

Taurine promotes differentiation and maturation of neural stem/progenitor cells from the subventricular zone via activation of GABAA receptors

Nadia Estefanía Gutiérrez-Castañeda

Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional

Jessica González-Corona

Universidad Nacional Autónoma de México

Ernesto Griego

Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional

Emilio J. Galván

Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional

Lenin D. Ochoa-de la Paz (✉ lochoa@unam.mx)

Universidad Nacional Autónoma de México

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Abstract

Neurogenesis, the formation of new neurons in the brain, occurs throughout the lifespan in the subgranular zone of the dentate gyrus and subventricular zone (SVZ) lining the lateral ventricles of the mammal brain. In this process, gamma-aminobutyric acid (GABA) and its ionotropic receptor, the GABA_A receptor (GABA_AR), play a critical role in the proliferation, differentiation, and migration process of neural stem/progenitor cells (NPC). Taurine, a non-essential amino acid widely distributed throughout the central nervous system, increases the proliferation of SVZ progenitor cells by a mechanism that may involve GABA_AR activation. Therefore, we characterized the effects of taurine on the differentiation process of NPC expressing GABA_AR. Preincubation of NPC-SVZ with taurine increased the expression of SOX-2 and microtubule-stabilizing proteins assessed with the doublecortin assay. Taurine, like GABA, stimulated a neuronal-like morphology of NPC-SVZ and increased the number and length of primary, secondary, and tertiary neurites compared with control NPC of the SVZ. Furthermore, neurite outgrowth was prevented when simultaneously incubating cells with taurine or GABA and the GABA_AR blocker, picrotoxin. Patch-clamp recordings revealed a series of modifications in the NPCs' passive and active electrophysiological properties exposed to taurine, including regenerative spikes with kinetic properties similar to the action potentials of functional neurons.

Introduction

Neurogenesis, the process of generating functional neurons from precursor cells, was traditionally associated with mammals' embryonic and perinatal developmental stages [1]. However, the milestone discovery by Altman and Das (1965) [2] demonstrating the presence of newborn neurons in the adult brain showed for the first time that active neurogenesis continues throughout the lifespan of the mammalian brain [2]. Today, active adult neurogenesis is acknowledged as a neurodevelopmental process that includes cell proliferation, migration, differentiation, cell fate decision, and functional integration into pre-existing neuronal networks [3]. Adult neurogenesis is a dynamic and accurate process regulated by multiple physiological, pharmacological, and pathological stimuli [4]. Previous studies demonstrated that pharmacological modulation of gamma-aminobutyric acid (GABA) transmission regulates neurogenesis in the adult brain [5, 6], suggesting that GABA-activated responses trigger a series of molecular and biochemical mechanisms relevant to the neurogenesis process.

Under physiological conditions, neurogenesis is restricted to two neurogenic regions: the subgranular zone of the dentate gyrus, an area that generates new granule cells [2]; and the subventricular zone (SVZ) of the lateral ventricles, where new neurons are generated and migrate through the rostral migratory stream to the olfactory bulb where they mature into interneurons [7].

It is well documented that GABA has a depolarizing activity in neural precursor cells during brain development, controlling cell division and contributing to neuronal migration and maturation. Moreover, neural stem/progenitor cells (NPC) express at least GABA_A and metabotropic GABA receptors (GABA_BR) in the subventricular and subgranular zones [8]; in these cells, GABA causes depolarization and affects

proliferation, migration, and neuronal maturation [9]. Electrophysiological studies have documented that the tonic release of GABA activates GABA_A receptors (GABA_AR) expressed in neuroblasts and stem cells [10–13]. In addition, GABA or the selective GABA_AR agonist, muscimol, activates a chloride current in neonatal NPC-SVZ [10]. In line with these findings, acute treatment of type B and C SVZ cells with muscimol decreases cell proliferation, assessed with a bromodeoxyuridine incorporation assay [14]. Interestingly, taurine, a neuroactive molecule and structurally related analog of GABA, mimics the GABA action by activating GABA_AR [15, 16]

Taurine (2-aminoethane sulfonic acid) is an abundant free amino acid found in the mammalian brain [17] and also present in the heart, retina, and muscle tissues [18]. Humans obtain taurine directly from meat consumption, as a secondary source, or via a reduced biochemical synthesis process [19]. Taurine is synthesized from cysteine, in which cysteine dioxygenase and cysteine sulfinic acid decarboxylase form hypotaurine. Hypotaurine is converted by the hypotaurine dehydrogenase enzyme to taurine [20]. Experimental evidence demonstrates that taurine acts as a trophic factor, stimulates proliferation, and promotes neurogenesis of NPC-SVZ cultures [21]. These observations suggest that taurine plays a central role in the process of proliferation, differentiation, and migration of progenitor cells during the embryonic development of the central nervous system. However, the mechanism by which taurine participates in NPC differentiation of the SVZ is unclear. Because taurine is one of the most abundant neuroactive molecules from an early CNS developmental stage and GABA_AR are present on SVZ progenitor cells, the present study aimed to evaluate the effect of taurine on the differentiation process of NPC-SVZ through interaction with GABA_AR.

Materials And Methods

Cell culture

All the animals were handled following the guidelines of the National Institutes of Health Guide for Care and Use of Laboratory Animals and with the approval of the Institutional Animal Care and Use Committee of the National University of Mexico (protocol # 068-2019/018-CIC-2019). The study was not pre-registered. Only 8-day-old CD1 mice were used to obtain NPC-SVZ, in total 100 animals were used for 4 experimental groups (25 animals per group). Animals were collected from a laboratory animal facility and decapitated as soon as they arrived at the laboratory, the brain was removed and the SVZ was isolated. No randomization was performed to allocate subjects in the study and no blinding was performed. The tissue obtained was mechanically dissociated, and placed in DMEM/F12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12. Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) medium, and centrifuged for 5 min at 1000 rpm. The supernatant was removed, and the button was resuspended and cultured in a growth medium (DMEM/F12 supplemented with B27, plus epidermal growth factor and fibroblast growth factor 2) to promote progenitor cells proliferation and SVZ-neurosphere formation. The culture was kept at 37 °C and 5% CO₂. After 7 days, primary neurospheres were disaggregated with trypsin for 5 min, and a trypsin inhibitor (1:1) was added to stop the reaction.

Once the cells were separated, they were recultured in a fresh growth medium for 4–5 days at 37 °C and 5% CO₂. For the differentiation process of NPC-SVZ, disaggregated cells of the secondary SVZ-neurospheres were cultured in 12 wells pretreated with poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA), and differentiation medium (DMEM/F12 supplemented with 1% fetal bovine serum [FBS]) and complemented with taurine (10 mM) or GABA (100 μM). To evaluate the role of GABA_AR in the differentiation process, cells were preincubated for 1 h with picrotoxin (5 μM, 50 μM, and 100 μM); after this time, the medium was stirred to withdraw the stimulus and a differentiation medium or treatment with taurine (10 mM) or GABA (100 μM) was applied. GABA was used as a positive control to compare the mechanism of taurine on NPC differentiation processes.

Immunofluorescence assay

Cells were fixed with cold 4% paraformaldehyde, washed with phosphate-buffered saline (PBS) + 0.1% bovine serum albumin (BSA; 3 x 5 min each), permeabilized/blocked with PBS + 0.1% BSA + 10% FBS + 0.3% Triton X-100 for 1 h at room temperature. The cells were subsequently incubated overnight with the primary antibodies nestin (Cell Signaling Technology Cat# 4760, RRID:AB_2235913) [1:300]; SOX-2 (Cell Signaling Technology Cat# 14962, RRID:AB_2798664) [1:100]; glial fibrillary acidic protein (GFAP; Cell Signaling Technology Cat# 12389, RRID:AB_2631098) [1:100]; and doublecortin (DCX; Santa Cruz Biotechnology Cat# sc-271390, RRID:AB_10610966) [1:100]. The primary antibodies were removed the following day, followed by 1-h incubation with the secondary antibodies Alexa Fluor-488 anti-rabbit 9 for GFAP and SOX-2) and Alexa Fluor-647 anti-mouse (for nestin and DCX). Next, the excess secondary antibodies were washed and removed from the preparation assembly. The nuclei were stained with Hoechst (20 μg/mL) included in the mounting solution (100/500 μL).

Quantitative analysis for cell markers

Microphotographs were obtained with the cell imaging multi-mode plate reader Cytation™ 5 (BioTek Instruments Inc., Winooski, VT, USA) using a green fluorescent protein (GFP) filter cube (excitation 469/35 nm; emission 525/39 nm; dichroic mirror 497 nm) and 465 nm LED; Texas Red filter cube (excitation 586/15 nm; emission 647/67 nm; dichroic mirror 605 nm) and 590 nm LED; and 4',6-diamidino-2-phenylindole (DAPI) filter cube (excitation 377/50 nm; emission 447/60 nm; dichroic mirror 409) and 365 nm LED. Microphotographs of five different fields were randomly selected from each coverslip, and the number of total (Hoescht positive) cells and cells positive for the different markers in the selected fields were quantified. Cell images were processed using Gen5™ software (BioTek Instruments Inc.). The results were expressed as the percentage of positive cells of the total cell number.

Morphometric analysis of DCX positive cells

Microphotographs of DCX positive (DCX+) cells were randomly obtained with a 20x magnification lens. DCX+ cells were analyzed for each experimental condition (control, taurine, and GABA). The number of primary, secondary, and tertiary neurites and the length of the primary neurites were determined with Gen 5 3.04. The difference between primary, secondary, and tertiary neurites was analyzed in the different experimental conditions. Primary neurites were defined as those that originated directly from the soma; secondary and tertiary neurites were defined as those that originated from primary and secondary neurites, respectively [22].

Whole-cell patch-clamp recordings

Dissociated NPC-SVZ from SVZ neurospheres were taken from a petri dish containing cell culture, mounted on a coverslip, and transferred to a submersion recording chamber. They were visualized with infrared differential interference contrast optics coupled to a Nikon FN1 microscope (Nikon Corporation, Minato, Tokyo, Japan). The NPC-SVZ were continuously perfused with Ringer's solution at the rate of 3–4 mL.min⁻¹ with the help of a peristaltic pump (120S, Watson-Marlow, Wilmington, MA, USA). The composition of Ringer's solution was the following (mM): 125 NaCl, 2.5 KCl, 1.25 Na₂HPO₄, 25 NaHCO₃, 2 MgCl₂, 2 CaCl₂, and 10 D-glucose; pH = 7.30–7.40; osmolarity = 280–290 mOsm. The recording chamber temperature was maintained at 32 ± 1°C with a single channel temperature controller (TC-324C, Warner Instruments, Hamden, CT, USA). For the whole-cell patch-clamp recordings of hippocampal interneurons included in this study, the parameters were published elsewhere [23]. The patch pipettes were pulled from borosilicate glasses using a micropipette puller (P57, Sutter Instruments, Novato, CA, USA). The puller was programmed to obtain pipettes with a tip resistance of 5–7 MΩ when filled with an intracellular solution containing (mM): 135 K⁺-gluconate, 10 KCl, 5 NaCl, 1 EGTA, 10 HEPES, 2 Mg²⁺-ATP, 0.4 Na⁺-GTP, 10 phosphocreatine, and pH = 7.20–7.30. Whole-cell recordings were performed with an Axopatch 200B amplifier (Molecular Devices, San José, CA, USA). Acquired data were digitized at a sampling frequency of 10 kHz and filtered at 5 kHz with a Digidata 1322A (Axon Instruments, Palo Alto, CA, USA). Signals were acquired and analyzed offline with pCLAMP 10.6 software (Molecular Devices).

Determination of intrinsic properties

The resting membrane potential (RMP) was determined after the initial break-in from giga-seal to whole-cell configuration. Once the cell was switched to current-clamp mode, a series of negative and positive current steps from -300 to rheobase (1 s, 30 pA increments) were injected to determine input resistance (R_N) and membrane time constant (τ_{memb}). R_N was calculated as the slope value of a first-order polynomial function $f(x) = mx + b$ fitted to the current–voltage relationship near RMP. τ_{memb} was determined by fitting a mono-exponential decay function to a voltage response elicited by injecting a negative current step (1 s, -30 pA). Membrane capacitance (C_m) was calculated as the ratio of τ_{memb} to R_N , as previously reported [24].

Determination of firing output properties

We analyzed the firing properties of recorded NPC-SVZ by constructing current–frequency plots. For these measurements, the firing frequency was plotted against the intensity of the injected current. Experimental data were fitted to a three-parameter logistic function.

Spike and action potential analyses

The spike threshold was calculated using the third derivative's first peak intersecting with the spike onset. The spike kinetic properties were analyzed by constructing phase plots. In this analysis, the first derivative of the action potential (AP) dV/dt ($\text{mV}\cdot\text{ms}^{-1}$) was plotted against the membrane potential, as previously reported [25].

Statistical analysis

No statistical method was used to determine sample size. Sample size was arbitrarily set to 100 (4 groups with 25 animals each). Statistical analysis was performed with GraphPad Prism 6. The graphs represent the mean \pm standard error of mean. Data normality was previously determined with a Kolmogorov–Smirnov test. Most of the data did not meet the normality criteria, so non-parametric tests were applied: Mann–Whitney U for comparing two groups and Kruskal–Wallis for comparing more than two groups. A one-way analysis of variance (ANOVA) test was applied, followed by Tukey's *post hoc* test in cases where normal distribution was observed. In cases where the comparison was between two groups, the Student's t-test was applied. In all cases, the accepted level of significance was $P < 0.05$.

Results

Characterization of NPC markers in SVZ-derived neurospheres

Cultured SVZ-neurospheres are widely used to mimic NPC's proliferative capacity, self-renewal, and differentiation into mature cell lineages; therefore, we used SVZ neurospheres to characterize the effects of taurine on the differentiation processes of NPC-SVZ. The neurospheres were obtained from 8-day-old CD1 mice and cultured in a growth medium with two passages (see *Materials and Methods* for details). Primary, secondary, and tertiary SVZ-neurospheres were formed at 7 ± 1 , 12 ± 1 , and 17 ± 1 days, respectively (Fig 1A–C). For this study, all the experimental manipulations were restricted to secondary SVZ-neurospheres (Fig 1B) to minimize biased interpretations given the cellular changes that occur during self-renewal (unorganized structure) or senescence (necrotic center) of SVZ-neurospheres [26].

We first determined the presence of progenitor and neuronal markers in the secondary SVZ-neurospheres. Immunofluorescence image analysis revealed nestin and SOX-2 immunopositivity (Figs 2A and 2B);

contrary to this observation, we did not detect DCX immunofluorescence (Fig 2C). The immunopositivity to SOX-2 and nestin confirms the progenitor origin of the NPC-SVZ, whereas the lack of DCX immunopositivity indicates a lack of differentiation to immature neurons.

Next, the secondary SVZ-neurospheres were disaggregated and the resulting NPC-SVZ were attached to coated poly D-lysine dishes. A differentiation medium replaced the growth medium for 7 days to induce cellular differentiation. Under this developmental condition, immunopositivity to SOX-2 was found in the differentiated NPC-SVZ, which exhibited a distinctive nuclear pattern (Supplementary Fig 1A). Furthermore, SOX-2 immunopositivity was present in disaggregated cells incubated with either taurine (10 mM) or GABA (100 μ M; Supplementary Figs 1B–1D).

Expression of neuronal markers in NPC-SVZ exposed to taurine or GABA

Next, the expression of the neuronal markers during the differentiation process was analyzed in disaggregated cells. Because previous studies have documented the expression of neuronal markers in the presence of GABA [27], we used this pharmacological condition as a positive control. Therefore, cells were exposed to taurine (10 mM) or GABA (100 μ M) for 7 days, and the immunocytochemical analyses were performed against DCX, a standard marker to identify the neuronal identity of differentiated cells [28]. Compared with control cells (Fig 3A), the cultures exposed to taurine or GABA (Figs 3B and 3C, respectively) exhibited increased immunopositivity to DCX (one-way ANOVA; $P < 0.05$), with no statistical difference in DCX expression between the treated groups (Fig 3D). Moreover, the cells exposed to taurine exhibited a clear morphological difference compared with control cells: small and elongated or multipolar somata with cytoplasmic outgrowths or neurites with a neuronal-like morphology (Fig 3B; right panel). Although cells exposed to GABA also exhibited neurites, these structures were less conspicuous than those observed with taurine (Fig 3C; right panel). These results suggest that taurine stimulates the expression of neuronal markers and has stronger induction properties than GABA to generate neurites in disaggregated NPC-SVZ.

Taurine induces neuronal-like characteristics in NPC-SVZ

Because the NPC-SVZ can differentiate to both glial and neuronal cells [29], we next determined if taurine or GABA influences the differentiation of the disaggregated NPC-SVZ to glia. Compared with control cells (Fig 4A), treatment with taurine (10 mM) or GABA (100 μ M; Figs 4B and 4C) promoted somatic elongation and neurite outgrowth that conferred a neuronal-like morphology of the treated cells. However, this morphology was restricted to DCX+ cells (middle panels in Figs 4B and 4C); more importantly, it was absent in cells immunopositive to GFAP (right panels in Figs 4A–C; see also Suppl. Fig 2). The DCX+ cells with neurite outgrowths exhibited rounded somata, had a length of $52.07 \pm 4.8 \mu\text{m}$, and a nuclei size of $12.18 \pm 1.05 \mu\text{m}$. By contrast, the GFAP+ cells exhibited several somatic shapes, including elongated,

stellate, or round morphology with abundant cytoplasm ($133.3 \pm 9,21 \mu\text{m}$) and elongated nuclei ($14.05 \pm 0.8 \mu\text{m}$) (right panels in Fig 4A and Suppl. Fig 2).

A morphometric analysis of DCX+ cells revealed differences in the differentiation process in each experimental condition. For instance, cells treated with taurine or GABA exhibited numerous secondary and tertiary neurites compared with control cells. The number of primary, secondary, and tertiary neurites in each experimental condition is summarized in the bar graph in Fig 4D (one-way ANOVA; $P > 0.05$). Regarding the length of primary neurites, we did not find a statistical difference in the three experimental conditions (Fig 4E).

We performed a Sholl analysis to achieve more accurate morphometry of the differentiated cells. Representative microphotographs of cells from the three experimental conditions and their corresponding digital reconstructions are depicted in Figs 5A–C. Concentric rings superimposed to the digital reconstructions for the Sholl analysis are depicted in Figs 5D–F. Control cells exhibited unipolar morphology with fewer neurites (Figs 5A and 5D). In sharp contrast, cells treated with taurine exhibited multipolar morphology and a robust number of neurites and arborizations (Figs 5B and 5E). Furthermore, cells treated with GABA exhibited a bipolar-like morphology, with a reduced number of neurites and no arborizations compared with those observed in taurine-treated cells. (Figs 5C and 5F). The number of neurite intersections are plotted against neurite length from the soma, thus summarizing neuronal complexity, in Fig 5G. As expected, the number of neurite intersections was larger in cells treated with taurine than those expressed in control cells or from cells treated with GABA. These morphometric analyses strongly suggest that taurine acts as a strong promoter of neurite complexity and contributes to the neuronal maturation of differentiated NPC.

Blockade of GABA_AR suppress the morphogenic process induced by taurine

Previous studies demonstrated that GABA_AR participate in the morphogenic process of NPC-SVZ [5, 30]. To investigate if GABA_AR are involved in the morphogenic process mediated by taurine, disaggregated NPC-SVZ were differentiated in increasing concentrations of the GABA_AR antagonist, PTX (5, 50, and 100 μM , respectively). We first corroborated that preincubation with the increasing doses of PTX did not induce cell damage. For this, a cell counting kit-8 assay determined that none of the PTX concentrations caused significant cell damage (data not shown). These observations also showed that 100 μM PTX caused a considerable reduction of neurites in cells exposed to taurine or GABA. Therefore, the number and length of neurites were analyzed in DCX+ cells exposed to 100 μM PTX. Differentiated cells were preincubated with 100 μM PTX for 1 h. Under these conditions, there was a significant decrease in the number of primary neurites in DCX+ cells treated with either GABA plus PTX (one-way ANOVA; $P < 0.01$; middle panel, Fig 6A and red bars in Fig 6B) or taurine plus PTX (one-way ANOVA; $P < 0.001$; bottom

panel, Fig 6A and blue bars in Fig 6B). We also determined that blockade of GABA_AR with 100 μM PTX reduced the number of secondary and tertiary neurites (one-way ANOVA; $P > 0.05$; Fig 6B).

Likewise, the length of primary neurites exhibited a significant decrease in the presence of PTX alone or combined with taurine or GABA (one-way ANOVA; $P < 0.05$ for all comparisons; Fig 6C). These experiments demonstrate for the first time that activation of GABA_AR is a necessary step in the taurine-mediated morphogenic process and neurite outgrowth of NPC-SVZ.

Taurine or GABA treatment induces electrophysiological responses in disaggregated NPC

Because our previous finding that cells differentiated with taurine or GABA express neuronal markers and exhibit a neuronal-like morphology, we next determined the passive and active electrophysiological properties of the NPC-SVZ with whole-cell patch-clamp recordings. We measured membrane properties from 21 cells divided into control, taurine- and GABA-treated cells ($n = 7$ for each group). Figs 7A1–3 show representative voltage responses to current injections obtained under the three experimental conditions. Compared with control cells, the decreased voltage deflections observed in GABA- or taurine-treated cells (see the continuous horizontal dash lines) indicate increased functionality of ion channels active near the RMP, possibly voltage-dependent and inward-rectifying potassium channels. From the current–voltage relationships, we determined that neither GABA nor taurine altered the RMP compared with control cells (RMP in: control cells = -67.1 ± 4.7 mV; GABA-treated cells = -63.3 ± 4.4 mV; taurine treated cells = -69.4 ± 4.6 mV; one-way ANOVA; $F_{(2,18)} = 0.456$; $P = 0.641$; Fig 7B). Consistent with the decreased voltage deflections as a result of increased ion channel activity near the RMP, we observed a marked decrease in the somatic R_N of NPC-SVZ treated with GABA or taurine compared with control cells (R_N in: control cells = 777.7 ± 87.66 MΩ; GABA-treated cells = 350.1 ± 38.2 MΩ [Tukey's test; $P < 0.001$]; taurine-treated cells = 307.3 ± 22.55 MΩ [Tukey's test; $P < 0.001$]; one-way ANOVA; $F_{(2,18)} = 21.040$; $P < 0.001$; Fig 7C). The changes in R_N were accompanied with an increase in the cell's C_m (C_m in: control cells = 34.6 ± 6.7 pF; GABA-treated cells = 75.9 ± 12.6 pF [Tukey's test; $P = 0.012$]; taurine-treated cells = 92.7 ± 8.7 pF [Tukey's test; $P < 0.001$]; one-way ANOVA; $F_{(2,18)} = 10.860$; $P < 0.001$; Fig 7D). Furthermore, the C_m was higher in cells treated with taurine than those treated with GABA, consistent with the cell's volume regulation being mediated by taurine [31, 32]. Lastly, we did not find differences in t_{memb} between the three experimental conditions (t_{memb} in: control cells = 25.7 ± 3.21 ms; GABA-treated cells = 23.9 ± 1.74 ms; taurine-treated cells = 27.8 ± 2.25 ms; one-way ANOVA; $F_{(2,18)} = 0.616$; $P = 0.550$; Fig 7E).

Next, we explored the active membrane properties of disaggregated NPC-SVZ. Increasing current steps were injected to test if a sustained membrane depolarization triggers AP. Control cells did not elicit an AP, as illustrated in Fig 7F1 (left panels), and only two control cells exhibited a short, voltage-sensitive 'spikelet' (upper panel, Fig 7F1) upon sustained depolarization. On the other hand, cells treated with GABA exhibited an increased proportion of cells that elicited at least one fast spike that resembled an actual AP

(middle panel, Fig 7F1). Contrary to these observations, repetitive AP discharge was observed in the taurine-treated cells (right panel, Fig 7F1). The number of cells that exhibited AP-like waveforms (solid fill) versus those with no response (grey fill) in the three experimental conditions is summarized in the upper pie charts of Fig 7F1. The proportion of: control cells eliciting a spikelet = 28.57 %; GABA-treated cells eliciting at least one spike = 57.14 %; taurine-treated cells eliciting at least one spike = 85.71%. Interestingly, the AP observed in the taurine-treated cells was followed by an afterhyperpolarization (AHP) that resembled the Ca^{2+} -dependent slow AHP that follows the AP of central neurons [33]. The firing pattern observed in the three experimental conditions and the resulting AHP is depicted in Fig 7F2. We also expressed the repetitive firing in terms of AP frequency. Fig 7G summarizes the AP frequency as a function of the injected current of control (black symbols), GABA- (red symbols), and taurine-treated cells (blue symbols). Together, these results support the hypothesis that taurine is a potent inductor of phenotypic differentiation of NPC maintained *in vitro* and show that taurine is more effective than GABA in transforming NPC-SVZ to a neuron-like phenotype with electrophysiological properties resembling those of central neurons.

Action potential properties of the differentiated NPC-SVZ

The voltage traces in Fig 7F2 show that incubation with GABA or taurine reshapes the spike waveform. This effect is more evident in the superimposed, magnified spikes in Fig 8A. As previously stated, the control 'spikelet' was smaller and slower, with an amplitude that barely exceeded the AP overshoot, lacked an AHP, and required strong depolarization for initiation. On the other hand, GABA- and taurine-treated cells exhibited fast and regenerative spikes with prominent overshoot followed by an AHP conductance (Fig 8A). Given these findings, we next analyzed the kinetic properties of the spikes and compared the resulting parameters with averaged values obtained from actual AP spikes from hippocampal interneurons. First, we determined the voltage membrane threshold (V_{th}) for spike initiation by computing the third derivative of the spike, a mathematical method that identifies an actual AP threshold [34] (Fig 8B1). Compared with control cells, we found that both GABA and taurine treatment shifts the spike V_{th} to a more hyperpolarized membrane potential, closer to the V_{th} of an actual AP (spike V_{th} in: control cells = -21.1 ± 2.00 mV; GABA-treated cells = -32.5 ± 2.50 mV [Tukey's test; $P = 0.022$]; taurine-treated cells = -35.2 ± 1.44 mV [Tukey's test; $P = 0.005$]; one-way ANOVA; $F_{(2,9)} = 9.352$; $P = 0.006$; actual AP $V_{th} = -42.33 \pm 1.54$ mV [Tukey's test; $P < 0.001$]; one-way ANOVA; $F_{(3,14)} = 15.840$; $P < 0.001$; Fig 8B2).

Next, we analyzed the kinetic parameters of the spikes utilizing a phase plot analysis. The left panel in Fig 8C shows an actual AP spike from a hippocampal interneuron, and the right panel, its resulting geometrical representation or phase plot; the superimposed phase plots from the three experimental conditions plus an actual AP (green trace) are superimposed in Fig 8D.

Compared with control cells, GABA and taurine treatment increased the spike amplitude (spike amplitude in: control cells = 27.1 ± 2.55 mV; GABA-treated cells = 66.9 ± 6.83 mV [Tukey's test; $P = 0.021$]; taurine-treated cells = 81.9 ± 3.58 mV [Tukey's test; $P = 0.001$]; one-way ANOVA; $F_{(2,9)} = 14.330$; $P = 0.002$; Fig 8E).

NPC-SVZ treated with taurine exhibited an amplitude closer to the actual AP amplitude (actual AP amplitude = 90.97 ± 2.12 mV [Tukey's test; $P < 0.001$]; one-way ANOVA; $F_{(3,14)} = 20.940$; $P < 0.001$; Fig 8E). Consistent with this finding, the spike half-width (h-w) was shorter in the GABA- and taurine-treated cells and in the latter group was closer to the actual AP h-w (spike h-w in: control cells = 3.4 ± 0.70 ms; GABA-treated cells = 2.9 ± 0.20 ms [Tukey's test; $P = 0.379$]; taurine-treated cells = 2.0 ± 0.09 ms [Tukey's test; $P = 0.009$]); one-way ANOVA; $F_{(2,9)} = 9.198$; $P = 0.007$; actual AP h-w = 1.7 ± 0.14 [Tukey's test; $P < 0.001$]; one-way ANOVA; $F_{(3,14)} = 12.910$; $P < 0.001$; Fig 8F). Likewise, the maximum depolarization slope (MDS) of the spike, a parameter that reflects activity of the AP's voltage-dependent Na^+ conductance, was increased in GABA- and taurine-treated cells (MDS in: control cells = 18.7 ± 1.80 mV/ms; GABA-treated cells = 32.8 ± 4.42 mV/ms [Tukey's test; $P = 0.049$]; taurine-treated cells = 58.1 ± 2.57 mV/ms [Tukey's test; $P < 0.001$]; one-way ANOVA; $F_{(2,9)} = 32.260$; $P < 0.001$; actual AP MDS = 88.7 ± 2.88 mV/ms [Tukey's test; $P < 0.001$]; one-way ANOVA; $F_{(3,14)} = 79.130$; $P < 0.001$; Fig 8G). Lastly, we also computed the maximum repolarization slope (MRS) as an indirect measure of voltage-dependent K^+ conductances underlying the AP repolarization phase. Compared with control cells, GABA and taurine treatment increased the speed of the MRS (MRS in: control cells = -22.7 ± 2.50 mV/ms; GABA-treated cells = -47.7 ± 4.32 mV/ms [Tukey's test; $P = 0.011$]; taurine-treated cells = -79.5 ± 3.08 mV/ms [Tukey's test; $P < 0.001$]; one-way ANOVA; $F_{(2,9)} = 48.870$; $P < 0.001$; actual AP MRS = -84.7 ± 3.56 mV/ms [Tukey's test; $P < 0.001$]; one-way ANOVA; $F_{(3,14)} = 42.630$; $P < 0.001$; Fig 8H). Together, these data demonstrate that taurine, like GABA, induces phenotypic plasticity and modulates electrophysiological properties of disaggregated NPC-SVZ. Moreover, our data strongly suggest that taurine has a stronger effect compared with GABA in the differentiation process of NPCs.

Discussion

In this work, we have provided experimental evidence regarding the multipotentiality of NPC-SVZ to generate cells that express neuronal markers and develop electrophysiological properties following treatment with the non-essential amino acid taurine. In line with this finding, we demonstrated the critical role of the ionotropic GABA_A R in the differentiation process mediated by taurine. The interaction between the GABA_A R and taurine promotes a neuron-like phenotype of NPC-SVZ, increases their dendritic complexity, and favors an electrophysiological behavior that resembles actual neuron activity, including the onset of repetitive firing.

We first confirmed that NPC-SVZ matured in neurospheres expressing nestin and SOX-2 markers, as reported by Sanchez-Mendoza (2016) [35]. Moreover, disaggregation of neurospheres to monolayer cultures treated with taurine increased the percentage of cells expressing the neuronal marker DCX, an observation previously reported for GABA [27] and confirmed in this study.

Ramos-Mandujano (2014) [36] proposed that taurine promotes mitochondrial efficiency and increases ATP availability, an immediate effect that increases neuronal proliferation. As an alternative to this proposal, we offered data demonstrating both the critical role that the GABA_AR has in the differentiation process of NPC-SVZ, and that blockade of this receptor suppresses differentiation. In agreement with our findings, the participation of GABA_AR in the neurogenic process has been previously observed in cortical precursor cells, in which activation of the GABA_AR triggers a sustained depolarization in postnatal and adult precursor cells, promoting axon elongation, neurite growth, and synaptogenesis [37–39].

Therefore, one possible way to explain the role of GABA_AR in the taurine-mediated NPC-SVZ differentiation process is that upon their activation, a GABA_AR-mediated sustained depolarization favors cytosolic calcium accumulation and activates Ca²⁺-dependent mechanisms associated with neurite formation [40].

However, additional experiments are required to confirm this. As previously stated, the critical role of GABA_AR in the neuronal differentiation of NPC-SVZ was demonstrated when disaggregated NPC-SVZ were simultaneously exposed to taurine and the GABA_AR blocker PTX. Blockade of GABA_AR decreased the number of cells immunopositive to DCX; furthermore, these cells exhibited fewer secondary and tertiary neurons. These observations strongly indicate that taurine modulates the differentiation processes of NPC-SVZ to neuronal cells via activation of GABA_AR. In this sense, it is important to emphasize that modulation of GABA_AR by taurine may promote other neurogenic regulatory mechanisms, including the decreased activity of glial cells [40]; activation of the sonic hedgehog signaling pathway [42]; increased mitochondrial activity [35, 43], or activation of GABA_BR.

Our Sholl analyses confirmed that taurine has a stronger differentiation effect than GABA. This observation was confirmed with the morphometric analyses that verified the more complex neurite branching and increased formation of secondary and tertiary neurites in taurine- versus GABA-treated cells. Thus, it can be assumed that taurine participates in the maturation and incorporation of NPC-SVZ to preformed neuronal circuits by promoting axonal differentiation, dendrite arborization, and formation of synaptic connections [37–39].

Another remarkable observation of this study is the emergence of defined electrophysiological properties of disaggregated NPC-SVZ exposed to taurine or GABA. Compared with control cells that barely exhibited neurite outgrowth, cells exposed to GABA, and more importantly taurine, exhibited changes in their passive and active membrane properties. Taurine caused a dramatic drop in the R_N and increased the activity of membrane conductances active near the RMP. Moreover, the proportion of cells generating repetitive APs was increased. In line with this observation, taurine-treated cells exhibited a well-defined AHP phase that resembles the Ca²⁺-activated K⁺ conductances that underlie and shape the AP output of mature neurons. Taurine caused NPC-SVZ cells to behave as an actual AP of a mature neuron by altering several kinetic parameters, including AP threshold, spike amplitude, and h-w, as well as AP depolarization

and repolarization rates. More importantly, these effects were more marked in cells treated with taurine than GABA, supporting the hypothesis that taurine represents a stronger morphogen than GABA.

Conclusion

We have provided *in vitro* evidence regarding the role of taurine as a key neurogenic factor in the differentiation process of NPC-SVZ. We have demonstrated that taurine induces functional neurons by a mechanism that requires the activation of GABA_AR. The morphological and electrophysiological changes mediated by taurine support the hypothesis that this non-essential sulfur-containing amino acid has functional implications in the neurogenesis process, including gene transcription, neural protein expression, and transport system activity. Given the non-active biochemical nature of taurine, our observations may provide a novel pharmacological tool to renew, increase, or maintain the pools of stem cells that participate in the neurogenesis process during or after a pathological condition, or for the treatment of the age-related reduction in adult neurogenesis.

Declarations

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Competing Interests:

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript

Author Contributions / Credit:

Gutierrez-Castañeda: Investigation, Formal analysis, Writing – original draft preparation. González-Corona: Investigation. Griego: Investigation, Formal analysis, writing. Galván: Resources, writing, review and editing. Ochoa-de la Paz: Conceptualization, Resources, Writing, Visualization, Project administration.

Data Availability:

The datasets generated during and/or analysed during the current study are available under request.

Ethics approval:

All the animals were handled following the guidelines of the National Institutes of Health Guide for Care and Use of Laboratory Animals and with the approval of the Institutional Animal Care and Use Committee

of the National University of Mexico (protocol # 068-2019/018-CIC-2019). The study was not pre-registered.

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Conflict of interest:

The authors declare no competing financial interests.

CrediT authorship contribution statement:

Gutierrez-Castañeda: Investigation, Formal analysis, Writing – original draft preparation. **González-Corona:** Investigation. **Griego:** Investigation, Formal analysis, writing. **Galván:** Resources, writing, review, and editing. **Ochoa-de la Paz:** Conceptualization, Resources, Writing, Visualization, Project administration.

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Figures

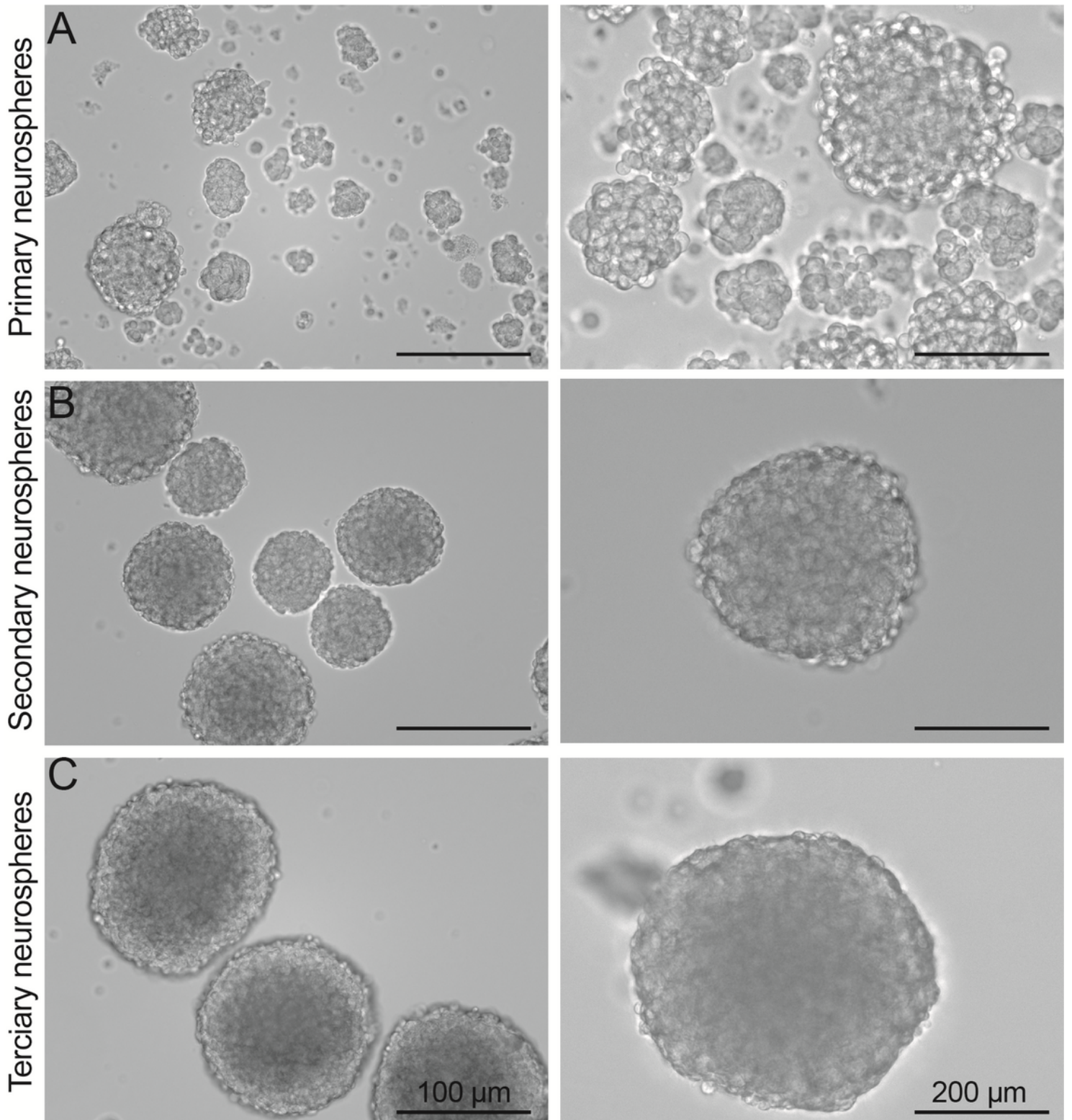


Figure 1

Neurospheres obtained from SVZ from a mouse brain. Representative bright-field microphotographs of A) primary, B) secondary, and C) tertiary neurospheres. Microphotographs in the left panels were taken with a 10x magnification lens; right panel, 20x. Neurospheres were formed at 7, 12, and 17 days, respectively.

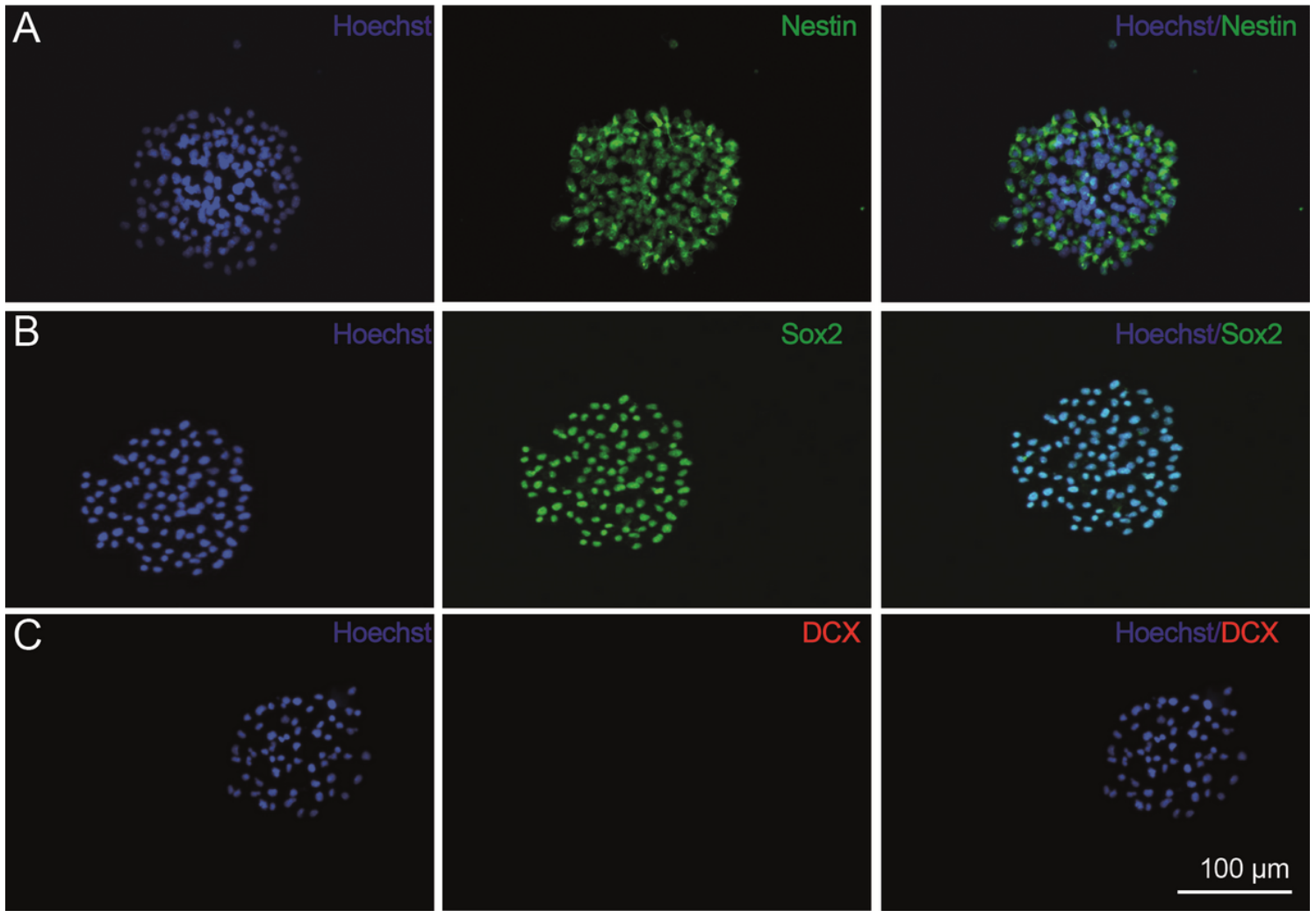


Figure 2

Expression of NPC markers in NPC-ZSV neurospheres. Representative immunofluorescence microphotographs of secondary neurospheres before differentiation. The nuclei were stained with Hoescht (blue signal). Neurospheres were immunopositive to **A)** nestin and **B)** SOX-2. **C)** Neurospheres did not exhibit immunopositivity for DCX. Microphotographs were taken with a 20x magnification lens. The scale bar applies to all panels.

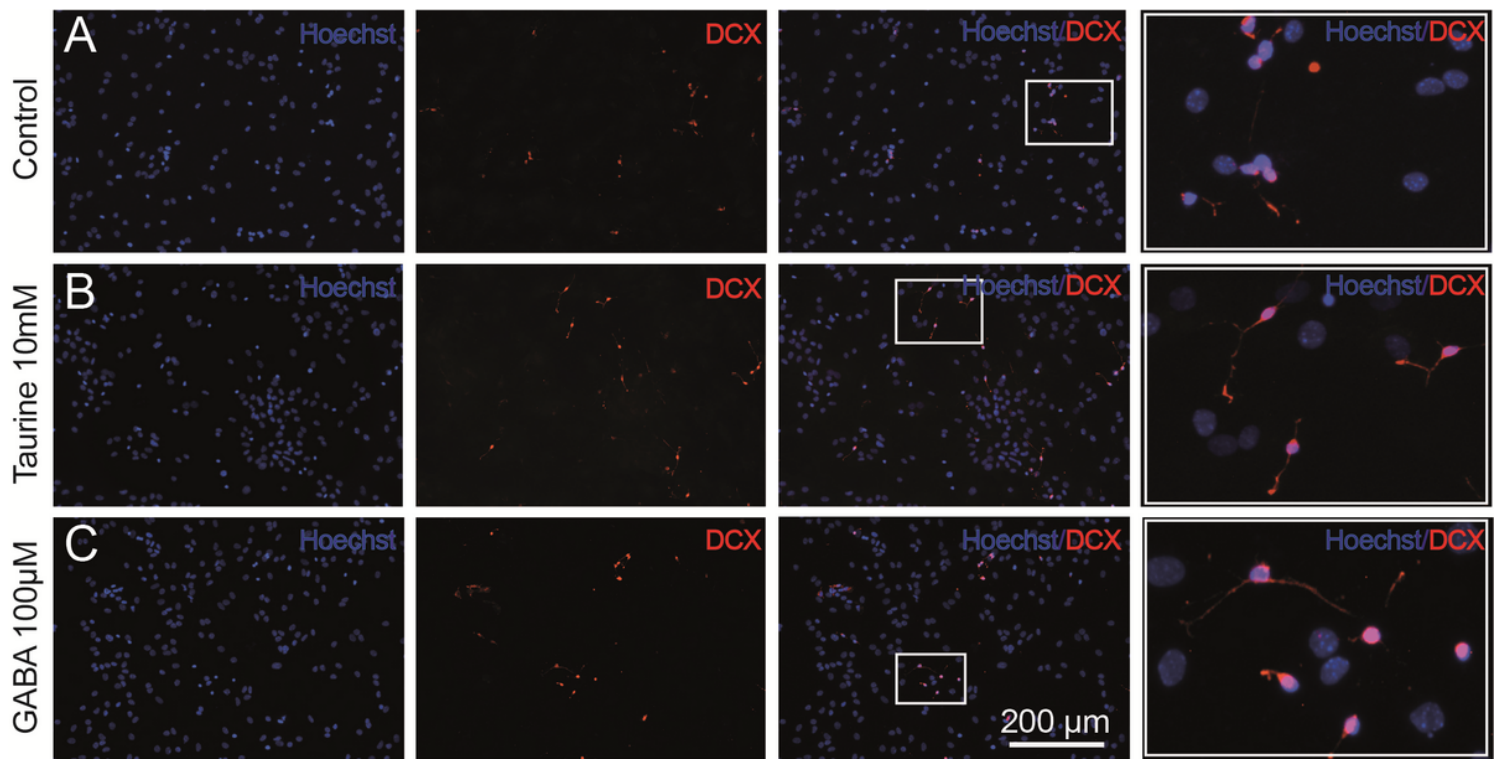


Figure 3

Immunolabeling for neuronal markers in disaggregated NPC-SVZ Immunopositivity of disaggregated cells to DCX was observed consistently in A) control cells, B) cells treated with 10 mM taurine, and C) cells treated with 100 μM GABA (positive control). The nuclei were stained with Hoescht (blue labeling). Right panels are magnifications obtained from the white boxes in the preceding panels showing the neurite outgrowths in the three experimental conditions. D) Boxplot graph showing a normalized count of DCX+ cells in each experimental condition. Treatment with taurine or GABA significantly increases the number of DCX+ cells. * $P < 0.05$.

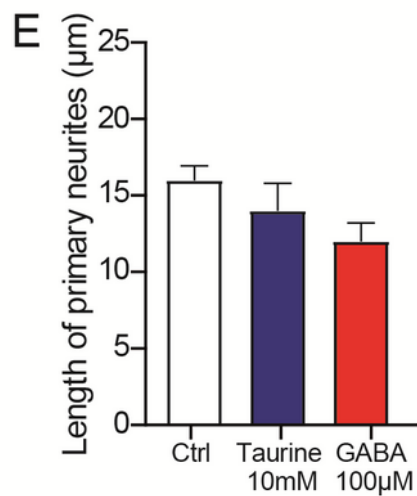
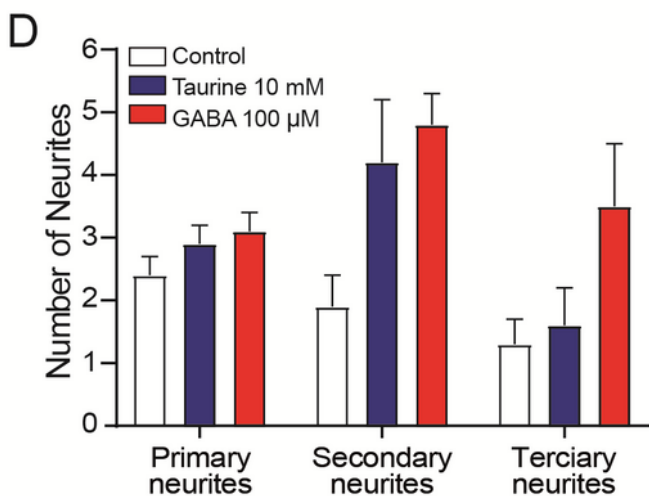
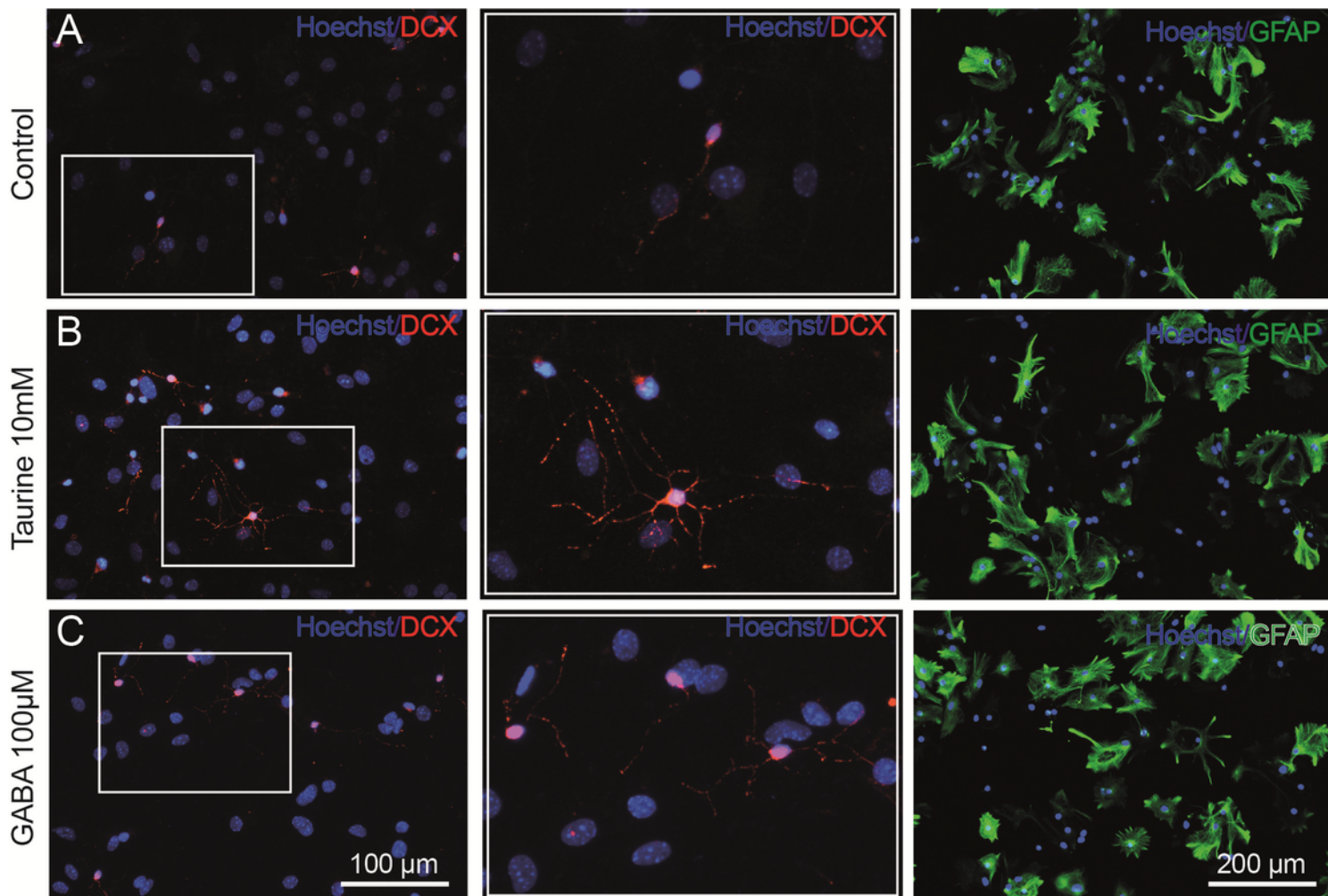


Figure 4

Neurite outgrowth is restricted to DCX+ and absent in GFAP+ cells. A–C) Left panels, disaggregated NPC-SVZ showing immunopositivity to DCX. Middle panels: corresponding magnified microphotographs from the left panels. Treatment with taurine or GABA stimulated the formation of neurites. Right panels: cells positive to GFAP lacked neurite outgrowths. D) Bar graph showing the average number of neurite

outgrowths measured from somata. E) Bar graph showing the average length of primary neurites in the three experimental conditions.

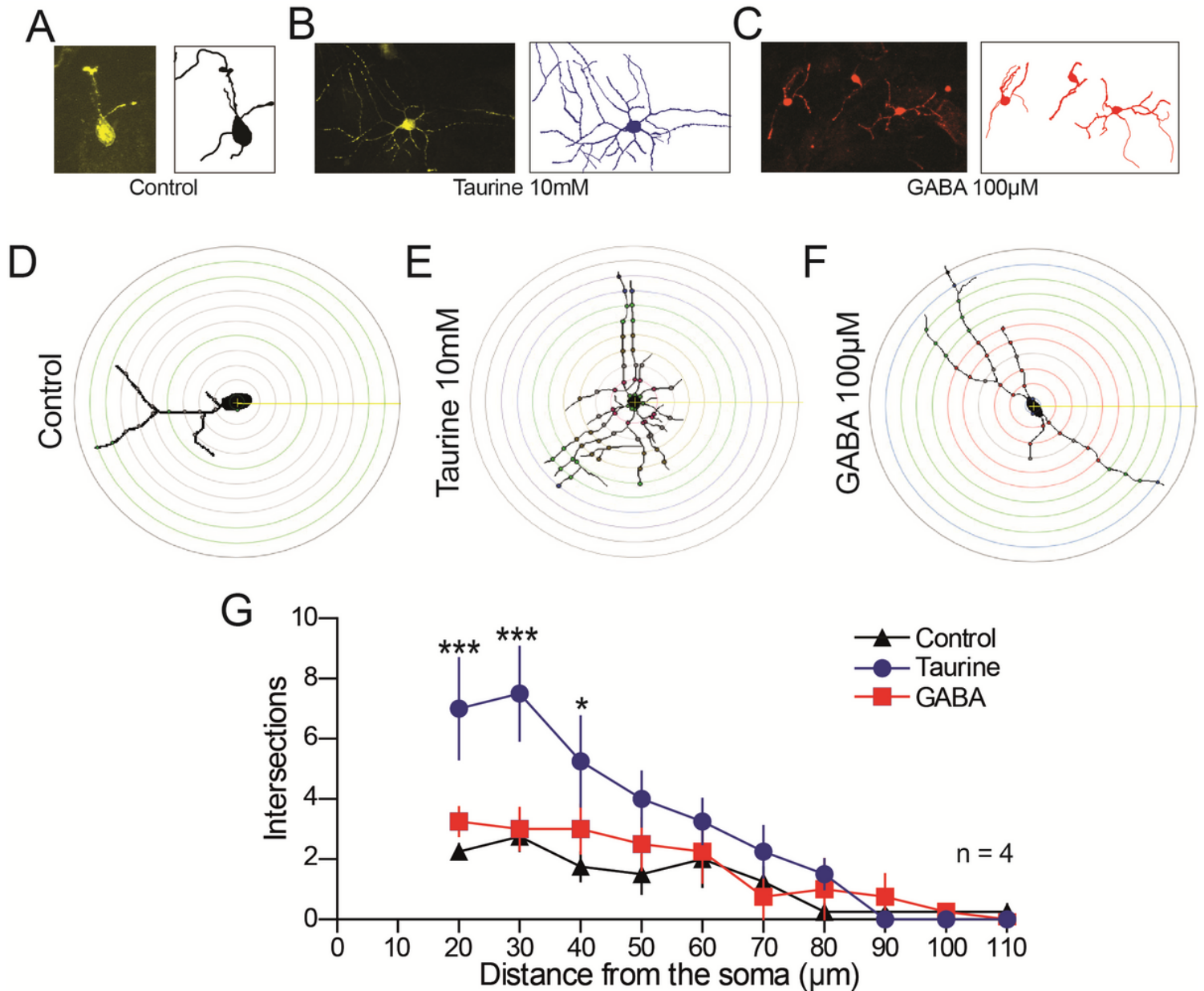


Figure 5

Representation of the Sholl analysis. A–C) Microphotographs of representative NPC-SVZ in the three experimental conditions showing the typical neurite outgrowth and their corresponding digital reconstructions. D–F) Schematic representation of the morphometric Sholl analysis performed on control, taurine- and GABA-treated cells. G) The number of intersections between neurite outgrowths and concentric rings (10 µm increments) from the center of the soma were used to determine primary, secondary, and tertiary ramifications (n=5). * $P < 0.05$; *** $P < 0.001$.

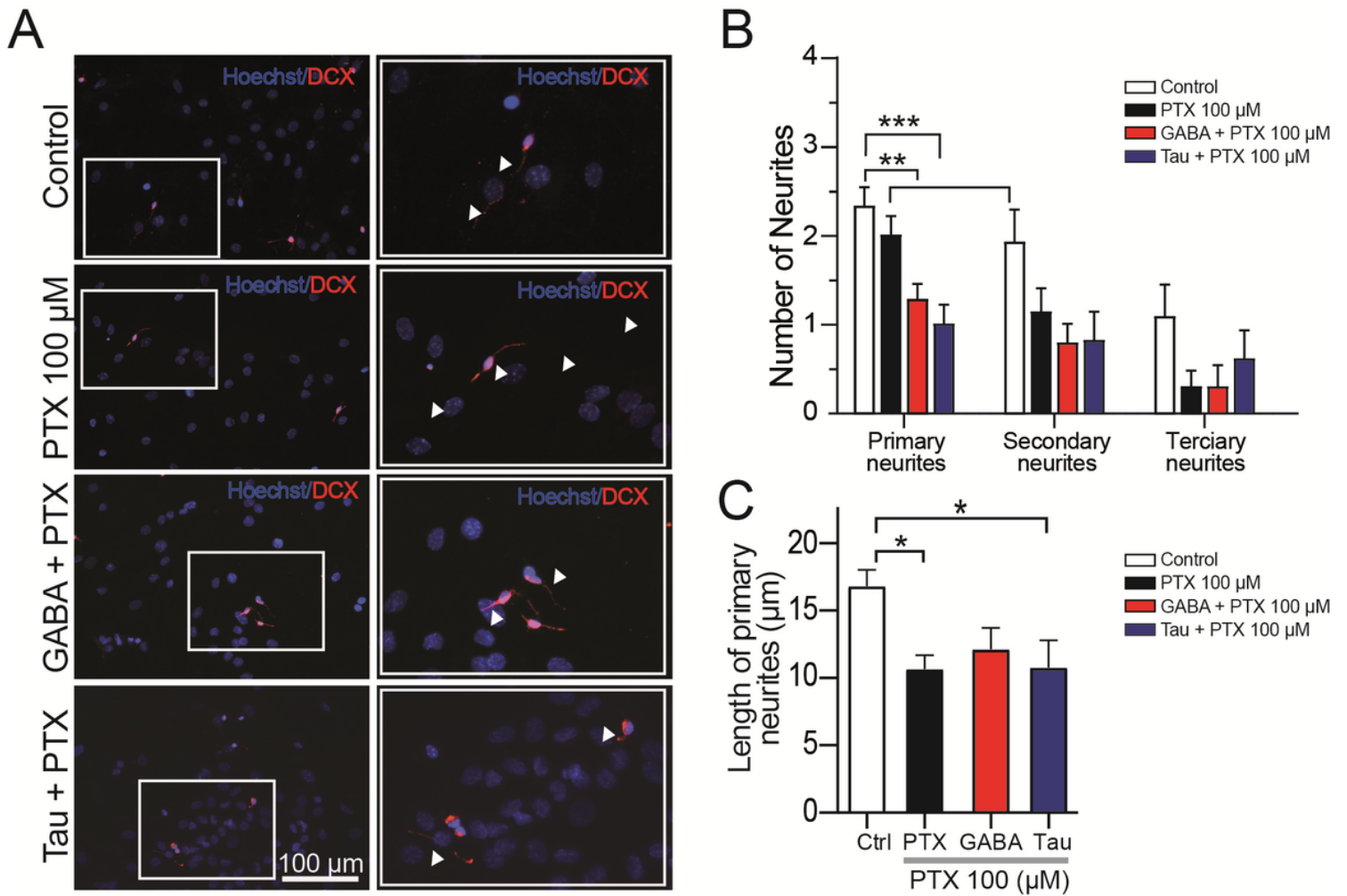


Figure 6

Effect of PTX on the differentiation and maturation process. A) Left panels, disaggregated NPC-SVZ immunopositive to DCX; the cells were incubated with 100 μM PTX. Right panels, corresponding magnifications showing the reduced number of neurites in the presence of PTX. Arrowheads indicate neurite outgrowth. B) Bar graph showing the average number of primary, secondary, and tertiary neurite outgrowths measured from the somata. C) Bar graph contrasting the average length of primary neurites in control and the three experimental conditions. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

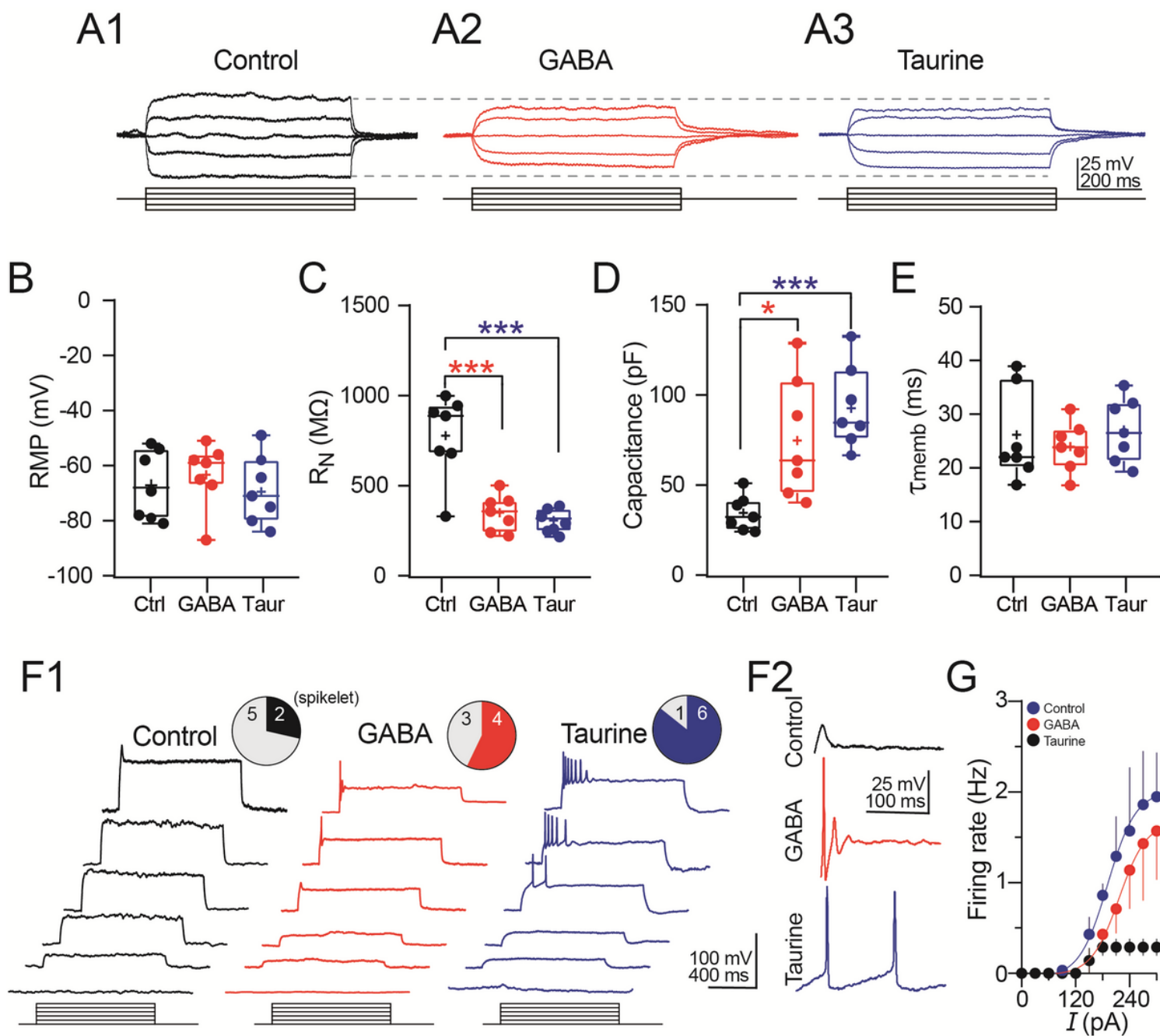


Figure 7

Electrophysiological properties of disaggregated NPC-SVZ treated with taurine or GABA. A1–3) Representative current–voltage responses elicited at -60 mV from control, GABA, and taurine-treated cells. Note the decreased voltage response of cells treated with GABA or taurine (continuous dashed lines). B–E) Box plots and individual experiments (circles) showing: B) RMP, C) somatic R_N , D) somatic C_m , and E) t_{memb} of control, GABA-, and taurine-treated cells. The solid line in the boxplots represents the median and the cross represents the mean. F1) Representative voltage responses elicited at -60 mV. Increasing depolarization triggered a ‘spikelet’ in control cells (left panels); in GABA- (middle panel), and taurine-treated cells (right panel), a brief burst of spikes was observed. The upper pie charts show the proportion of cells capable of eliciting a spike in response to depolarizing current injection. F2)

Magnification of representative spikes obtained in the three experimental conditions. GABA- and taurine-treated cells exhibited a well-defined AHP conductance following individual AP. G) Plot of the averaged maximum spike firing frequency response to depolarizing current steps for each experimental condition. * $P < 0.05$; *** $P < 0.01$ or higher statistical significance; $n = 7$ disaggregated cells for each experimental condition.

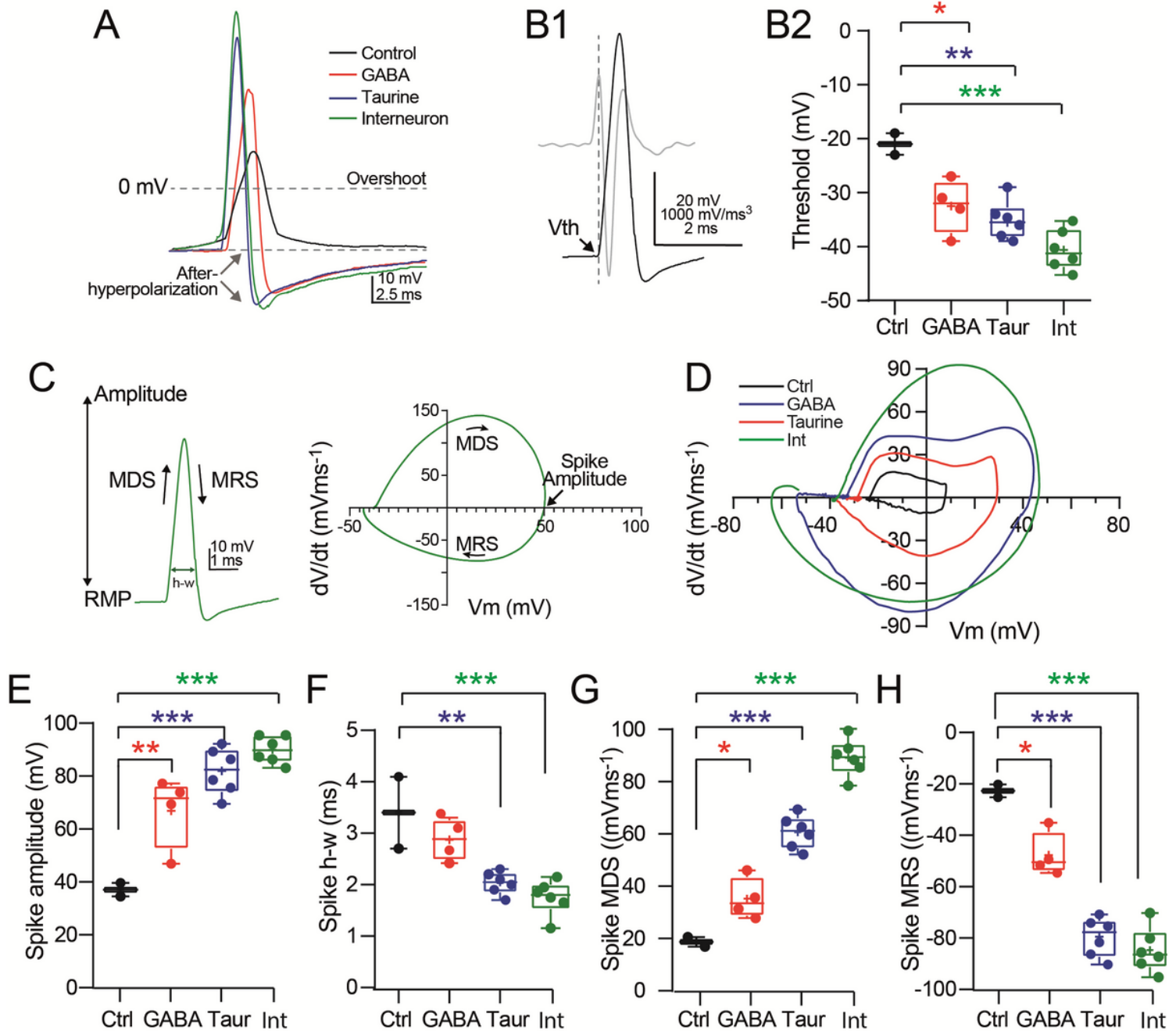


Figure 8

Kinetic properties of NCP-SVZ spikes. A) Superimposed spikes from control, GABA-, and taurine-treated cells. The green trace corresponds to an actual AP from a hippocampal interneuron recorded from an

acute hippocampal slice. Note the control spike's slow onset, reduced overshoot, and lack of AHP. In contrast, GABA and taurine treatment increased the spike's onset, overshoot, and repolarization. B1) Representative examples of the first and third derivatives (grey and black trace, respectively) of an actual AP or spike. The V_{th} for spike initiation was determined by the third derivative changes from negative to positive. B2) Box plots and individual experiments showing V_{th} for each experimental group, including the values obtained from an actual AP (green box and symbols). GABA and taurine treatment shifted V_{th} to a more hyperpolarized membrane potential. C) Example of an actual AP (from a hippocampal interneuron) and the resulting phase plot indicating the kinetic parameters measured. MDS = maximum depolarization slope; MRS = maximum repolarization slope; h-w = spike half-width. D) Superimposed phase plots from control, GABA-, and taurine-treated cells and an actual AP. E-H) Box plots and individual experiments (symbols) summarizing the kinetic parameters obtained from spikes in each experimental condition. GABA and taurine treatment: E) increased spike amplitude; F) decreased spike h-w, G) increased spike MDS, and H) decreased MRS. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n = 7 disaggregated cells for each experimental condition.

Supplementary Files

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