REGULAR ARTICLE

Taurine stimulates proliferation and promotes neurogenesis of mouse adult cultured neural stem/progenitor cells

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Abstract This study reports an effect of taurine (1–10 mM) increasing markedly (120%) the number of neural precursor cells (NPCs) from adult mouse subventricular zone, cultured as neurospheres. This effect is one of the highest reported for adult neural precursor cells. Taurine-containing cultures showed 73–120% more cells than controls, after 24 and 96 h in culture, respectively. Taurine effect is due to enhanced proliferation as assessed by BrdU incorporation assays. In taurine cultures BrdU incorporation was markedly higher than controls from 1.5 to 48 h, with the maximal difference found at 1.5 h. This effect of taurine reproduced at every passage with the same window time. Taurine effects are not mimicked by glycine, alanine or GABA. Clonal efficiency values of 3.6% for taurine cultures and 1.3% for control cultures suggest a taurine influence on both, progenitor and stem cells. Upon differentiation, the proportion of neurons in control and taurine cultures was 3.1% (±0.5) and 10.2% (±0.8), respectively. These results are relevant for taurine implication in brain development as well as in adult neurogenesis. Possible mechanisms underlying taurine effects on cell proliferation are discussed.

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Introduction

Taurine is a sulfur amino acid, present in large amounts in most cell types and tissues as a free amino acid. It is not a protein amino acid and participates in only few metabolic reactions (Huxtable, 1992). Taurine has a function as osmoregulator (Pasantes-Morales, 2007) and exhibits a protective effect in a variety of detrimental situations, often acting as antioxidant (Schaffer et al., 2009). Early studies on brain taurine point to its involvement in brain development. Taurine levels in the immature brain are 3–4-fold higher than in the adult brain, a decrease consistently observed regardless of the species variations in brain taurine content (Agrawal et al., 1968;
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Sturman and Gaull, 1975; Miller et al., 2000). Taurine deficiency disturbs development in cats (Sturman, 1993) leading to a delay in cell division and migration in cerebellum and visual cortex (Sturman et al., 1985; Palackal et al., 1986). Similarly, taurine deprivation in newborn monkeys results in abnormalities in the visual cortex characterized by delayed migration of neurons and poor differentiation of pyramidal cells (Neuringer et al., 1990). Furthermore, studies in vitro in cultures of dissociated cerebellum showed that cell taurine depletion significantly reduces cell migration (Maar et al., 1995). It seems therefore that a deficiency of taurine disturbs the developmental precise program of cellular proliferation, migration and differentiation. In further support to an involvement of taurine in brain development, a recent study reports that the detrimental influence of gestational protein restriction on fetal brain is prevented by supplementation of taurine during pregnancy (Liu et al., 2011).

The mechanism(s) of this taurine requirement for brain development are poorly understood. The influence of taurine may be exerted on cell division, migration or differentiation. These possibilities can be examined in a preparation of neural precursor cells (NPCs) collectively including neural progenitor and stem cells, which are characteristically proliferative and self renewing, and maintain the ability to differentiate into neural cell lineages (Reynolds et al., 1992; Rietze and Reynolds, 2006). NPCs actively proliferate in cultures as floating spherical clusters called neurospheres. This is a widely used technique to characterize a variety of NPCs features. In the proper conditions, i.e. free-serum media supplemented with specific growth factors, NPC cultures can be expanded through several passages. If then switched to serum-containing media, NPCs differentiate into astrocytes, neurons and oligodendrocytes, which are formed in different proportion, being astrocytes the most abundant cell type formed (Ahmed, 2009). A previous study from our laboratory described an effect of taurine increasing proliferation of NPCs generated from embryonic mouse mesencephalon (Hernández-Benítez et al., 2010a) but there is no information about taurine effects in NPCs from the adult brain.

Interest in studies about adult NPCs is raised by the possibility of increasing and manipulating neurogenesis in the adult brain. Neurogenesis persists in some regions of the adult brain derived from NPCs located at the SVZ of the lateral ventricles (Ming and Song, 2011), and it is enhanced by brain injuring conditions such as ischemic stroke, seizures or in neurodegenerative diseases (Arvidsson et al., 2002; Nakatomi et al., 2002; Parent, 2007; Vandenbosch et al., 2011). NPCs may then be a source of neuronal replacement during brain repair. It is therefore of interest to investigate whether taurine has an influence on proliferation or differentiation of NPCs obtained from the adult brain. The present study on NPCs obtained from the SVZ of adult mice brain, examined whether taurine affects cell viability and proliferation and evaluated its effect on the proportion of astrocytes and neurons generated after differentiation.

Results

Neural precursor cells (NPCs) of the SVZ of mice adult brain, cultured as neurospheres were used in this study. NPCs from adult tissue are currently defined by self-renewal, positive reaction to nestin and multipotent differentiation. We found that i) neurospheres are formed from the dissociated SVZ and expanded through several passages, ii) cells forming neurospheres from passages 1 to 6 ranged 90.4–95.5% positive to nestin, 0.92–2.2% positive to GFAP and 1.2 to 2.3% positive to β-III-tubulin, and iii) under the appropriate conditions, NPCs differentiate into neurons (β-III-tubulin-positive cells), and astrocytes (GFAP-positive cells) with a marked reduction (up to 2.9%) of nestin-positive cells (Supplementary Figs. 1A–C). It can be then reasonably concluded that our cultures contain essentially NPCs organized as neurospheres. As shown by the range of variation in the quantitative composition of cells, no significant difference was found through the several passages, 1–6.

Figure 1  Effect of taurine on the number of NPCs grown as neurospheres. Neurospheres generated in EGF/FGF2 plus or minus taurine, were mechanically disaggregated and the cells were directly counted. A. Cells from primary neurospheres (P1) or from secondary neurospheres at different culture passages (P2–P4). At each passage (P) the number of NPCs was counted. Bars represent the percentage increase in NPCs grown in the presence of 10 mM taurine (black bars) over the control condition (empty bars). The number of cells in empty bars was set at 100% to homologate differences between primary and secondary passages. B. Increase with time of the number of NPCs from neurospheres grown in the control medium (□) or in the presence of 10 mM taurine (■). Values are means ± SE, n ≥ 5 experiments, significantly different at *P < 0.05.
The effect of taurine on NPCs was examined in primary neurosphere cultures as well as on the secondary neurospheres formed after successive culture passages, up to 4–6 passages. Primary neurospheres originate directly from the excised SVZ, and cultured in a conditioned medium without FBS. Growth and proliferation of NPCs forming neurospheres are sustained by the growth factors EGF and FGF2. No neurospheres were formed in cultures with taurine but without growth factors.

Addition of taurine to cultures markedly increased the number of NPCs. All secondary cultures start with a similar number of cells (about $200 \times 10^3$), which is referred to as the initial cell number. After 96 h, taurine cultures contained about 120% more cells than control cultures. This effect of taurine was consistently observed in primary cultures as well as in secondary cultures at all passages (Fig. 1A). The increase with time (1–4 days) of NPC number in cultures in the presence or absence of taurine is shown in Fig. 1B. After 24 h taurine cultures have significantly more cells (73%) than controls. This difference further increased with time, and at 48, 72 and 96 h, taurine cultures have, respectively, 85%, 116% and 120% more cells than controls (Fig. 1B).

The effect of taurine increasing NPC number may result from a protective action improving cell viability or and from an increase in proliferation. This was examined by measuring viability by the Trypan blue exclusion and MTT assays, and proliferation by BrdU incorporation. Fig. 2A shows the time course of BrdU incorporation, in cells cultured with or without taurine. BrdU incorporation was monitored at short times in culture, 1.5, 3, 6 and 12 h, and thereafter every 24 h up to 96 h (Fig. 2A). In control cultures, the percentage of BrdU$^+$ cells found immediately after neurosphere disaggregation in the previous passage was 14% (initial point of the curve) and this number remained essentially unchanged during the first 12 h in the new culture. During the next hours (24–48 h) the number of BrdU$^+$ cells increased up to 24% and thereafter a decline was observed (Fig. 2A). The BrdU incorporation pattern in the presence

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

**Figure 2** Viability and BrdU incorporation of NPCs formed over the time in culture in the presence or absence of taurine. A. Time-course of cellular BrdU incorporation, after a 1.5 h pulse (see Materials and methods). Results are expressed as percentage of BrdU$^+$ cells over total cells counted in control cultures (□) or in taurine containing cultures (■). The initial point in the curve corresponds to the percentage of BrdU$^+$ cells found after 96 h in culture, immediately after neurosphere disaggregation (∆, ▲). B. Cell viability determined by the Trypan blue exclusion assay as described in Materials and methods. C. Cell viability assayed by MTT. Bars represent means±SE of 4–12 experiments. Significantly different from their respective controls at *P*<0.05.
of taurine was markedly different than in controls. The percentage of BrdU in cells from dissociated neurospheres was about 12%, but once seeded for a new culture, a striking and fast increase in BrdU incorporation occurred, so that as early as 1.5 h of culture the number of BrdU+ cells has doubled attaining 26% of NPCs. At this time a BrdU incorporation peak was observed (Fig. 2A). BrdU incorporation rate slightly increased in the following days up to 48 h, and declined thereafter. Upon neurosphere disaggregation to start a new cycle of expansion, the effect of taurine increasing BrdU incorporation occurs again, with the same window time, and this sequence of events reproduces at every passage. Comparing the BrdU incorporation curve in control and taurine cultures, it is evident that taurine was markedly increasing the number of NPCs in DNA synthesis phase during the first hours after seeding (Fig. 2A). In contrast, taurine did not affect cell viability, examined by Trypan blue exclusion and MTT assays. No difference was found at any time between 1.5 and 96 h between taurine and control cultures (Figs. 2B, C). Apoptotic death estimated by TUNEL was found to be lower than 3%, in cultures either in the presence or absence of taurine (results not shown).

Taurine effect increasing NPC number was examined at taurine concentrations ranging 0.1 to 30 mM. Maximal effect was found at 10 mM, but some increase was observed already at 0.5 mM although it was significant only from 1 mM (Fig. 3A). Other amino acids such as GABA, glycine or alanine, at the same concentration, did not modify NPC number. Only the amino acids structurally similar to taurine, hypotaurine and β-alanine, exhibited some effect increasing NPC number although it is clearly lower than that of taurine (Fig. 3B). In embryonic NPCs, GABA shows a proliferative effect at low doses (Nakamichi et al., 2009). In adult NPCs, GABA (1–10 mM) induced a slight decrease in cell number (Fig. 3C).

NPCs is used as a generic term including stem cell and progenitor cells. To investigate whether the effect of taurine above described occurs on the neurosphere stem cells as well as on progenitor cells, NPCs were seeded at low-density on a 96 wells plate (25 cells per well; 0.25 cells/μL) and the total number of neurospheres generated after 7 days was counted. Under these conditions, control cultures generated 31 (±4) neurospheres per plate while in the presence of taurine 86 (±14) neurospheres per plate were generated (Fig. 3C).

Figure 3
Concentration-dependent effect of taurine and other amino acids on NPC number. Neurospheres were generated in control condition (empty bars), in taurine condition (black bars) or in the presence of other amino acids (spotted bars), at the indicated concentrations (in mM). After 5 days in culture, neurospheres were disaggregated and the number of NPCs was counted. A. Concentration-dependent effect of taurine. B. Effect of the amino acids: hypotaurine, β-alanine, glycine and L-alanine (at 10 mM) on NPC number. C. Concentration-dependent effect of GABA on NPC number. All results are means ± SE, n ≥ 4 experiments. Significantly different from control at *P<0.01, significantly different from taurine bar ** P<0.05 and ● P<0.01.
formed, an increase of 177% (Fig. 4). The clonal efficiency in cultures with or without taurine was calculated and results showed a markedly higher efficiency in the presence of taurine than in controls (Fig. 4).

Taurine concentration in the SVZ of the lateral ventricles from which NPCs were obtained was 14.5 μmol/g protein, but the taurine content in cells forming neurospheres in the absence of taurine is of only 2 μmol/g protein. Upon taurine addition to the culture medium, this concentration strikingly increased up to 240 μmol/g protein (Fig. 5B), a level higher than that of other adult brain regions (Fig. 5A), and comparable to that found in fetal brain. This ability to concentrate taurine indicates the presence of an efficient mechanism for taurine uptake into the cell. In most cell types, taurine accumulates by the operation of a well characterized energy-dependent transporter TauT. The molecular expression of TauT in NPCs was detected by immunocytochemistry. As shown in Fig. 6A TauT is expressed in essentially all (92.8±2.6%) NPCs. The functional expression of TauT was also examined. Fig. 6B shows taurine uptake by NPCs incubated with increasing concentrations of unlabelled taurine in a range of 1 to 200 μM and 0.8 μCi/mL of 3H-taurine. Total taurine uptake include a nonsaturable and a saturable component. The non-saturable component was identified by measuring 3H-taurine uptake when cells were incubated in a Na+-free medium (NaCl replaced by KNO3). Subtraction of this non-saturable component from total uptake revealed the saturable component. The kinetic constant values of the saturable taurine uptake calculated from the Lineweaver–Burk equation were a Vmax of 493 pmol/mg protein/min and a Km of 41.7 μM (Fig. 6B).

Differentiation of NPCs was induced by replacing the culture medium by a medium without growth factors and containing 1% FBS. Under this condition, NPCs generated astrocytes and neurons. Astrocyte was the most abundant cell type, present in a proportion of 89.6% (±1.3%). The presence of taurine in the differentiation medium only marginally decreased the percentage of astrocytes (85.6±1.0%). The proportion of neurons, identified by β-III-tubulin, was only 3.1% (±0.5%). In the presence of taurine the number of β-III-tubulin-positive neurons increased up to 10.2% (±0.8%) (Figs. 7A, B, C). Neurons positive to the mature neuron marker MAP2 in control cultures were 1.8% (±0.4%), and increased to 5.1% (±0.5%) in taurine cultures (Supplementary Fig. 2). These results correspond to cells growing in 1% FBS medium during one week. The proportion of β-III-tubulin and of MAP2 positive neurons decreased at longer times in culture of 2–3 weeks, and the effect of taurine also proportionally decreased (Supplementary Fig. 3). Other differentiation media were tested in this study, as follows: 1. B27 replacing FBS, 2. ascorbic acid replacing FBS, 3. medium without FBS, 4. neurobasal medium plus B27. In the ascorbic acid medium and medium without factors, only a few number of cells survived. In the B27 medium the number of cells in the culture, including neurons decreased by 50%.

Figure 4 Clonal efficiency in taurine and control cultures. Secondary neurospheres were gently dissociated and seeded at low density (25 cells/well) on 96-well dishes, in the presence or absence of taurine. After seven days in culture, the number of formed neurospheres was counted and the clonal efficiency was calculated. Bars (±SE) represent the mean of neurospheres/plate in each condition. Significantly different *P<0.05.

Figure 5 Taurine concentration of adult mouse brain tissues and of NPCs. Taurine levels were measured by HPLC as described in Materials and methods. A. Taurine content of adult neural tissues. Fresh tissue samples were isolated from cortex, olfactory bulbs and from the lateral ventricles and processed for HPLC. B. Neurosphere taurine content. Neurospheres generated in the EGF/FGF condition plus or minus taurine were collected and treated for HPLC measurements. Bars represent net taurine concentration, i.e. after subtraction of taurine values found in cells exposed to 10 mM taurine and immediately washed and processed for quantification. Data are means±SE of 3–4 experiments.
expected, being this medium directed to neuronal growth. However, the number of neurons in this medium decreased by 45% as compared to the FBS medium. Taurine significantly increased the proportion of neurons formed in neurobasal/B27 and in medium without FBS, but to a lower extent than in the 1% FBS medium.

Discussion

The present study reports an effect of taurine markedly increasing the number of NPCs obtained from the SVZ of adult mouse brain, and grown in culture as neurospheres. The taurine-induced increase in NPC number, of over 120%, is one of the highest reported for any other molecule or condition in NPCs from adult brain, including melatonin, dopamine or neuropeptides (O’Keeffe et al., 2009; Sotthibundhu et al., 2010; Thiriet et al., 2011). We recently reported an effect of taurine increasing the number of embryonic NPCs (Hernández-Benítez et al., 2010a), but the effect now observed in NPCs derived from the SVZ is over 5-fold higher than that in embryonic cells. The reason for this difference is so far unclear, and could be related to the fact that embryonic cells are growing in an environment rich in taurine, while in the adult brain, taurine levels are much lower.

Taurine concentrations of 1–10 mM, required for its action in increasing NPC number are within the range of taurine content found in most cells types, including brain cells (Huxtable, 1992; Wójcik et al., 2010). Taurine content of NPCs cultured in a taurine-free medium is very low, but upon addition of taurine there is a fast accumulation, increasing the cell taurine content up to attain the fetal brain levels. This is likely occurring via the taurine specific carrier TauT. We found this transporter expressed and functionally active in NPCs derived from the adult brain. TauT is present in essentially all animal cells, where it is in charge of taurine uptake from plasma, and has a critical role in maintaining the intracellular taurine levels characteristic of each cellular type. The kinetic constant values for TauT found in adult NPCs cells are within the range found in most cell types, including embryonic NPCs (Hernández-Benítez et al., 2010b).

The increase in the number of NPCs observed in cultures containing taurine is not due to an effect enhancing cell survival but rather results from an action on cell proliferation, as evidenced by the BrdU incorporation assay. Taurine seems not to be a mitogen per se, since in the absence of the growth factors, no neurospheres are formed in the taurine containing medium. It is only when the proliferative activity has initiated that taurine stimulates proliferation.

Figure 6 Molecular and functional expression of TauT in NPCs. A. TauT reactivity was detected by immunocytochemistry in NPCs from disaggregated neurospheres, using a primary anti-TauT antibody, followed by the secondary antibody Alexa fluor 568 (red). Nuclei were counterstained with Hoechst (blue). Reactivity was visualized by epifluorescence microscopy. The photomicrographs are from a representative assay. B. Kinetic analysis of 3H-taurine uptake by NPCs. Cells were incubated during 30 min with 3H-taurine (29 nM), and unlabeled taurine over a concentration range of 1–200 μM. The lines represent the total taurine uptake (●), the uptake measured in a Na+/Cl−-free medium (NaCl replaced by KNO3) (non-saturable component) (■) and the uptake resulting from subtracting the Na+/Cl−-independent uptake (saturable component) (▲). Uptake rates were calculated from the amount of radioactivity in the cells after the uptake period and the unlabeled taurine in the incubation media. C. Lineweaver–Burk plot of the saturable component and values of Vmax and Km calculated using the Lineweaver–Burk equation. Results are means±SE of 13 experiments.
Assays of BrdU incorporation as used in this study showed a progressive increase in the number of cells with DNA replicative activity up to 48 h in culture, when the activity declines, suggesting the transit to the phases of the cell cycle. Taurine markedly influenced the magnitude and time course of DNA synthesis in NPCs. In the presence of taurine, substantially more NPCs exhibit DNA replicative activity at a significant shorter time than in controls and this effect results in the notable increase in cell number found in taurine cultures already after 24 h of a new passage. After this time the difference in BrdU incorporation between taurine and control cultures decreases, and no difference is observed anymore at 72 h. but the initial difference in the number of formed cells persists through all culture days, so that taurine cultures contain more cells than controls, at all times.

We aimed here to elucidate whether the effect of taurine increasing proliferation occurs on stem cells as well as on neural progenitor cells. Neurospheres are formed by aggregates of neural progenitor cells around one or several stem cells, but may also be formed by clusters of progenitor cells only (Rietze and Reynolds, 2006). The protocol of low-cell density was used in the present study to test whether taurine effect enhancing proliferation occurs in stem cells. A low plating density of 5–50 cells/μL has been considered as a clonal condition, i.e. a condition in which each one of the formed neurospheres is presumably generated from a stem cell (Campos, 2004). The cell density used in the present study of 25 cells/well (0.25 cells/μL in 100 μL) is among the lowest reported for NSCs, and the clonal efficiency obtained of 1.3%, is not far from the 2% calculated for the single-cell clonal efficiency (Engstrom et al., 2002). Our results show that taurine markedly enhances the number of neurospheres formed, with a clonal efficiency of 3.6%. Although this approach is not providing rigorous evidence of a clonal condition, the low dilution of the assay minimizes
the possibility of neural progenitor coalescence, and increases the possibility of neurosphere formation due to the presence of stem cells. From these considerations there is a reasonable possibility that the effect of taurine is exerted on both, stem cells and neural progenitor cells.

The mechanism(s) of the stimulatory effect of taurine on cell proliferation here described are still unknown (Pasantes-Morales and Hernández-Benítez, 2010). A possibility, based on the time course of DNA replicative activity observed in the presence or absence of taurine, is that the mechanical disaggregation of neurospheres creates adverse conditions for DNA synthesis, and that this is prevented by taurine. Taurine cell protective activity is well documented although its mechanism is still unclear. Activity as antioxidant, a modulator of calcium homeostasis, or a general effect on membrane stability, are suggested possibilities (Schaffer et al., 2009). Taurine may influence proliferation via an effect on the redox state of the cells, which is known to regulate DNA replication and cell proliferation in various cell types, including NPCs (Kim and Wong, 2009). The antioxidant properties of taurine, reported in a variety of cells and tissues (Schaffer et al., 2009), by preventing redox imbalance in NPCs, may preserve favorable conditions for proliferation (Sharma et al., 2008; Kim and Wong, 2009). However, unpublished studies from our laboratory did not find any effect of general antioxidants increasing proliferation of NPCs as taurine did, a result arguing against an antioxidant action of taurine as the mechanism underlying its effect on NPCs proliferation.

Another possibility is related to a possible interaction of taurine with the GABA<sub>A</sub> receptor. High concentrations of taurine as those showing its proliferative effect may act as agonists of the GABA<sub>A</sub> receptor (Albrecht and Schousboe, 2005) and through this mechanism could regulate proliferation. This is also an unlikely possibility since even when GABA stimulates proliferation in certain conditions in embryonic NPCs (Fukui et al., 2008), in adult cells GABA decreases proliferation, as confirmed in the present study, an action opposite to that of taurine. Taurine-induced cell swelling may possibly be related to its proliferation regulatory effect. There is evidence showing that an increase in cell volume can regulate proliferation (Stutzin and Hoffmann, 2006). The large amounts of taurine accumulated by NPCs may lead to cell swelling and via this mechanism, affecting proliferation. However, other amino acids such as glycine, alanine and GABA also tested at high concentrations may as well accumulate into the cells and induce similar swelling (Andäng et al., 2008), and nevertheless, did not exhibit the same effect as taurine in cell proliferation.

Alternately, taurine may be affecting the rate of cell migration and through this mechanism accelerate cell proliferation. By increasing cell adhesion or and accelerating migration on the plate, cells get close faster and this proximity may then generate a propitious condition for proliferation, as has been suggested (Mori et al., 2006). In any event, the time course of the taurine effects on BrdU incorporation suggests its influence on mechanisms related to the progress of the DNA replication phase of the proliferation cell cycle. Taurine might exert this action by directly influencing the chromatin structure, via electrostatic or hydrophobic interactions with the DNA. There is evidence showing the presence of high taurine levels in nuclei of other cell types (Lobo et al., 2000a,b). Thus, taurine could favor a more propitious chromatin configuration for DNA synthesis, and via this action regulate NPC proliferation (Buche et al., 1989). This possibility is now under investigation in our laboratory.

In addition to its effect on cell proliferation, taurine induced a significant increase in the proportion of neurons formed during the NPC differentiation process. The mechanism of this action is so far unknown.

The present results in NPCs from the present study may be relevant for the taurine role in brain development. Since the early studies of Sturman et al. (1985), it is known that taurine deficiency impairs brain development, disturbing the sequence of cell mitosis, migration and organization. After three decades, the reason for this taurine requirement for optimal brain development remains obscure. An in vitro system as that used in the present study offers a useful experimental model to clarify the role of taurine on brain developing cells. Taurine may also be required for neurogenesis in the adult brain. One of the few regions where neurogenesis persists in the adult brain is the SVZ of the lateral ventricles which provide new neurons to the olfactory bulb during adulthood. Cells from the SVZ proliferate and migrate via the rostral migratory stream to the olfactory bulb where they differentiate into neurons, mostly into granule cells, which integrate into the functional circuitry of the bulb (Lledo et al., 2008). Of relevance for taurine implication in neurogenesis, the olfactory bulb contains the highest taurine levels in the adult brain, and in contrast to most brain regions, there is no taurine decline during brain maturation (Miranda-Contreras et al., 2000). The high levels of taurine in the olfactory bulb may then respond to a requirement for the optimal proliferation, migration and differentiation of the NPCs in the adult brain.

Altogether, results of the present study point to the NPCs as an interesting system to examine in detail the mechanisms by which taurine participates in brain development and of its presumed requirement for neurogenesis in the adult brain. In another context, they provide a rationale for considering the use of taurine as a factor to enrich niches for considering the use of taurine as a factor to enrich niches of neurogenesis when the cell replacement therapy is the strategy of choice for brain injuries and diseases.

**Materials and methods**

**Materials**

DMEM/F12 (Dulbecco’s Modified Eagle Medium) with L-glutamine and 15 mM HEPES, Pen Strep (penicillin streptomycin), fetal bovine serum (FBS), epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), glutamax 100×, goat serum (GS), Neurobasal medium, B27 medium, antibodies Alexa fluor 488 (chicken anti-rat) and Alexa fluor 568 (goat anti-rabbit) were from GIBCO, Invitrogen. Anti-β-III-tubulin, progesterone, sodium selenite, putresine (1-4-diaminobutane-dihydrochloride), human transferrin, human insulin, 5-bromo-2'-deoxyuridine (BrdU), bisbenzimide H 33258 (Hoechst), o-phthalaldehyde (OPA), D-(+)-glucose, Triton 100×, brilliant blueG, MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS), γ-aminobutyric acid (GABA), β-alanine,
l-alanine, glycine, hypotaurine, ascorbic acid and poly-L-lysine hydrobromide, were from Sigma Aldrich. Monoclonal rat anti-bromodeoxyuridine antibody (Rat Mab anti-BrdU), rat anti-GFAP, polyclonal mouse nestin tail and MAP2, were from Accurate Chemical & Scientific Corporation, ZYMED Invitrogen, COVANCE and Santa Cruz Biotechnology, respectively. DeadEndTM Fluorometric TUNEL System was from Promega. Bovine serum albumin (BSA) was from Calbiochem. Ethanol and methanol were from Reactivos Quimica MEYER. Paraformaldehyde (CH2O)n, potassium acetate, and hydrochloric acid (HCl) were from J.T. Baker and taurine (ultra > 99.5%) was from in BioChemika Fluka.

**Cultures**

Animals used in this study were cared for in accordance with the Norma Oficial Mexicana NOM-062-ZOO-1999. SVZ from CD1 adult mouse (6-8 weeks) was dissected and transferred into ice-cold culture medium containing: DMEM/F12, 1% glutamax 100×, 25 μg/mL insulin, 100 μg/mL transferrin, 20 nM progesterone, 60 μM putresine, 30 nM sodium selenite, 50 μM penicillin and 50 μg/mL streptomycin. The tissue was triturated with a micropipette and the supernatant was collected and centrifuged at 1000 rpm/5 min. The pellet was resuspended with culture medium, and cells were seeded in the presence of EGF and FGF2 (20 ng/mL each) in sterile 6-well plates (4×10^4 cells/well) or plus 10 mM taurine. Cultures were incubated at 37 °C in humidified 5% CO2/95% air atmosphere. EGF (20 ng/mL) was added to cultures every 48 h. After 12 days, primary neurospheres were collected and centrifuged at 1000 rpm/5 min. The pellet was resuspended with culture medium, and cells were seeded in the presence of EGF and FGF2 (20 ng/mL each) in sterile 6-well plates (4×10^4 cells/well) or plus 10 mM taurine. Cultures were incubated at 37 °C in humidified 5% CO2/95% air atmosphere. EGF (20 ng/mL) was added to cultures every 48 h. After 12 days, primary neurospheres were collected and centrifuged at 1000 rpm/5 min, disaggregated and re-seeded at 2×10^5 cells/well in sterile 6-well plates. Secondary neurospheres were obtained after 4 days. Unless otherwise indicated, all the experiments were carried out with secondary cultures between 2 and 6 passages. For studies on taurine dose-dependent effect or to test other amino acids, cells were seeded in EGF/FGF2 medium at 5×10^4 cells/well in 24-well plates, and grown in media containing the indicated concentration of amino acids. The number of cells in cultures was measured by direct counting after 4 days on a Neubauer chamber. In all cultures, viability was determined by Trypan blue exclusion assay.

For lineage analysis of undifferentiated NPCs, the neurospheres were disaggregated, cells plated (100×10^3) on pre-treated poly-L-lysine 96-well microplates. Cells were incubated at 37 °C in humidified 5% CO2/95% air atmosphere for 2 h, and then fixed and processed by immunocytochemistry. For NPC differentiation the neurospheres were disaggregated, washed and cells (50×10^3) were plated on pre-treated poly-L-lysine cover slips, and cultured in a medium without growth factors, containing 1% FBS in the presence or absence of 10 mM taurine. Cultures were incubated at 37 °C in humidified 5% CO2/95% air atmosphere during 7, 14 or 21 days and then fixed and processed for immunocytochemistry.

**MTT assay**

Neurospheres were mechanically dissociated and cells were seeded on poly-L-lysine-treated in 48-well plates (2×10^5 cells/well) with EGF/FGF2 medium in the presence or absence of 10 mM taurine. After the indicated culture conditions, MTT reconstituted in PBS (1 mg/mL) was added to obtain 0.1 mg/mL final concentration, and cells incubated during 60 min at 37 °C. After incubation, the formazan reaction products were dissolved with DMSO, wells stirred for 5 min on a plate shaker and the optical density measured in an UV-2100 Spectrophotometer (Cole Parmer) at 570 nm.

**TUNEL assay**

The TUNEL assay was carried out using the DeadEndTM Fluorometric TUNEL System. After the indicated treatments cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton 100x during 5 min and incubated in darkness with the TUNEL reaction mixture (equilibration buffer, terminal deoxynucleotidyl transferase, and nucleotide mix) for 60 min at 37 °C. Nuclei were counterstained with Hoechst.

Cells were observed in an epifluorescence microscope Olympus IX71 and microphotographs obtained from the QCapture Pro 6.0 software. The percentage of TUNEL-positive cells was calculated over the total of cells counted.

**Low density assay**

Neurospheres were mechanically disaggregated to obtain a single cell suspension. The cells were seeded on 96-well microplates (25 cells/well) containing EGF/FGF2 medium (100 μL/well) in the presence or absence of 10 mM taurine. Cultures were incubated at 37 °C in humidified 5% CO2/95% air atmosphere, and after 7 days the formed neurospheres were counted.

**BrdU incorporation and immunocytochemical detection**

For assays of BrdU incorporation, a 1.5 h-pulse of 10 μM BrdU was applied to cultures, before the indicated time. Cells were collected, washed and resuspended with culture medium, and plated on poly-L-lysine-treated 96-well microplates. Ninety minutes after plating, cells were fixed in 4% cold paraformaldehyde for 15 min, washed (3-times 5 min each) with PBS plus 0.1% BSA, incubated 15 min with preheated PBS plus 2N HCl solution (37 °C), washed and permeabilized/blacked with a solution containing PBS plus 0.1% BSA+ 10% GS + 0.3% Triton 100 ×, during 1 h, at room temperature. Cells were incubated overnight at 4 °C with [1:100] anti-BrdU antibody followed by 1 h incubation with [1:200] Alexa fluor 488 to visualize the signal. Nuclei were counterstained with 2 μg/mL Hoechst diluted in PBS. Microphotographs were obtained by direct epifluorescence microscope Olympus IX71 using the QCapture Pro 6.0 software and data analyzed with Adobe Image 7.0 software. The percentage of BrdU-positive cells was calculated over the total of cells counted.

**Immunocytochemistry**

For cell lineage analysis cultured cells were fixed in 4% cold paraformaldehyde (15 min), washed (3-times, 5 min each) with PBS+0.1% BSA, permeabilized/blacked with PBS+0.1% BSA+10% GS+0.3% Triton 100 ×, during 1 h, at room
measurements of taurine concentration

Taurine levels were measured in tissue samples obtained from adult mouse brain cortex and olfactory bulbs or in neuronspheres cultured in the presence or absence of taurine. Samples were collected and washed with PBS, then resuspended in 150 μL of PBS and sonicated (30 s) with a Branson Sonifier-250. For HPLC measurements, 10 μL of each sample was mixed with 400 μL ethanol during 20 min, centrifugated and filtered across 0.22 μm sterile membrane. 10 μL of the filtered was derivatized with the same volume of o-phthalaldehyde and injected into a Beckman liquid chromatograph system. An ODS column (4.6×250 mm internal diameter) was used, and the column effluent was monitored with a fluorescence detector (emission at 460 nm and excitation at 330 nm). The mobile phase was methanol/potassium o-phthaldialdehyde and injected into a Beckman liquid chromatograph system. An ODS column (4.6×250 mm internal diameter) was used, and the column effluent was monitored with a fluorescence detector (emission at 460 nm and excitation at 330 nm). The mobile phase was methanol/potassium acetate (0.1 M, pH 5.5) and was run at a rate of 1.5 mL/min in a linear gradient (15 min duration). Protein content was determined by the Bradford procedure.

Taurine uptake experiments

Secondary neuronspheres were disaggregated and plated on poly-L-lysine-treated 48-well microplates (2×10^3 cells/well) with culture media and incubated at 37 °C in humidified 5% CO2/95% air atmosphere during 24 h. For the uptake well) with culture media and incubated at 37 °C in humidity.

30 min with 3H-taurine (0.8 μM, 10 μL HEPES, 300 mOsm, pH 7.4, and cells were incubated for clonal analysis of epidermal growth factor-responsive neural progenitors. J. Neurosci. Methods 117, 111–121.


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References


Data analysis

All results are expressed as means±standard error (SE). A t-test was used to detect significant differences at *P<0.05. Data in Fig. 3 were analyzed by one-way ANOVA with Tukey’s HSD post hoc test.

Supplementary materials related to this article can be found online at doi:10.1016/j.scr.2012.02.004.