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Homeostatic plasticity in hippocampal slice cultures involves changes in voltage-gated Na^+ channel expression

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Abstract

Neurons preserve stable electrophysiological properties despite ongoing changes in morphology and connectivity throughout their lifetime. This dynamic compensatory adjustment, termed 'homeostatic plasticity', may be a fundamental means by which the brain normalizes its excitability, and is possibly altered in disease states such as epilepsy. Despite this significance, the cellular mechanisms of homeostatic plasticity are incompletely understood. Using field potential analyses, we observed a compensatory enhancement of neural excitability after 48 h of activity deprivation via tetrodotoxin (TTX) in hippocampal slice cultures. Because activity deprivation can enhance voltage-gated sodium channel (VGSC) currents, we used Western blot analyses to probe for these channels in control and activity-deprived slice cultures. A significant upregulation of VGSCs expression was evident after activity deprivation. Furthermore, immunohistochemistry revealed this upregulation to occur along primarily pyramidal cell dendrites. Western blot analyses of cultures after 1 day of recovery from activity deprivation showed that VGSC levels returned to control levels, indicating that multiple molecular mechanisms contribute to enhanced excitability. Because of their longevity and in vivo-like cytoarchitecture, we conclude that slice cultures may be highly useful for investigating homeostatic plasticity. Furthermore, we demonstrate that enhanced excitability involves changes in channel expression with a targeted localization likely profound transform the integrative capacities of hippocampal pyramidal cells and their dendrites.

Keywords

Homeostatic plasticity; Hippocampal slice culture; Voltage-gated sodium channel; Excitability; Activity deprivation; Dendrite

1. Introduction

Multiple forms of plasticity occur in the brain and are likely governed by independent mechanisms [21]. Synapse-specific plasticity, such as long-term potentiation (LTP) and depression (LTD), has been well established in the nervous system both in vivo and in vitro and much is known about this form of plasticity at the molecular level [31]. Global changes in

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synaptic strength can also shape neural function. For example, homeostatic plasticity is characterized by an enhanced or depressed neural excitability in the opposite direction of ambient activity levels that occurs over days and is present in all synapses [41]. This compensatory capacity of neurons to modulate their overall sensitivity to activity across their complex dendritic arborizations has been theorized to play a critical role in maintaining network stability during development and in the mature, learning brain [18].

To date, homeostatic plasticity has been shown to exist in dissociated cortical cell cultures [15,42], acute cortical slices [12], and hippocampal primary cell cultures [6]. However, important questions involving the molecular mechanism of homeostatic plasticity have been difficult or impossible to address with these preparations. For example, acute slice preparations last only for hours and thus preclude reversibility experiments, which take days. Furthermore, while dissociated cell cultures can be maintained for days to weeks in vitro, they lack the in vivo-like cytoarchitecture of brain slices, which have been shown to affect important mediators of plasticity such as the localization of ion channels [36].

Here, we show that the hippocampal slice culture preparation, an in vivo-like neural network capable of maintenance in vitro for weeks [38], provides a solution to these problems. Using hippocampal slice cultures, we show that homeostatic plasticity can be investigated at the network, cellular and molecular level. First, by measuring field potentials, we show that compensatory plasticity occurs, as evidenced by a change in excitability following 48 h of activity blockade from TTX exposure; a finding that confirms and extends observations made in dissociated cell culture [42]. Second, by Western blot analyses, we show that this enhanced excitability occurs with an upregulation of voltage-gated Na^+ channel (VGSC) expression. Previous work with dissociated cell culture shows that TTX-induced hyperexcitability occurs with increased Na^+ channel currents [11]. Finally, using immunohistochemistry, we show that this upregulation of VGSCs occurs along pyramidal cell dendrites, a localization that could dramatically effect the information processing these cells. Taken together, these results demonstrate that slice cultures may be an ideal preparation for the study of both acute and long-term effects of homeostatic plasticity. Furthermore, this form of compensatory plasticity involves changes in channel expression with a targeted localization that is likely to fundamentally alter properties of pyramidal cell dendrites.

2. Methods

2.1. Slice culture preparation

Hippocampal slice cultures are widely accepted experimental models (for review, see Refs. [1,16]) and were prepared and maintained as described previously [27,38]. Briefly, postnatal 6- to 8-day-old Wistar rat pups were anesthetized via CO_2 exposure, their brains were rapidly removed and placed in Gey's balanced salt solution supplemented with D-glucose (6.5 mg/ml) chilled to 4 °C. Both hippocampi were removed, placed on 3.5% agar-coated Teflon™ plates and sectioned perpendicularly to their septotemporal axis (450 μm thick) using a McIlwain tissue chopper. Slices that displayed an intact dentate gyrus (DG) and pyramidal cell layer were transferred to uncoated 30-mm Millicell-CM tissue culture inserts (Millipore™, Bedford, MA) in six-well culture dishes (Becton Dickinson, Lincoln Park, NJ) containing Basal Medium Eagle Medium (50%), Earle's Balanced Salt Solution (25%), horse serum (23%), 10 μg/ml gentamycin, 1 mM L-glutamine, 250 μg/ml Fungizone, and glucose to a final molarity of 42 mM, replaced twice a week. All media constituents were obtained from Invitrogen (Grand Island, NY) or Sigma (St. Louis, MO). Cultures were maintained at 37 °C with 5% CO_2/ balanced humidified air in a standard incubator (Heraeus Instruments, South Plainfield, NJ). Experimental cultures were incubated in media containing 10 μM TTX (Sigma) for 48 h, replaced at 24 h. Media for control cultures were changed and replaced identically to experimental cultures without drug added.

Slices were maintained in vitro for 21–35 days prior to use, a period at least over which synaptic functional activity remains stable [28,35,38]. Furthermore, pyramidal cell viability (assessed by NeuN expression) and astrocytic reactivity (assessed by glial fibrillary acidic protein expression, a marker of tissue injury, degeneration and inflammation) remain unchanged over this period [35]. However, dendritic spine density of pyramidal cell apical dendrites in slice cultures [32] resembles that seen in 15-day-old counterparts in vivo [23], perhaps due to reduced synaptic input. Nonetheless, hippocampal slice cultures show intrinsic patterns of GABAergic transmission that are similar to that seen in vivo [39]. Furthermore, AMPA-type and NMDA-type glutamate receptors and other synaptic proteins in hippocampal slice cultures remain stable for at least 4 weeks in vitro [2]. Thus, we presumed that Na^+ channel expression and distribution remain stable over the period of our experiments. Indeed, the above findings and our experimental results support this contention.

Hippocampal slice cultures, like acute brain slices, are deafferented. However, the considerably longer survival of hippocampal slice cultures allows sufficient time for some synaptic reorganization. For example, some CA3 and CA1 pyramidal cells synapse back onto CA3 pyramidal neurons [10]. CA1–CA1 synapses and synapses from CA3 to DG neurons are also seen [22]. Nonetheless, these aberrant connections do not seem to be a dominant confounding factor for the use of hippocampal slice cultures. This conclusion follows from the fact that the basic trisynaptic loop (i.e., DG–CA3–CA1) is structurally [45] and functionally [22] preserved in hippocampal slice cultures. Furthermore, hippocampal slice cultures can show CA1 and CA3 selective neurotoxicity to irreversibly injurious insults as is seen in vivo [26].

2.2. Stimulating and recording technique

Field potentials were used as a measure of postsynaptic responses [20] (Fig. 1). Inserts containing six slice cultures were placed in a 35-mm culture dish, mounted on an inverted microscope and perfused at a rate of 1 ml/min with a normal Ringer's solution of (in mM): NaCl (124), KCl (2), $NaHCO_3$ (26), $CaCl_2$ (2.5), $MgCl_2$ (1), KH_2PO_4 (1.2), glucose (6), adjusted to pH 7.4 with < 5% CO_2/75% N_2/20% O_2 at 37 °C. To prevent dehydration in the recording chamber, slice cultures were covered with a thin layer of light mineral oil. Under low-power transillumination, a bipolar stimulating electrode made of twisted, insulated wire (90% platinum/0% iridium, 125 μm diameter) was gently positioned on the surface of the DG. An extracellular microelectrode (tip diameter 4–6 μm) filled with 150 mM NaCl was placed in the pyramidal neuron layer of CA3 to record field potentials. A 1 M KCl agar bridge ground electrode was placed outside the insert in the perfusate. Stimulating pulses were 200 μs in duration and 20–50 V. Field potentials were monitored after 30 min equilibration in the setup via an A-1 Axoprobe amplifier system (Axon Instruments, Foster City, CA), digitized with a 1200 B series Digidata system (Axon Instruments), processed with Axoscope software (Axon Instruments) and recorded using a Pentium III PC. All field potential figures and slow potential figures were composed with Origin (Microcal Software, Northampton, MA) and CorelDraw software (Corel, Ontario, Canada). Field potential records were smoothed by a Savitzky–Golay smoothing filter in Origin.

2.3. Western blot analysis

Samples consisting of 6–12 slice cultures for each experimental condition were removed from inserts using a clean, fine paintbrush, rapidly transferred directly into ice cold lysis buffer adopted from Gong et al. [19] containing 20 mM sodium phosphate, 1 mM magnesium chloride, 0.5 mM EDTA, 300 mM sucrose, 1.1 μM leupeptin, 0.7 μM pepstatin A, 0.23 mM PMSF, 76.8 nM aprotinin (all chemicals were obtained from Sigma) and immediately homogenized with a Fisher Scientific model 100 sonic dismembrator (Fisher Scientific, Atlanta, GA) before rapid freezing on dry ice and storage at −80 °C. Total protein concentrations were determined in duplicate by the Bradford method on a Smart-spec 3000

spectrophotometer (Bio-Rad, Hercules, CA). Samples were gently heated to 37 °C for 15 min in sample buffer, loaded at a total protein concentration of 1 μg/μl per lane and run on a 5% gel and transferred to nitrocellulose. After incubating for 1 h in 5% blotto [nonfat powdered milk in PBS (7.6 pH) plus 0.5% Tween 20 (PBS–t)], the membrane was incubated overnight at 4 °C in a pan-specific primary antibody VGSCs (1:1000, catalog #S 8809; Sigma). After three 10-min washes in 5% blotto, the membrane was incubated for 1 h with horseradish peroxidase-conjugated goat antimouse secondary antibody (1:1000; Biosource, Camarillo, CA) in 5% blotto. After three 10-min washes in PBS–t, the immunoblots were visualized with the ECL immunodetection system (Amersham Pharmacia, Bucking-hamshire, England) and developed using Kodak X-OMAT film and developer (Eastman Kodak, Rochester, NY). Control and activity-deprived tissue samples were run in triplicate per gel and subsequently quantitated by densitometric computer-based analysis using Un-Scan-It (Silk Sciences, Orem, UT). Tissue samples ($n = 3$ per group) were run in triplicate. Each condition was paired with its own control per gel and results tested for significance using a one-way Student's *t*-test.

2.4. Immunohistochemistry

Slice cultures were fixed with 4% paraformaldehyde in PBS (7.4 pH) for 30 min at 4 °C, gently removed from the inserts with a fine paintbrush and placed in 10 mM phosphate-buffered saline (PBS) solution (7.4 pH) containing 0.01% sodium azide at 4 °C until processed. Using an adapted histology protocol for primary cell culture [33], slices were incubated for 1 h in a blocking solution (Pt-NGS) containing PBS, 0.3% triton-X and 10% normal goat serum (Colorado Serum, Denver, CO). Slice cultures were then incubated overnight at 4 °C in blocking solution containing the pan-specific antibody to VGSCs (1:100; Sigma) then washed 3 times for 10 min each in blocking solution. Next, slice cultures were incubated for 1 h in horseradish peroxidase-conjugated goat antimouse secondary antibody (1:200) in blocking solution. The peroxidase label was visualized by incubation in PBS containing 0.05% diaminobenzidine dihydrochloride and 0.01% H_2O_2 for 5 min. Slice cultures were mounted on gelatin-coated slides, air-dried, dehydrated in graded alcohols, cleared in xylenes, and coverslipped. Images were acquired using a CoolSNAP digital camera (Roper Scientific, Trenton, NJ) and MetaMorph Imaging system (version 4.6; Universal Imaging, Downington, PA).

2.5. Cell death assay

Slice cultures were incubated for 24 h in media containing 1 μM Sytox™ Green (Molecular Probes, Eugene, OR). Fluorescence was visualized using conventional fluorescence microscopy (FITC filter).

3. Results

3.1. Activity deprivation causes robust enhancement of excitability

To evaluate excitability in control cultures, we recorded extracellular field potential responses in hippocampal slice cultures after 30 min of equilibration in the setup. DG is the clearest visual landmark in the slice culture and thus useful in obtaining recording positions which were highly reproducible across preparations. DG stimulation evoked a single population spike and excitatory postsynaptic field potential (fEPSP) in area CA3 of each control culture examined (Fig. 2A top trace). There was a significant increase ($P < 0.001$, $n = 7$; ANOVA and post hoc Tukey) in the number of population spikes evoked in activity-deprived slice cultures compared with controls (Fig. 2A, bottom trace and Fig. 2B). The duration of evoked responses also showed evidence of TTX-induced increased excitability. For example, the DG evoked response ended in ~50 ms under control conditions but lasted more than several hundred milliseconds after TTX treatment. This robust enhancement of excitability following activity deprivation

confirms and extends homeostatic plasticity previously observed in hippocampal [6] and cortical [42] dissociated cell cultures and acute cortical slices [12].

3.2. Activity-deprived slice cultures are sensitive to stimulation frequency

In control cultures, a single CA3 population spike and fEPSP was evoked after DG stimulation in all interstimulus intervals (ISIs) tested. In contrast, activity-deprived slice cultures were found to be highly sensitive to stimulation frequency. Spike number was inversely related to stimulation frequency in these cultures. An average of 7 spikes, but up to 20, could be evoked when the ISI was ≥10 min. Spike number decreased, as ISI was decreased to 1 min, a condition under which multiple spikes nonetheless still could be evoked in activity-deprived cultures. We did not decrease the stimulation interval below 1 min to avoid approaching the stimulation frequency (0.5 Hz) that has been shown to induce hippocampal LTD [37]. To quantify the effect of the ISI on excitability, we systemically varied it in the following manner. An experiment consisted of recording responses to five stimulations with a 10-min ISI followed by five stimulations with a 5-min ISI, then five stimulations with a 3-min ISI and finally five stimulations with a 1-min ISI (i.e., ~2.5 h full recording time including initial rest period). There was a rest period preceding the first recording for each ISI equal to that particular ISI. To confirm that the above progressive reduction in excitability was truly stimulation frequency-dependent, we repeated the 10-min interval protocol at the end of the experiment. Excitability rebounded toward that seen with the initial 10-min stimulation paradigm (Fig. 2B). All stimulation frequencies were significantly different from controls ($P \leq 0.001$, $n = 7$; ANOVA and post hoc Tukey).

3.3. Homeostatic plasticity is reversible

To be physiologically useful, adjusting excitability should itself be plastic. We tested for reversibility of homeostatic plasticity by returning cultures that had been exposed to TTX for 48 h to normal media for 1, 2 and 3 days and assessed excitability using our varying-ISI paradigm. As illustrated in Fig. 2C, excitability gradually returned to control levels in a time-dependent manner by 3 days, indicating that TTX-induced enhanced excitability in hippocampal slice cultures can reverse itself in response to removal of drug.

3.4. Homeostatic plasticity is not associated with cell death

Cell death can occur with excess excitability in hippocampal slice cultures [34]. To confirm that neither TTX itself nor the increased excitability induced by TTX exposure caused cell death [4], we assayed for dead cells before, during, and after 48 h of TTX incubation using Sytox™ Green, a nucleic acid marker of dead cells. Typical of hippocampus in vivo, some neural cells continuously die throughout adulthood in the DG [24], a feature also evident in hippocampal slice cultures (Fig. 2D, arrows). Apart from this phenomenon of normal hippocampal tissue, no cell death was observed during (Fig. 2D, middle panel), nor up to and including 7 days after recovery from 48 h of TTX exposure (data not shown). For comparison, robust pyramidal cell death was observed after 30 min of incubation in 10 μM NMDA (at 37 °C) followed by 24 h return to normal media, an in vitro model of excitotoxicity [7,28]. Thus, activity deprivation in hippocampal slice cultures causes a reversible enhanced excitability not associated with cell death.

3.5. Mechanisms homeostatic plasticity

Electrophysiological evidence that the amplitude of Na^+ currents are significantly enhanced after 48 h of TTX exposure in primary cortical cultures [11] prompted us to investigate whether similarly induced changes in VGSC expression might contribute to the observed enhanced excitability in hippocampal slice cultures. Accordingly, we used a pan-specific VGSC antibody that recognizes the intracellular III–IV loop of the VGSC alpha subunit common to all channel

isoforms to examine changes in channel expression and location after TTX exposure [33]. Western blot analyses were performed following the methods of Gong et al. [19]. As shown in Fig. 3A, 48 h of TTX exposure triggered increased expression of VGSCs compared to controls. Densitometric analyses from five separate experiments showed this increased expression of VGSCs was significantly greater than controls [$P < 0.001$; one-way *t*-test compared to control, anticipating increased expression or null (Fig. 3B)]. Immunostaining for VGSCs further demonstrated that an upregulation of VGSCs occurred in the stratum oriens, which consists mainly of basilar dendrites of CA3 pyramidal neurons. Furthermore, increased immunostaining was evident in the plexiform layer of the DG and to a lesser extent in the stratum lucidum of CA3, two other hippocampal areas consisting primarily of dendrites (Fig. 3C).

Further Western blot analyses of VGSCs in slice cultures returned to normal media for 1, 2 and 3 days after activity deprivation ($n = 3$ per time point) were not significantly different from the control, as represented in Fig. 3D for results seen for 1 day. This result confirms that enhanced excitability in hippocampal slice cultures following activity deprivation cannot be due to VGSC expression alone, as shown in dissociated cell cultures by Turrigiano et al. [42] who found enhanced AMPA and NMDA receptor currents (in addition to sodium currents [11]) after TTX-induced activity deprivation.

4. Discussion

4.1. Induction and reversibility of homeostatic plasticity

We measured excitability by field potential analyses and showed that a single bipolar DG stimulus evoked a single population spike and fEPSP that lasted tens of milliseconds in CA3 of untreated control slice cultures. The level of excitation evoked by a single stimulus was significantly enhanced by prior activity deprivation. After 48 h of TTX, cultures showed an average of 6–8 and up to 20 spikes. Furthermore, such evoked responses now lasted hundreds of milliseconds.

The longevity of the slice culture preparation enabled us to further investigate the duration of this enhanced excitability induced by TTX treatment. We found the enhanced excitability observed in activity-deprived slice cultures to be reversible over a time course similar to its induction (days). Activity-deprived slice cultures returned to normal media for 1 and 2 days persisted in exhibiting electrophysiological evidence of enhanced excitability. Recovery to baseline excitability was complete by 3 days after activity deprivation. This particular time course of reversibility suggests to us that the enhanced excitability observed may be a modulation of protein expression, which occurs over hours to days [8], as opposed to other common forms of modifying excitability such as phosphorylation of proteins [14], a biological event with a time course typically from seconds to minutes.

TTX disables action potential generation by VGSCs. All events downstream of the action potential generation that effect excitability—from raised intracellular calcium levels at presynaptic terminals triggering vesicle fusion and neurotransmitter release to postsynaptic receptor activity and subsequent activation of second messenger systems—conceivably may be altered during TTX treatment. Morphological changes (i.e., spines) may also be involved in altered excitability, although different laboratories have generated conflicting data regarding the effect of TTX treatment on neural morphology [13,32,43].

4.2. Cell death not associated with activity deprivation or recovery

Many brain diseases involve levels of excess excitability that are associated with neurotoxicity [3]. For example, in a recent study using hippocampal slice cultures, Lahtinen et al. [29]

demonstrated that among the many parameters affected by injury in an in vitro model of ischemic stroke, selectively blocking excessive neural firing during or soon after injury selectively prevented cell death. Our results show for the first time that neither TTX treatment nor, more notably, return to normal media (when excess excitability is unmasked), causes cell death (using our culturing strategies [27,28,35]). Absence of cell death supports the notion that compensatory enhanced excitability is a physiological response, rather than a byproduct of cellular toxicity.

4.3. Homeostatic plasticity and VGSCs

Viewed broadly, the capacity of neurons to compensate for chronic changes in synaptic drive is supported by data from drosophila and vertebrate neuromuscular junction, invertebrate central pattern generators, and mammalian cortex [9]. Specifically, these data demonstrate that neurons are capable of modifying their ionic conductances to recalibrate intrinsic excitability. Forty-eight hours of activity deprivation via TTX significantly increases the inward Na^+ current without changing its voltage dependence [11]. One possible interpretation of this finding—an enhancement of Na^+ current without a change in current kinetics—is an increase in channel density. Although VGSC density has been shown to change in response to channel blockade in nonneural cells [5,40] and after axotomy in neurons [44], the mechanisms mediating changes in VGSCs expression by activity are incompletely understood [25]. VGSCs are an attractive candidate for homeostatic plasticity principally because they serve as the molecular generators of action potentials—the ultimate, common pathway for neural output.

When we probed for VGSCs by Western blot analyses, we found that 48 h of activity deprivation via TTX was sufficient to significantly upregulate VGSC expression in slice cultures compared with controls. Further analysis by Western blot of cultures returned to normal media for 1, 2 and 3 days post-TTX incubation did not reveal a significant difference in VGSC expression between activity-deprived and control slice cultures. This result is open to several interpretations. Because enhanced excitability persisted after VGSC expression returned to control levels, we favor the idea that multiple mechanisms contribute to the compensatory excitability observed electrophysiologically, and these mechanisms possess different sensitivities to activity. Among contributing mechanisms, if VGSC expression is more tightly regulated, expression may return to control levels earlier than other candidate proteins involved in controlling excitability. VGSC expression may itself catalyze other cellular changes acting in concert to change excitability.

Coupled expression of multiple ion channels has been directly demonstrated in a recent report of homeostatic plasticity in lobster stomatogastric ganglion (STG) [30]. MacLean et al. [30] found that injection into STG neurons of a mutant *shal* RNA, encoding a nonfunctioning K^+ channel, was as competent at elevating hyperpolarization-activated inward current levels as was the RNA for the functional K^+channel protein. That is, the presence of the nonfunctional K^+ channel *itself* is sufficient to change expression of another ion channel. Likewise, the presence of extra VGSCs may influence the regulation of other proteins. Thus, in addition to directly contributing to control of excitability, VGSC expression may similarly serve to catalyze expression changes in other proteins that contribute to excitability. Might chronic enhancement of activity catalyze a *decrease* in VGSC density? As has been demonstrated in primary neural cell culture, increased VGSC activity, achieved through direct channel activation by neurotoxins, results in downregulated sodium channel expression in at least two different ways: (1) a rapid internalization upwards of 50% of surface-expressed VGSCs and accompanying prolonged channel activation, and (2) a significant decrease in the levels of mRNA encoding two brain-specific VGSC α subunits [17]. Thus, our work contributes to the general idea that homeostasis of neural excitability may involve altered expression of VGSCs, both the up and down, via multiple mechanisms.

Finally, our finding that upregulated VGSCs were primarily localized to basilar dendrites of pyramidal neurons (stratum oriens), plexiform layer of the DG and to a lesser extent, stratum lucidum, suggests that rather than merely enhancing excitability, targeted localization of VGSC to dendrites is likely to fundamentally alter the information processing capacities of these cells. Taken together, our results demonstrate that the hippocampal slice culture preparation can serve as a useful model for investigating homeostatic plasticity at the network, cellular and molecular level. In addition, synthesis of VGSCs may be one mechanism by which enhanced excitability is achieved in compensation for network activity blockade.

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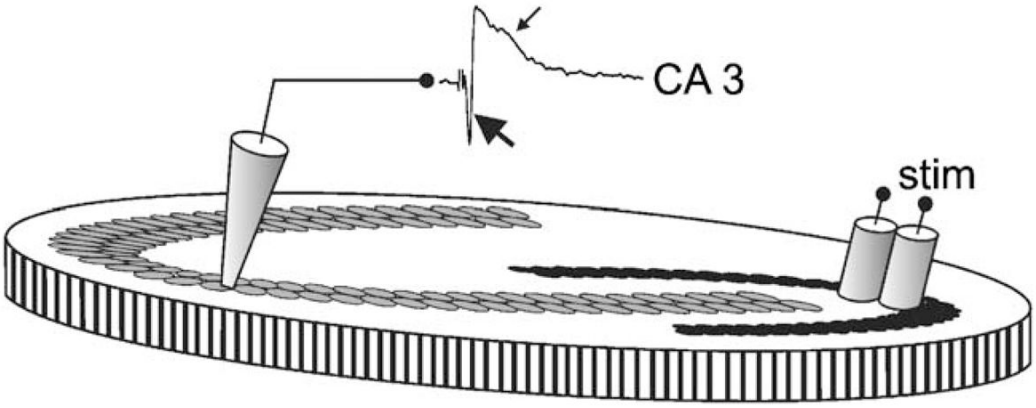

Fig. 1.

Schematic of electrophysiological recording paradigm. Bipolar stimulating electrode (stim) in the DG was used to evoke population spike in the CA3 pyramidal layer. The latter was recorded by a microelectrode placed in the interstitial space that recorded the typical negative (population spike) then positive (fEPSP), marked by large arrow and small arrow, respectively.

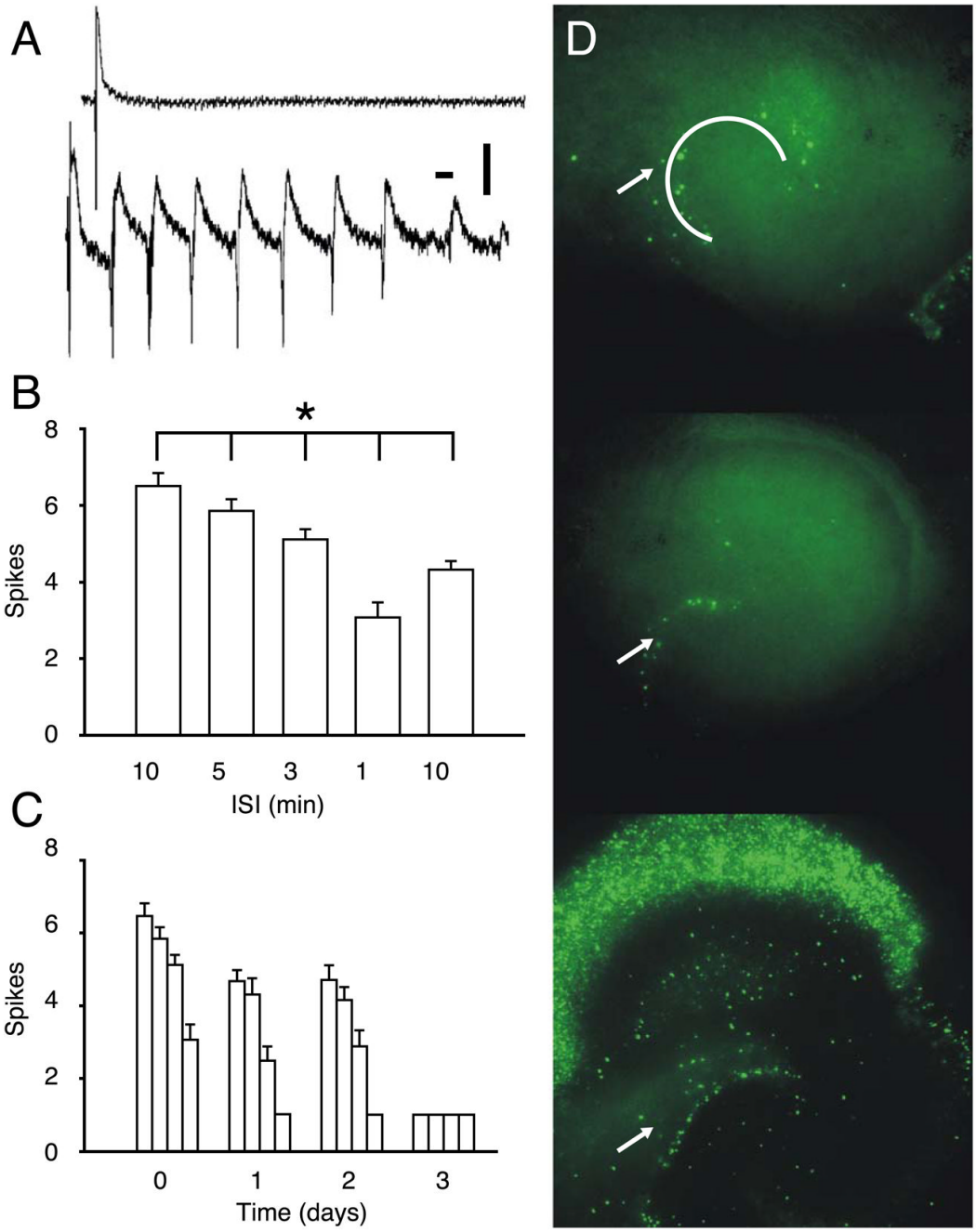

Fig. 2.

TTX treatment reversibly increases excitability of CA3 neurons in hippocampal slices cultures without cell death. (A) Increased excitability is evident in extracellularly recorded responses from area CA3 to a single stimulatory pulse in dentate gyrus (DG), recorded after allowing cultures to rest in the electrophysiological setup for 30 min. Upper trace shows typical response in controls and lower trace shows typical multiple spikes produced by same stimulus in activity deprived cultures. Vertical calibration represents 2 mV and the horizontal calibration represents 50 ms. (B) Interstimulus interval (ISI) affects spike number with the most robust response occurring at the longest ISI. Each bar represents average (n = 7) number of spikes evoked. Cultures were always tested for their responses to stimulation in the same order (ISI of 10, 5,

3, and 1 min) and were then retested for their response to stimulation at an ISI of 10 min (last bar). All responses were significantly ($P \leq 0.001$) greater than those evoked in control cultures at each stimulation interval tested, as indicated by asterisk. We rejected spikes less than 2 mV in amplitude when counting. (C) Enhanced excitability is completely reversed for all ISIs by 3 days after 48 h of activity deprivation. Four bars grouped in each day represent, from left to right, the response to stimulation at intervals of 10, 5, 3, and 1 min. (D) Sytox™ measurement of cell death in slice cultures. Top panel is representative of control cultures ($n = 6$) exhibiting only bright green stained cells indicative of normal cell death in DG (emphasized here by white elliptical line and arrow; with arrow alone used for similar purposes in two lower panels). Middle panel is representative of both TTX-exposed slice cultures and cultures exposed to TTX and returned to normal media for up to 7 days showing no increased cell death compared with controls. For positive control, the bottom panel shows robust cell death in CA1 24 h after 30 min of incubation in media containing 10 μM NMDA.

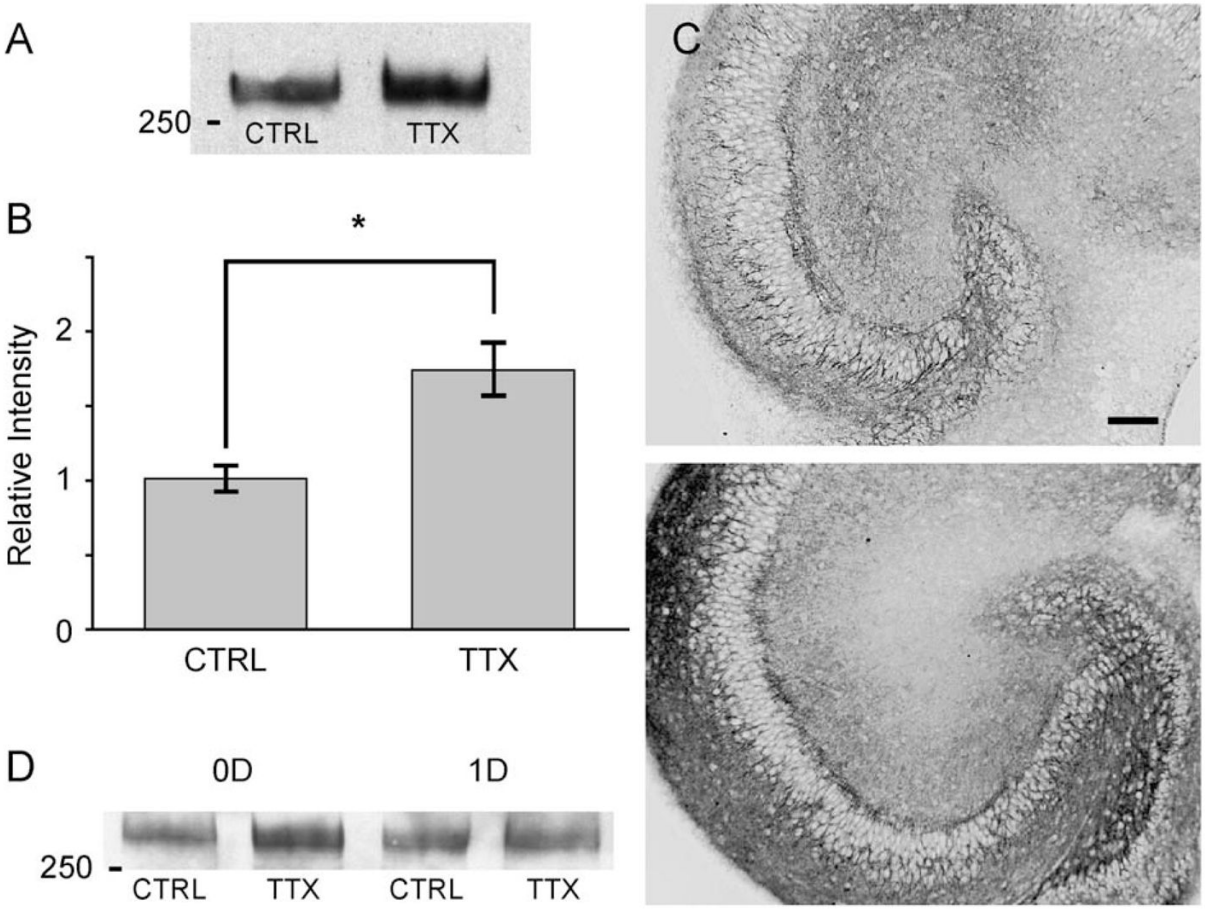

Fig. 3.

VGSCs are upregulated in activity-deprived slice cultures as revealed by Western blot and immunohistochemical analyses. (A) Representative Western blot of VGSC expression illustrates an increase in channel expression from control (CTRL) to 48 h activity-deprived (TTX) cultures. (B) Quantification of increased VGSC expression by densitometric analysis shows a significant (*: $P < 0.001$) increase in VGSC expression in TTX treated cultures compared with controls ($n = 5$ per group). All measurements were normalized to the control average. (C) Representative low-power images of VGSC immunostaining in control (upper) versus activity-deprived (lower) cultures. Images show CA3 to left and dentate gyrus (DG) to right. TTX treatment triggered increased immunostaining that was concentrated to the stratum oriens of CA3 that consists mainly of the basilar dendrites from pyramidal neurons (leftmost aspect of images). In addition, increased immunostaining also was evident in the plexiform layer of the DG and to a lesser extent in the stratum lucidum of CA3, two other hippocampal areas enriched with dendrites. (D) No significant difference in VGSC expression was seen between TTX-treated and respective control cultures 1, 2 and 3 days after exposure to TTX ($n = 3$). Representative Western blot of VGSC expression in control (CTRL) and activity-deprived (TTX) cultures recovered for 1 day are shown. Analogous results were seen for cultures recovered 2 and 3 days (data not shown). Calibration bar in C is 100 μm.