Interestingly, at the highest concentration (1 μM) TZD was ineffective, suggesting that at that concentration, the drug may activate mechanisms or receptors that potentially counteract those promoting neuro-genesis at lower concentrations. Similar effects were obtained by the drug on the population of MAP-2$^+$/nestin$^+$ neuroblasts (data not shown). We also tested the effects of maximally effective concentrations of TZD (100 nM) and 5-HT (1 μM), in comparison with vehicle, as a function of exposure time (2, 12, 24, and 48 h). In the presence of vehicle, the number of neurons (and neuroblasts, data not shown) progressively increased with time. When we analyzed the number of newly generated MAP-2$^+$/nestin$^−$ neurons, in comparison with vehicle, 5-HT promoted a significant increase at 12 and 24 h but not at 48 h (12 h, p < 0.05; 24 h, p < 0.001, ANOVA, Figure 2b). Compared with 5-HT, TZD proneurogenic effects appeared slightly delayed but longer lasting and more pronounced, with a significantly higher number of neurons generated at 24 and 48 h (% of MAP-2$^+$/nestin$^−$ cells: 24 h, 5-HT 6.1 ± 0.7, TZD 8.9 ± 0.4, p < 0.01 vs 5-HT-treated cells; 48 h, 5-HT 9.1 ± 0.7, TZD 14.3 ± 1.3, p < 0.001 vs 5-HT treated cells, ANOVA, Figure 2b).

In absence of growth factors, ahNPCs can also differentiate toward non-neuronal lineages. Interestingly, 24 h exposure of ahNPCs to TZD (0.0003−1 μM) affected neither the percentage of newly generated GFAP$^+$ astrocytes (Figure 2c) nor the percentage of NG2$^+$ oligodendrocyte precursors (Figure 2d). In order to investigate whether TZD may affect survival of NPCs and their progeny, we also analyzed apoptotic and necrotic rate in our cell culture. As shown in Figure 2e, the percentage of apoptotic cells was not significantly different in TZD- versus vehicle-treated conditions. Similarly, no difference was observed in the number of necrotic cells, as established by disrupted membrane integrity in vehicle- vs TZD-treated ahNPC cultures (Figure 2f). Some antidepressants are also positive modulators of NPC proliferation.[15] We tested the
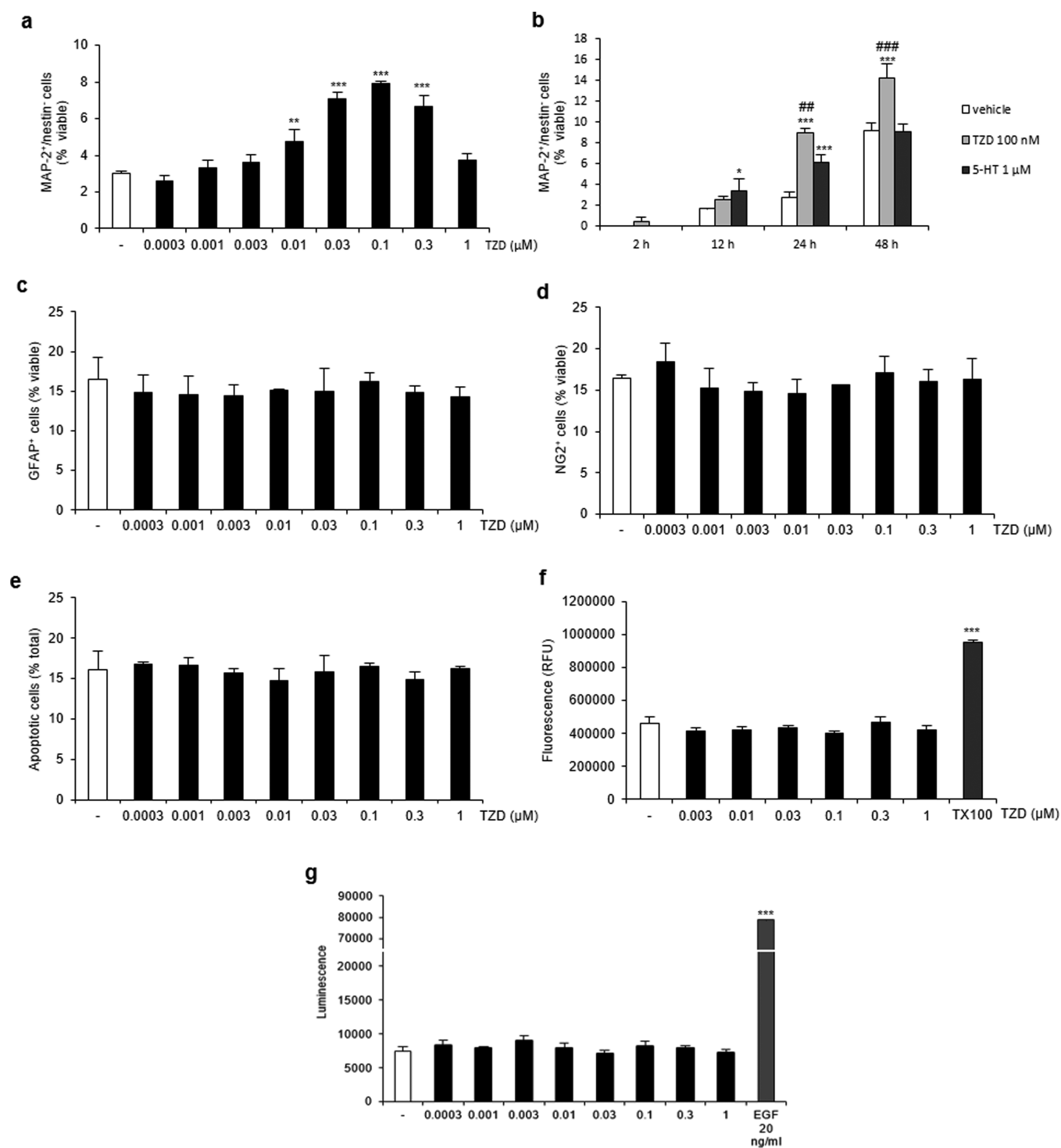
Figure 2. Effects of TZD on murine ahNPC differentiation, survival, and proliferation. Adult hippocampal NPCs were treated with TZD at the indicated concentrations or vehicle for 24 h. (a) TZD effects on the percentage of MAP-2$^+$/nestin$^-$ cells generated from ahNPCs. (b) Time course analysis of the proneurogenic effects of TZD and 5-HT. The percentage of MAP-2$^+$ cells generated in the presence of TZD (100 nM) and 5-HT (1 μM) was assessed after 2, 12, 24, and 48 h of treatment. Data are expressed as mean ± SD: *$p < 0.05$, ***$p < 0.001$ vs vehicle-treated cells; ##$p < 0.01$, ###$p < 0.001$ vs 5-HT-treated cells (One-way ANOVA, Tukey's *post hoc*). (c,d) Evaluation of TZD effect on the percentage of newly generated GFAP$^+$ astrocytes (c) and NG2$^+$ oligodendrocyte precursors (d). (e) Assessment of TZD effect on the apoptotic rate of ahNPCs and their progeny. Data are expressed as the percentage of apototic cells over the total number of cells. (f) Evaluation of TZD cytotoxic effect; 0.8% Triton X-100 was used as positive control (TX100). (g) Evaluation of TZD effect on ahNPC proliferation. Epidermal growth factor (EGF, 20 ng/mL) was used as positive control. Data are expressed as mean ± SD: ***$p < 0.001$ vs vehicle-treated cells (one-way ANOVA, Tukey's *post hoc*).

effect of TZD on the proliferation rate of ahNPCs, using epidermal growth factor (EGF) as a positive control. At all tested concentrations, 72 h-exposure to TZD had no effect on

ahNPC proliferation rate (Figure 2g). Overall, these data suggested that (i) TZD effects on ahNPC neuronal differentiation do not occur at the expense of NPC differentiation

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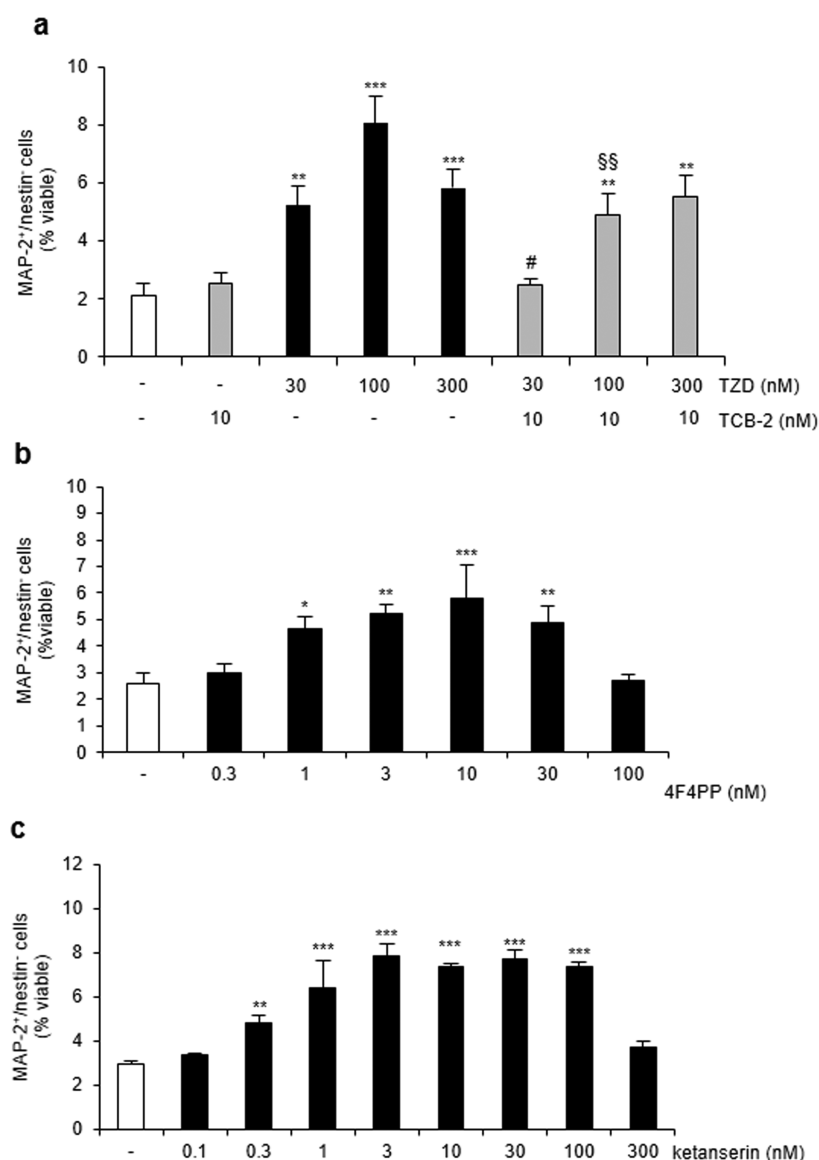
Figure 3. TZD proneurogenic activity in murine ahNPCs is mediated by 5-HT$_{2a}$ antagonism. Effects of the selective 5-HT$_{2a}$ agonist TCB-2 (10 nM) on TZD-mediated increase of newly generated MAP-2$^+$ cells. (b) Effects of 4F4PP (0.3−100 nM), selective 5-HT$_{2a}$ antagonist, and (c) ketanserin (0.1−300 nM), a nonselective 5-HT$_2$ antagonist, on ahNPC differentiation toward the neuronal lineage. Data are expressed as mean ± SD: *$p <$ 0.05, **$p <$ 0.01, ***$p <$ 0.001 vs vehicle-treated cells; #$p <$ 0.05 vs 30 nM TZD-treated cells. $^{§§}p <$ 0.01 vs 100 nM TZD-treated cells (one-way ANOVA, Tukey's *post hoc*).

toward glial lineages and (ii) the effect of TZD is merely differentiative in nature, since neither neuroprotective nor proliferative activity was elicited by the drug on ahNPC cultures.

The Proneurogenic Effects of TZD Are Mediated by 5-HT$_{2a}$ Antagonism. We investigated the receptor subtypes involved in the proneurogenic effect of TZD. TZD binds with high affinity to 5-HT$_{2a}$, α_1, and 5-HT$_{1a}$ and with lower affinity to 5-HT$_{2c}$, α_2, and 5-HTT.[20,21] We first evaluated the involvement of 5-HT$_{2a}$ receptors in TZD-mediated effect. When tested in ahNPC cultures, 10 nM TCB-2, a selective 5-HT$_{2a}$ agonist [$K_{i(5-HT2a)}$, 0.73 nM], produced a rightward shift in TZD concentration−response curve, while the compound was inactive *per se* (Figure 3a). These data suggested that the proneurogenic effects elicited by TZD are mediated by its antagonistic activity at 5-HT$_{2a}$ receptors. To further support the contribution of 5-HT$_{2a}$ receptors in TZD proneurogenic effects,

1−30 nM 4F4PP, a 5-HT$_{2a}$ selective antagonist, [$K_{i(5-HT2a)}$ 4 nM] and 0.3−100 nM ketanserin [a 5-HT$_{2a}$ and 5-HT$_{2c}$ antagonist, $K_{i(5-HT2a)}$ 3.6 nM, $K_{i(5-HT2c)}$ 200 nM], were both capable of promoting neuronal differentiation of ahNPCs in a concentration-dependent manner (ANOVA; Figure 3b,c). Interestingly, as shown for TZD, also for these drugs, we observed an inverted U-shaped response curve.

At first sight, the finding that antagonism at 5-HT$_{2a}$ receptors may promote neurogenesis may be surprising, given the vast plethora of data supporting the view of 5-HT as an endogenous positive modulator of adult hippocampal neurogenesis,[32−34] in line with its proneurogenic effect in our experimental model. However, literature data on a role of 5-HT specific receptor subtypes in the regulation of neurogenesis also support the idea of distinct and even opposite effects on neurogenesis.[35] In particular, *in vivo* data suggested oppositional effects of serotonin receptors 5-HT$_{1a}$ and 5-HT$_2$ in the regulation of
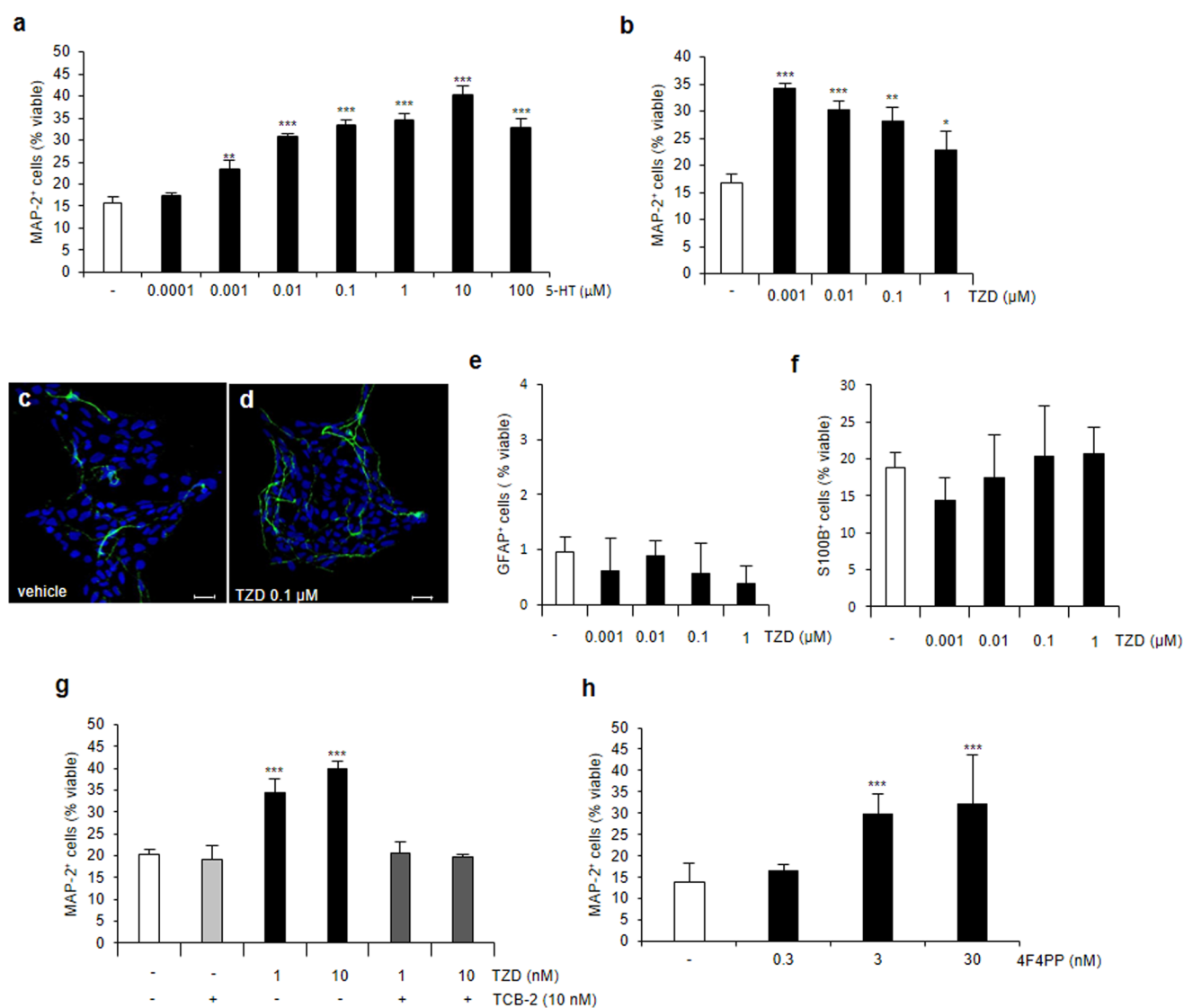
Figure 4. Neuronal differentiation of human iPSC-derived NPC is increased in the presence of 5-HT and of TZD: drug activity is mediated by 5-HT$_{2a}$ antagonism. (a) Effect of 28 day-long 5-HT treatment ($0.0001-100$ μM) on the percentage of MAP-2$^+$ cells generated from human NPCs, compared to vehicle. (b) Effect of 28 day-long TZD treatment ($0.001-1$ μM) on the percentage of MAP-2$^+$ cells generated from human NPCs, compared to vehicle. (c, d) Representative confocal microscopy images for MAP-2 immunolabeling (green) of human NPCs in the presence of vehicle (c) and TZD 100 nM (d). Nuclei are counterstained with Hoechst (blue). Magnification = $\times$40; Scale bar 20 μM. Effect of TZD treatment on the differentiation of human NPCs toward astroglial lineages (e, f), as indicated by the percentages of GFAP (e) and S100B (f) immunopositive cells. (g) Effect of the selective 5-HT$_{2a}$ agonist TCB-2 (10 nM) on TZD-mediated effects. (h) Evaluation of the percentage of *in vitro* generated MAP-2$^+$ cells in the presence of the selective 5-HT$_{2a}$ antagonist 4F4PP ($0.3-30$ nM). Data are expressed as mean $\pm$ SD: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs vehicle-treated cells (one-way ANOVA, Tukey *post hoc*).

adult hippocampal neurogenesis.[35] Unfortunately, in several reports these data appear contradictory, in part probably due to limited selectivity of utilized agonists and antagonists. Klempin et al.,[35] by using cinanserin as a nonselective 5-HT$_2$ antagonist *in vivo*, demonstrated that it acutely produces an increase in cell proliferation in hippocampus. When the same authors tested the nonselective 5-HT$_2$ agonist α-methyl-5-HT, a net decrease in the number of BrdU$^+$ cells was found. Phenotypic analysis for 5-HT$_2$ agonist treatment revealed a net decrease in type 1, 2a, and 2b cells after acute treatment and a significantly reduced number of newborn neurons after 7 days of treatment. Surprisingly, the same authors showed that *in vitro* blockade of 5-HT$_2$ receptors by cinanserin in NPC cultures produced a significant reduction in the number of βIII-tubulin positive cells, which represent newly generated neuroblasts.[35] Even

more confusing, a similar result was obtained upon incubation with the nonselective agonist α-methyl-5-HT. Kemplin's data are in contrast to those obtained by Banasr et al.[33] who showed that the nonselective 5-HT$_{2a}$ and 5-HT$_{2c}$ antagonist ketanserin produced a decrease in the number of BrdU-labeled cells whereas the 5-HT$_{2a}$ and 5-HT$_{2c}$ agonist DOI did not change cell proliferation. Similarly, Jha et al.[28] demonstrated an enhanced adult hippocampal progenitor proliferation as a result of a sustained blockade of the 5-HT$_{2a}$ and 5-HT$_{2c}$ receptors by ketanserin treatment in mice. In our experimental setting, we could confirm that TZD but also a selective 5-HT$_{2a}$ antagonist promote neuronal differentiation of ahNPCs and that TZD proneurogenic effects are counteracted in the presence of a selective 5-HT$_{2a}$ agonist. Based on these findings, herein we suggest that 5-HT$_{2a}$ antagonism may have a
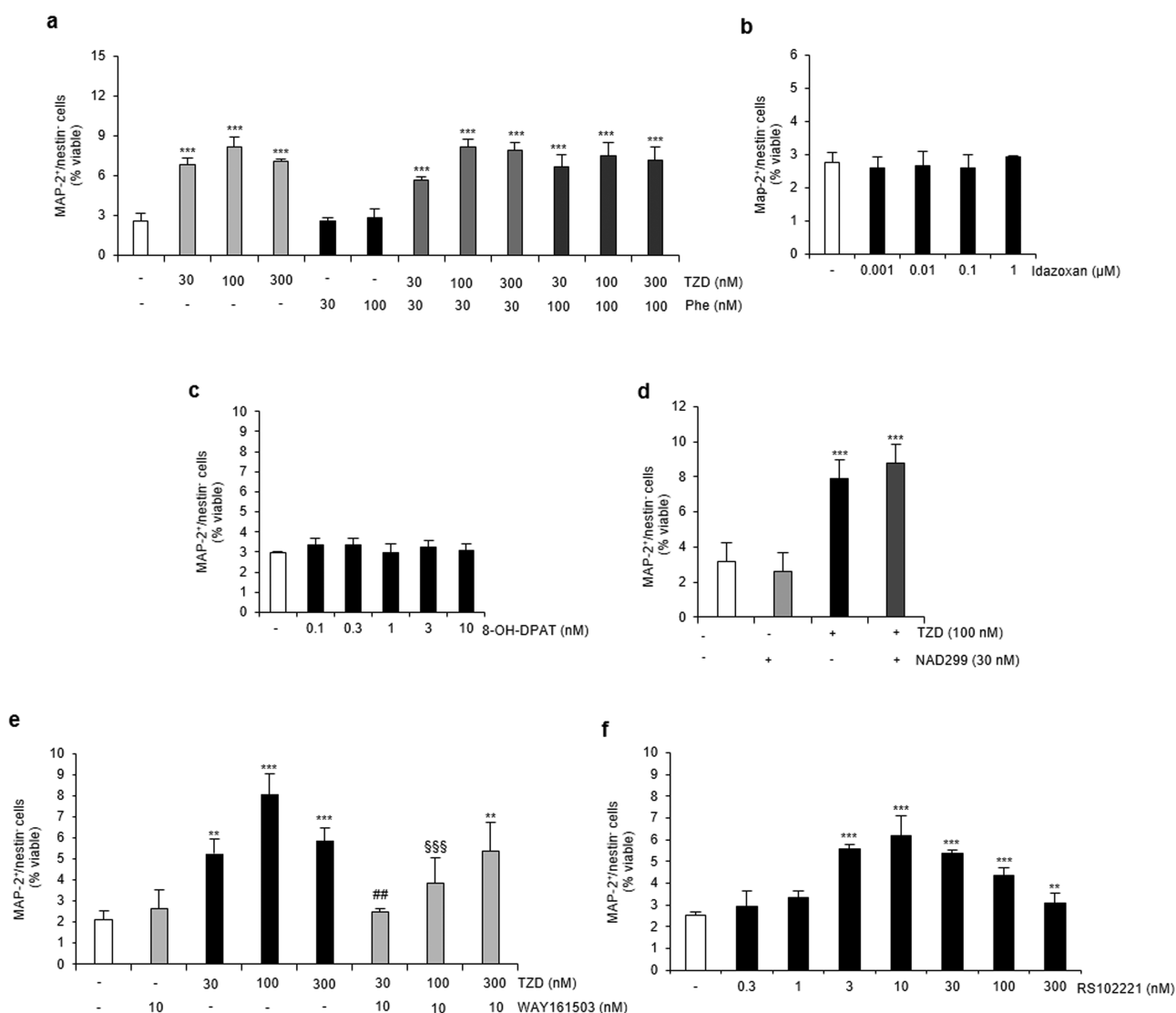
Figure 5. 5-HT$_{2c}$, but not 5-HT$_{1a}$ and α_1/α_2 adrenergic receptors, contribute to the proneurogenic effects of TZD in murine ahNPC. (a) Effect of the α_1 agonist phenylephrine hydrochloride (Phe) on TZD proneurogenic effects. (b) Effect of the α_2 antagonist idazoxan (0.001−1 μM) on neuronal differentiation of ahNPCs. (c) Effect of the 5-HT$_{1a}$ agonist 8-OH-DPAT (0.1−10 nM) on neuronal differentiation of ahNPCs. (d) Evaluation of the effect of pretreatment of ahNPCs with the selective 5-HT$_{1a}$ antagonist NAD299 (30 nM) on TZD-mediated proneurogenic effects. (e) Effect of the selective 5-HT$_{2c}$ agonist WAY161503 (10 nM) on TZD-mediated increase of newly generated MAP-2$^+$ cells. (f) Evaluation of the percentage of newly generated neurons in the presence of the 5-HT$_{2c}$ antagonist RS102221 (0.3−300 nM). Data are expressed as mean ± SD: **$p <$ 0.01, ***$p <$ 0.001 vs vehicle-treated cells; $^{\#\#}p <$ 0.01 vs 30 nM TZD-treated cells; $^{\S\S\S}p <$ 0.001 vs 100 nM TZD-treated cells (one-way ANOVA, Tukey's *post hoc*).

significant role in neuronal differentiation of ahNPCs, at least *in vitro,* and that TZD may act, at least in part, via this mechanism to promote neurogenesis. Recently, another novel pharmacological activity was described for TZD in a model of human neuronal cells, namely, its neuroprotective effects against inflammatory insults.[36] Interestingly, also this TZD-mediated effect involved antagonism at 5-HT$_{2a}$ receptors, since it was counteracted by (*R*)-DOI.[36]

5-HT$_{2a}$-Mediated Proneurogenic Effects of TZD in Human Induced Pluripotent Stem Cell (iPSC)-Derived NPCs. Very little is known about the effects elicited *in vitro* by antidepressant drugs in human neural progenitor cells. We tested the proneurogenic activity of 5-HT on NPCs generated from a human iPSC line. Human iPSC-derived NPCs were differentiated in the absence of growth factors and in the presence of vehicle or 5-HT (0.0001−100 μM) for 28 days. A

significant increase in the percentage of MAP-2$^+$ cells was observed at all tested 5-HT concentrations (ANOVA, Figure 4a). In the same experimental setting, we evaluated the effect of TZD. As shown in Figure 4b, similarly to 5-HT, TZD significantly increased the percentage of newly generated MAP-2$^+$ cells (0.1 μM TZD, $p <$ 0.01, ANOVA, Figure 4b−d). As demonstrated in murine cells, TZD produced no significant effect on human NPC differentiation toward the astroglial lineage, as measured by GFAP (Figure 4e) and S100B immunostaining (Figure 4f). Based on the results obtained on murine ahNPCs, we then tested the effect of TZD (1−10 nM) in the presence of the selective 5-HT$_{2a}$ receptor agonist TCB-2 (10 nM). TCB-2 completely counteracted the effect of TZD (Figure 4g), suggesting that, as in murine ahNPCs, also in human NPCs TZD promotes neuronal differentiation via 5-HT$_{2a}$ antagonism. To further confirm this hypothesis, the
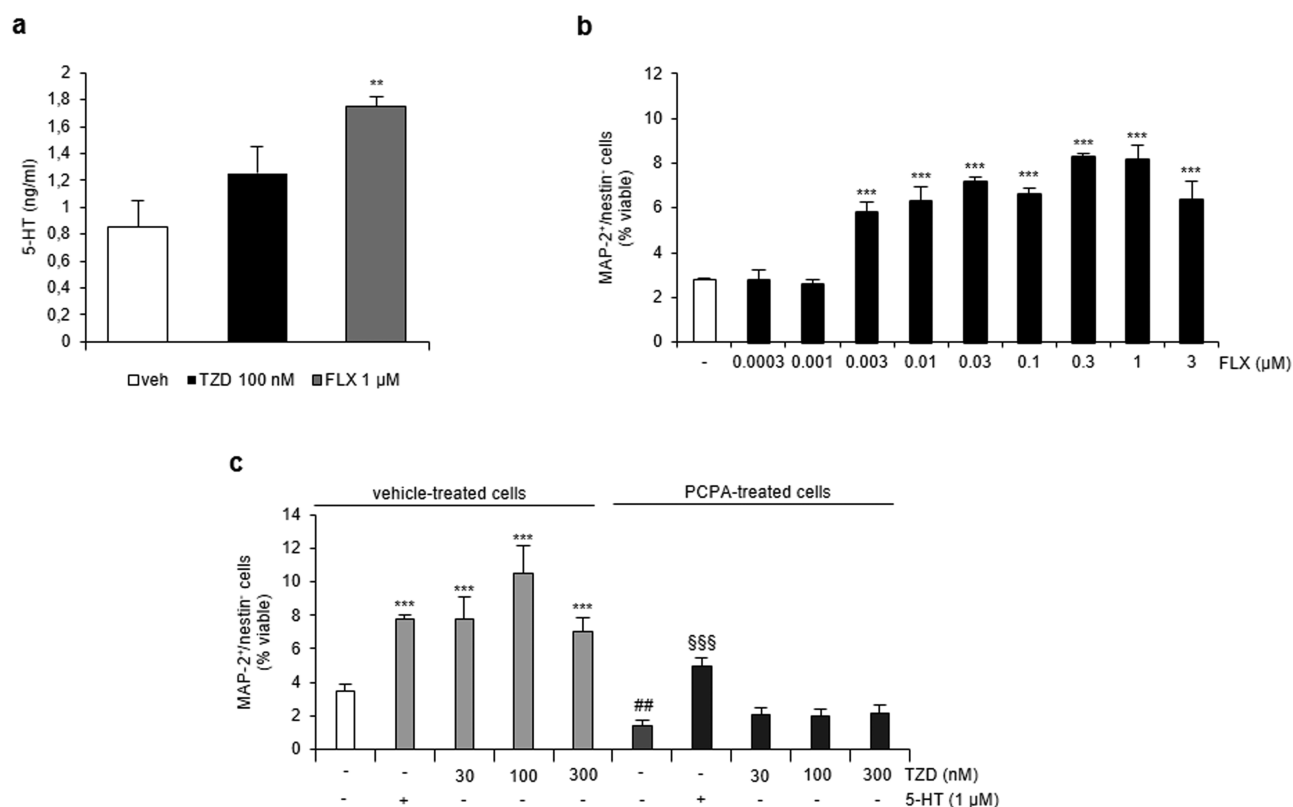
Figure 6. TZD proneurogenic activity requires synthesis of endogenous 5-HT by murine ahNPC. (a) LC-ESI-MS analysis of extracellular 5-HT content in our *in vitro* model under basal conditions and after treatment with 1 μM fluoxetine (FLX) and 100 nM TZD. (b) Effect of fluoxetine (0.0003−3 μM) on the percentage of MAP-2$^+$ cells generated from ahNPCs. Data are expressed as mean $\pm$ SD: ***$p < 0.001$ vs vehicle-treted cells (one-way ANOVA, Tukey's *post hoc*). (c) Effect of irreversible inhibition of tryptophan hydroxylase by *para*-chlorophenylalanine (PCPA, 50 μM, 3 day-treatment of proliferating ahNPCs) on the proneurogenic activity by TZD. Evaluation of exogenous 5-HT (1 μM) treatment on neuronal differentiation of both PCPA-treated and vehicle-treated ahNPCs. Data are expressed as mean $\pm$ SD: ***$p < 0.001$ vs basal condition in vehicle-treated cells; §§§$p < 0.001$ vs basal condition in PCPA-treated cells (one-way ANOVA, Tukey's *post hoc*); ##$p < 0.01$ vs basal condition in vehicle-treated cells (Student's *t* test).

selective 5-HT$_{2a}$ antagonist 4F4PP (3−30 nM) significantly increased the percentage of MAP-2$^+$ cells generated *in vitro*, when compared to vehicle-treated cells (ANOVA, Figure 4h).

Evaluation of the Involvement of Other Receptor Subtypes in TZD-Mediated Proneurogenic Effects: A Potential Role for 5-HT$_{2c}$ Receptors. In light of the multimodal mechanism of action of TZD, we further expanded our investigation. TZD indeed binds with high affinity also α_1 and 5-HT$_{1a}$ receptors.[21] Previous data in our laboratory suggested that α_1 antagonists like doxazosin could promote *in vitro* neuronal differentiation of ahNPCs.[30] However, when the α_1 agonist phenylephrine hydrocloride (30−100 nM) was tested in the presence of TZD, it did not counteract its proneurogenic effects in ahNPC cultures (Figure 5a). Although with low affinity, TZD also exerts antagonistic activity at α_2 receptors.[21] Blockade of this receptor subtype has been demonstrated to accelerate the proneurogenic effects of chronic antidepressant treatment.[37] Based on these observations and on a previous report of a noradrenergic tone in our cellular model,[30] we then tested idazoxan (0.001−1 μM), an α_2 antagonist [$K_{i(\alpha 2a)}$ 10 nM; $K_{i(\alpha 2b)}$ 100 nM]. In our experimental setting, idazoxan was devoid of proneurogenic effects on ahNPCs (Figure 5b). Altogether these data strongly suggested the lack of involvement of both α_1 and α_2 receptors in TZD proneurogenic activity *in vitro*.

A vast array of information suggested that 5-HT$_{1a}$ may be the predominant receptor for antidepressant activity and, more recently, also for serotonin-mediated regulation of hippocampal neurogenesis *in vivo*.[38−40] Although our cellular model expressed 5-HT$_{1a}$ receptor mRNA (data not shown), when murine ahNPCs were exposed to the 5-HT$_{1a}$ agonist 8-OH-DPAT [$K_{i(5-HT1a)}$ 1.78 nM; 0.1−10 nM], we did not observe any proneurogenic effect (Figure 5c), suggesting that 5-HT$_{1a}$ receptors may not be involved in neuronal differentiation elicited by TZD and even by 5-HT. To further corroborate this finding, the selective 5-HT$_{1a}$ antagonist NAD299 [$K_{i(5-HT1a)}$ 0.6 nM; 30 nM] had no effect on the proneurogenic response elicited by 100 nM TZD (Figure 5d). NAD299 alone had no effect on ahNPC neuronal differentiation (Figure 5d). Of note, also human NPCs expressed 5-HT$_{1a}$ mRNA, but as in murine ahNPCs, we could not observe any effect of NAD299 on TZD-mediated effects on these cells (data not shown). The lack of involvement of 5-HT$_{1a}$ receptors in TZD-mediated neuronal differentiation of both murine and human NPCs is in agreement with previously published data suggesting that 5-HT$_{1a}$ receptor subtype is not involved in modulation of ahNPC differentiation but rather in their proliferation. In line with our results, two 5-HT$_{1a}$ agonists, 8-OH-DPAT and ipsapirone, failed to regulate neuronal differentiation of NPCs *in vitro*.[35] Conversely, *in vivo* blockade of 5-HT$_{1a}$ receptors significantly reduced the number of proliferating ahNPCs in rat dentate gyrus.[41] Moreover, after serotonin depletion by *para*-chlorophenylalanine (PCPA), an irreversible inhibitor of the synthetizing enzyme TPH2, treatment with 8-OH-DPAT could
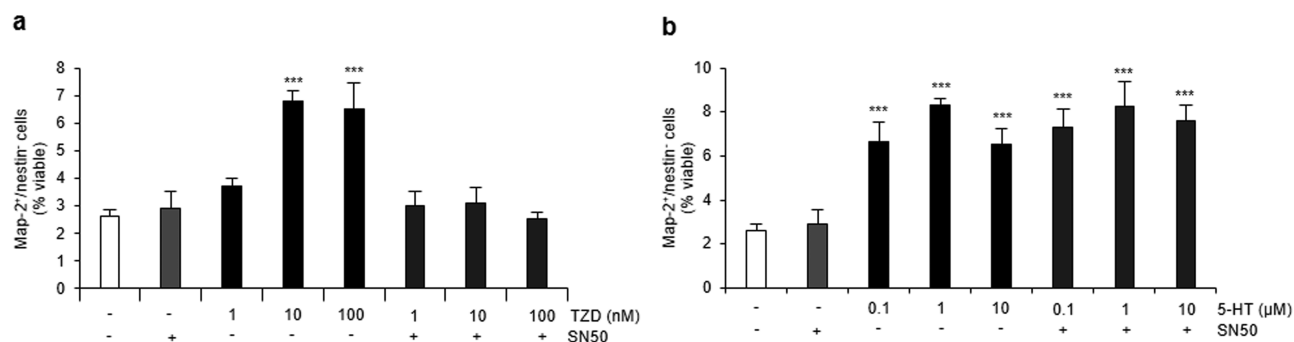
Figure 7. NF-κB p50 nuclear translocation is required for TZD but not for 5-HT proneurogeic effect. (a) Effect of TZD after inhibition of NF-κB p50 nuclear translocation. Data are expressed as mean $\pm$ SD: ***$p < 0.001$ vs vehicle treated cells. (b) 5-HT proneurogenic activity in the presence of NF-κB p50 nuclear translocation inhibition. Data are expressed as mean $\pm$ SD: ***$p < 0.001$ vs vehicle treated cells (one-way ANOVA, Tukey's post hoc).

rescue the number of proliferative ahNPC BrdU$^+$ in the dentate gyrus.[33] Similarly, *in vitro*, PCPA treatment and a 5-HT$_{1a}$R antagonist hampered proliferation but not differentiation of ahNPCs.[42] Again, NPC proliferation was restored after treatment with 5-HT or a 5-HT$_{1a}$ receptor agonist.[42] Interestingly, in a different model of human neuronal cells where TZD elicited neuroprotective effects against inflammatory insults, the antidepressant drug activated ERK phosphorylation and BDNF gene transcription, and this effect appeared to be mediated by activation of 5-HT$_{1a}$ receptors.[36]

TZD binds, although with low affinity, also 5-HT$_{2c}$ receptors. When tested in murine ahNPC cultures, the selective 5-HT$_{2c}$ agonist WAY161503 $[K_{i(5-HT2c)}$ 4 nM] produced a rightward shift in TZD concentration−response curve (Figure 5e). WAY was inactive *per se* (Figure 5e). These data suggested that the proneurogenic effects elicited by TZD are also mediated by its antagonistic activity at 5-HT$_{2c}$ receptors. To confirm our observation, we differentiated murine ahNPCs in the presence of 0.3−300 nM RS102221, a 5-HT$_{2c}$ selective antagonist $[K_{i(5-HT2c)}$ 10 nM]. RS102221 significantly increased the percentage of MAP-2$^+$ cells (10 nM RS102221, $p < 0.001$, ANOVA, Figure 5f). These *in vitro* results are in line with the observation that the antidepressant drug agomelatine can promote hippocampal neurogenesis both via melatonergic agonistic activity and 5-HT$_{2c}$ antagonism in rodents.[23] Altogether these data suggest (i) the lack of involvement of 5-HT$_{1a}$, α_1 and α_2 receptors in TZD proneurogenic activity *in vitro* and (ii) a possible involvement of 5-HT$_{2c}$ antagonism in TZD effect, at least in murine cells. Indeed we failed to demonstrate 5-HT$_{2c}$ mRNA expression in human iPSC-derived NPCs (data not shown).

The *in Vitro* Proneurogenic Effects of TZD Require 5-HT Synthesis by Murine ahNPCs. The fact that TZD exerted its effects via 5-HT$_{2a}$ and 5-HT$_{2c}$ antagonism supported the idea of an endogenous serotoninergic tone in our *in vitro* model and also raised the question of serotonin source both under basal conditions and in response to TZD. We measured, by LC-ESI-MS, extracellular 5-HT concentration in our murine cultures. Values were, on average, 0.86 ± 0.19 ng/mL (Figure 6a), corresponding to 4.9 ± 1.1 nM. Interestingly, murine and human ahNPCs not only express several 5-HT receptors but also 5-HTT.[35,42] TZD is a low affinity 5-HTT blocker,[21] and this drug activity can result in an increased availability of extracellular 5-HT. Interestingly, a slight, although not statistically significant, increase in 5-HT extracellular concentration was observed in the presence of a proneurogenic

concentration of 100 nM TZD compared to vehicle (TZD 1.25 $\pm$ 0.20 ng/mL, vehicle 0.86 ± 0.19 ng/mL, $p = 0.07$, Figure 6a). Under the same experimental conditions, the SSRI antidepressant fluoxetine (0.003−3 μM) promotes neuronal differentiation of murine ahNPCs in a concentration-dependent manner ($p < 0.001$, ANOVA, Figure 6b). The proneurogenic effect of fluoxetine correlated with a significant increase in 5-HT extracellular concentration, compared with vehicle-treated conditions (1 μM FLX 1.76 $\pm$ 0.1 ng/mL, $p < 0.01$ vs vehicle, t test), suggesting that our cellular model indeed does express functional 5-HTT (Figure 6a). Undifferentiated ahNPCs also express both isoforms of tryptophane hydroxylase, TPH1 and TPH2.[42] We performed a three-day treatment of proliferating ahNPCs with 50 μM PCPA to irreversibly inhibit TPH and deplete ahNPCs of endogenous serotonin.[42] As expected, extracellular 5-HT levels became undetectable by LC-ESI-MS in culture medium of PCPA-treated compared to vehicle-treated cells. Although literature data report that PCPA treatment significantly decreases proliferation of ahNPCs both *in vitro* and *in vivo*,[42,43] in our hands, blockade of 5-HT synthesis caused a small but not statistically significant reduction (about −23%) in the number of recovered PCPA-treated NPCs compared to vehicle-treated cells, suggesting a more limited effect of 5-HT depletion on ahNPC proliferation. Similarly, PCPA treatment did not affect the survival of ahNPCs (% dead cells/total cells, 12.9 ± 2.5 and 16.2 ± 2.9 in vehicle- and PCPA-treated cells, respectively, $p > 0.05$, t test). After PCPA or vehicle treatment, ahNPCs were washed and plated at the same cellular density, under conditions promoting neuronal differentiation in the presence of either TZD (30−300 nM) or the corresponding vehicle. The percentage of newly generated MAP-2$^+$ cells was then determined in vehicle- and PCPA-treated cells. Under basal conditions, we observed a statistically significant reduction in the percentage of MAP-2$^+$ cells in PCPA-treated cells compared to vehicle-treated cells (Figure 6c, −59.1%, $p < 0.01$, t test). This effect cannot be ascribed to a reduction in cell viability since we did not observe significant differences in the apoptotic or necrotic rate of ahNPCs and their progeny under the same experimental conditions (data not shown). Even more interestingly, 30−300 nM TZD promoted neuronal differentiation in vehicle-treated but not in PCPA-treated ahNPCs (Figure 6c). As a control, exogenously added 5-HT (1 μM) promoted neurogenesis in both vehicle- and PCPA-treated ahNPCs ($p < 0.001$ vs vehicle, ANOVA Figure 6c), suggesting that PCPA-treated cells were not affected in their ability to respond to proneurogenic signals.

Of note, although not significantly different, the percent of increase of MAP-2$^+$ cells over basal differentiating conditions was higher in PCPA-treated compared to vehicle-treated ahNPCs (+260.9 ± 83.9 and +127.2 ± 32.3% in PCPA- and vehicle-treated cells, respectively, $p = 0.062$, t test; Figure 6c). Altogether, these data strongly support the finding that the effects of TZD on neuronal differentiation of ahNPCs are mediated by drug antagonism at 5-HT$_2$ receptors, in the presence of a serotoninergic tone, which, in our experimental setting, is not significantly affected by the antidepressant drug.

Nuclear Translocation of NF-κB p50 Is Required for the Proneurogenic Effects Elicited by TZD. Based on the current results, our working hypothesis is that 5-HT exerts proneurogenic and antineurogenic effects by interacting with different serotonergic receptor subtypes. While the 5-HT receptors responsible for the serotonin proneurogenic effect in the present model remain to be clarified, we investigated which downstream signaling pathways could play a role in the TZD effect mediated by 5-HT2$_a$ and 5-HT2$_c$ receptor subtypes. Previous data from our laboratory established the relevance of NF-κB proteins, and in particular of the p50 subunit, in the *in vitro* and *in vivo* proneurogenic effect of several drugs[26,27] as well as of neural mediators.[44−46] Based on these data, we tested the involvement of NF-κB p50 in TZD-mediated proneurogenic effects on ahNPCs. Interestingly, cotreatment of ahNPCs with SN50 (10 μg/mL), an inhibitor of NF-κB p50 nuclear translocation, completely counteracted the proneurogenic effects of 1, 10, and 100 nM TZD (Figure 7a).

However, unlike TZD, in same experimental setting the enhancement of the proneurogenic effect obtained by adding external 5-HT did not require NF-κB p50 nuclear translocation (Figure 7b). These results would need additional studies to further characterize the signaling pathways that operate downstream the different 5-HT receptor subtypes and their balance in producing the proneurogenic effects of 5-HT and trazodone.

CONCLUSIONS

This *in vitro* study suggests, for the first time, a new pharmacological activity of TZD, namely, its ability to promote neuronal differentiation of murine and human NPC. An extensive pharmacological characterization of receptors involved in TZD proneurogenic activity in murine cells revealed that 5-HT$_{2a}$ and 5-HT$_{2c}$ antagonism is mainly responsible for that effect. Interestingly, also in iPSC-derived human NPCs, the antidepressant drug promotes neuronal differentiation via blockade of 5-HT$_{2a}$ receptors. This piece of information is quite important since most *in vitro* studies evaluating the activity of clinically relevant drugs on neurogenesis have been performed in rodent cells. Moreover in our hands, unlike the SSRI fluoxetine, the proneurogenic effects of TZD did not appear to correlate with an increase in 5-HT extracellular concentration, as a result of 5-HTT blockade. Finally, our data add further complexity to the role played by serotoninergic neurotransmission in the regulation of adult hippocampal neurogenesis. Previous *in vivo* data suggested oppositional effects of serotonin receptors 5-HT$_{1a}$ and 5-HT$_2$ in the regulation of adult hippocampal neurogenesis.[35] Although obtained *in vitro*, our data also support the concept that serotonin, acting on different receptor subtypes, may promote but also counteract via 5-HT$_2$ receptors neuronal differentiation of adult NPCs. Despite the widespread view of 5-HT as an endogenous positive modulator of hippocampal neurogenesis,

it is not surprising that homeostasis within this process may be reached also by oppositive effects via different receptor subtypes. In our experimental setting, we could not demonstrate that 5-HT$_{1a}$ receptors may play a role in the positive modulation of ahNG either in murine or in human cells, so at this stage we cannot point to the receptor subtype that mediates the *in vitro* proneurogenic effects of 5-HT in these cells. Based on literature reports, 5-HT$_4$ is known to promote neurogenesis both *in vitro* and *in vivo*,[47,48] but mRNA for that receptor subtype was not expressed in our cellular model (data not shown). The other receptor subtype that has been proposed to play a role in neurogenesis is the ionotropic 5-HT$_3$, although conflicting results have been obtained since both activation and blockade of that receptor have been proposed to promote neuronal differentiation of ahNPCs.[49,50] Again, mRNA for this receptor subtype was not detected in our model (data not shown).

Interestingly, at least in murine cells, in our experimental setting the time course of TZD proneurogenic effects was also different compared to that of 5-HT, again pointing to differences in the overall effects on neurogenesis of molecules that act via distinct receptor subtypes. The different time course profile of TZD and 5-HT could be also potentially explained by changes in receptor subtype expression by ahNPCs and their progeny over time *in vitro*. Such changes could even be triggered differently by TZD or 5-HT during *in vitro* cell maturation stages. For these reasons, future studies will need to further explore the differential contribution of distinct 5-HT receptors on NPCs and their progeny and their overall impact on adult hippocampal neurogenesis.

METHODS

Animals. Male C57BL/6 (C57BL/6, The Jackson Laboratories, US) mice were acclimated under light- and temperature-controlled conditions in high efficiency particulate air (HEPA)-filtered Thoren units (Thoren Caging System, Hazelton, PA). Mice were housed in numbers of 3−4/cage with *ad libitum* access to food and water at the animal facility of Piemonte Orientale University. Animal care and handling were carried out according to protocols approved by the European Community Directive and the local Institutional Animal Care and Use Committees (IACUC).

Drugs. All drug concentrations were chosen based on binding affinity to their target receptors. The source of compounds was as follows: 5-hydroxytryptamine hydrochloride (5-HT), trazodone hydrochloride (TZD), ketanserin (+) tartrate salt, DL-*p*-chlorophenylalanine (PCPA), phenylephrine hydrochloride (Phe), and fluoxetine hydrochloride were purchased from Sigma-Aldrich (Milan, Italy); 4F4PP oxalate, RS102221 hydrochloride, *R*-(+)-8-hydroxy-DPAT hydrobromide (8-OH-DPAT), WAY161503 hydrochloride, TCB-2, NAD299 hydrochloride, and idazoxan hydrochloride were purchased from Tocris (Bioscience, Bristol, UK).

Isolation and Culture of Adult Hippocampal Neural Progenitor Cells (ahNPCs). Adult murine hippocampal NPC isolation was performed as previously described.[30] Briefly, for each culture preparation, three adult male mice (3−4 month-old) were euthanized by cervical dislocation, and their hippocampi were dissected. Tissue was digested with a Papain Dissociation System (Worthington DBA, Lakewood, NJ). Cells were plated in NUNC EasyFlask 25 cm^2 (Thermo Scientific, Waltham, MA) and cultured in serum-free complete proliferation medium [Neurobasal-A with B27 supplement, 2 mM glutamine (Gibco, Life Technologies, Monza, IT), 20 ng/mL recombinant human epidermal growth factor (rhEGF), 10 ng/mL recombinant human fibroblast growth factor-2 (rhFGF-2, PeproTech, Rocky Hill, NJ), and 4 μg/mL heparin sodium salt (Sigma-Aldrich)]. Passage 1 (P1) neurospheres were dissociated for the first time after 10 days in vitro (DIV). From P2, dissociation was

performed every 5 days, and NPCs were replated at a density of 12 000 cells/cm^2. NPCs were used for proliferation and differentiation experiments from P5 to P30. Proliferating NPCs were routinely tested for their undifferentiated state by expression of SOX2 (Sry-related HMG box transcription factor) and nestin (type VI intermediate filament). For irreversible inhibition of tryptophane hydroxylase (TPH), cells were treated with 50 μM of PCPA or vehicle for 72 h starting from the second day after dissociation. At day 5, cells were dissociated and plated for differentation.

Human iPSC-Derived Neural Progenitor Cell Culture. Human NPC derived from human induced pluripotent stem cells (hiPSCs) generated by genome footprint-free episomal reprogramming method were purchased from 101 Bio (Palo Alto, CA). hNSCs were cultured in DMEM/F12 complete medium supplemented with B27 and N2 (Gibco), GlutaMax (Gibco), 20 ng/mL rhEGF, 20 ng/mL rhFGF-2, 2 μg/mL heparin, and 100 U of penicillin and 100 μg/mL streptomycin. Every 4 days (80% confluence), hNSCs were dissociated with StemPro Accutase (Thermo Fisher, Life Technologies) at 37 °C for 4 min. Single cells were harvested by centrifugation at 1000g, counted, and seeded onto Geltrex LDEV-Free hESC-qualified (Invitrogen, Life Technologies) coated dishes (3.5 cm, BD Falcon) at a density of 52 000 cells/cm^2. hNSC cultures were maintained at 37 °C, 95% humidity, 5% CO$_2$, and received daily medium changes. From P5 to P14 cells were used for differentiation.

Mouse Hippocampal NPC Proliferation and Differentiation. For evaluation of cell proliferation, dissociated NPCs were seeded onto flat bottom 96-well plates (Falcon) at a density of 4000 cells/well. Standard medium for basal conditions contained Neurobasal-A, B27, 2 mM glutamine, 10 ng/mL hbFGF-2, 4 μg/mL heparin, and 100 U of penicillin and 100 μg/mL streptomycin. Cells were treated with indicated drug concentration or corresponding vehicle and incubated in a humidified atmosphere at 37 °C with 5% CO$_2$. Complete proliferation medium with rhEGF (20 ng/mL) was included as positive control. After 72 h, proliferation rates were determined using CellTiter-Glo luminescent cell viability assay (Promega), according to manufacturer's instructions. Values were normalized to standard medium values. For NPC differentiation, the detailed procedure was previously described.[43] Briefly, single NPC cells were plated onto laminin-coated Lab-Tek 8-well Permanox chamber slides (Nunc) in differentiation medium containing Neurobasal-A, B27, and 2 mM glutamine at a density of 43 750 cells/cm^2. NPCs were treated with indicated drug concentrations or corresponding vehicle for 24 h. To investigate the involvement of different receptors in trazodone or serotonin effects, cells were treated for 30 min with selective agonists or antagonists before addition of the drug. For inhibition of NF-κB p50 nuclear translocation, NPCs were treated with 10 μg/mL SN-50 peptide (Calbiochem) for 30 min before addition of 5-HT or TZD.

hNSC Differentiation and Drug Treatment. hNSCs were cultured onto Geltrex coated Lab-Tek 8-well Permanox chamber slides (Nunc) at a density of 25 000 cells/cm^2 in 200 μL of complete medium. Cells were allowed to attach for 6 h, then medium was replaced by differentiation medium [DMEM/F12, B27 and N2 supplements, Glutamax, nonessential amino acids (NEAA, Sigma-Aldrich), 200 nM ascorbic acid (Stemcells technologies, FR), and 1349 g/L glucose (Sigma-Aldrich)] containing vehicle or drug treatment. Every 4 days medium was half changed with fresh differentiation medium.

Immunolocalization Studies in Differentiated Murine and Human NPCs. After differentiation, cells were fixed with 4% paraformaldehyde/4% saccharose solution (Sigma) and processed for immunocytochemistry as detailed elsewhere.[30] Primary antibodies were as follows: anti-microtubule-associated protein-2 (MAP-2; rabbit polyclonal, 1:600; Millipore, Milan, IT), anti-nestin (chicken monoclonal, 1:1500; Neuromics, DBA, IT), anti-glial fibrillary acidic protein (GFAP, mouse polyclonal, 1:600, Millipore), anti-chondroitin sulfate proteoglycan (NG2, rabbit polyclonal, 1:500, Millipore), anti-S100B (rabbit polyclonal, 1:1000, Dako, DBA, IT). Secondary antibodies were as follows: Alexa Fluor 555-conjugated goat anti-rabbit (1:1400), Alexa Fluor 488-conjugated goat anti-chicken (1:1400), Alexa Fluor 555-conjugated goat anti-mouse (1:1,600),

and Alexa Fluor 488-conjugated goat anti-rabbit (1:1400) (all from Molecular Probes, Life Technologies). Nuclei were counterstained with 0.8 ng/mL Hoechst, diluted in PBS. In each experiment, five fields/well (corresponding to about 150−200 cells/well) were counted with a 60× objective by a Leica DMIRB inverted fluorescence microscope. Positive cells for each marker were counted, and their percentage was calculated over total viable cells.

Cytotoxicity Assay. To assess the potential cytotoxic effect of trazodone on NPC and their progeny, cells were plated for differentiation on laminin-coated flat bottom 96-well plates (Falcon) at the density of 43 750 cells/cm^2. Cells were treated with indicated trazodone concentration or vehicle for 24 h. Cytoxicity was evaluated using CellTox Green Cytotoxicity Assay (Promega) according to manufacturer's instructions. Positive control for membrane damage was 0.8% Triton X-100 (Sigma) and four replicates for each condition were measured. Fluorescent signal was read by VICTOR Multilabel Plate Reader platform. The cytotoxicity results are expressed as relative fluorescent units (RFU). Apoptotic rate was determined as previously described.[44]

LC-ESI-MS Analysis. LC-ESI-MS analyses were carried out using a Thermo Finnigan LCQ Deca XP Plus system equipped with a quaternary pump, a Surveyor AS autosampler, and a vacuum degasser (Thermo Finnigan, San Josè, CA). The liquid chromatography was performed on a Phenomenex Luna HILIC column (150 mm × 2 mm, 3 μm) with a Phenomenex Luna HILIC security guard column (4 mm × 2 mm), at 25 °C, at 200 μL/min flow rate, under isocratic elution. The mobile phase was composed of a 90:10 ratio of acetonitrile and water (both with 0.2% v/v of formic acid). The injection volume was 5 μL, and the run time was 20 min. The MS detection of serotonin was performed in positive ionization mode. Quantitation was carried out using single reaction monitoring (SRM) mode to monitor the transition m/z 177 > 160 (collision-induced dissociation, CID 22 eV). The principle instrument parameters were set as follows: ion spray voltage, 5.2 kV; source current, 80 μA; capillary temperature, 350 °C.

Sample Preparation for LC-ESI-MS Analysis. Vehicle- or PCPA-treated ahNPCs were differentiated in the presence of vehicle, trazodone, or fluoxetine. Differentiation media were collected after 24 h and centrifuged for 10 min at 16 000g. Acetonitrile (140 μL) with 0.2% of formic acid was added to 60 μL of collected media. Proteins were precipitated by centrifugation for 10 min at 11 300g. An aliquot of 180 μL of supernatant was evaporated to dryness in a rotational vacuum concentrator (for about 4 h at 40 °C). The sample was reconstituted in 80 μL of water/acetonitrile with 0.2% of formic acid (3:7 vol/vol), before the injection. For quantification of serotonin in differentiation medium, a calibration curve was prepared in blank matrix (differentiation medium treated with acetonitrile with 0.2% of formic acid, 3:7 vol/vol) in the appropriate range.

Statistical Analysis. All experiments were run in triplicate using different cell preparations and repeated at least three times. Data were expressed as mean ± SD and analyzed by one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test or by Student's *t* test. Statistical significance level was set for p values <0.05.

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Author Contributions
M.G. and L.P. participated in research design. V.B., F.M., G.M, M.G., and L. P. conceived methodologies, designed experiments, and analyzed results. V.B., E.X., and R.S. performed cellular experiments. M.B. and E.D.G. performed LC-ESI-MS experiments. V.B., M.G., E.D.G., and P.L.C. analyzed results. M.G. wrote the manuscript with input from all coauthors. All authors participated in discussion and proofreading of the manuscript.

■ ABBREVIATIONS

5-HT, 5-hydroxytryptamine, serotonin; ahNG, adult hippocampal neurogenesis; ahNPC, adult hippocampal neural progenitor cell; BDNF, brain-derived neurotrophic factor; BrdU, bromodeoxyuridine; FLX, fluoxetine; MAP-2, microtubule-associated protein 2; MDD, major depressive disorder; PCPA, *para*-chlorophenylalanine; 5-HTT, serotonin transporter; SGZ, subgranular zone; SNRI, serotonin–norepinephrine reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor; TCA, tryciclic antidepressant; TPH, tryptophan hydroxylase; TZD, trazodone

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