GLIA 41:128–136 (2003)

Astrocytes Selectively Enhance N-Type Calcium Current in Hippocampal Neurons

MARY MAZZANTI[1] AND **PHILIP G. HAYDON**[2*]
[1]*Department of Zoology and Genetics, Iowa State University, Ames, Iowa*
[2]*Department of Neuroscience, University of Pennsylvania, Philadelphia, Pennsylvania*

KEY WORDS glia; synaptogenesis; development; primary neuronal culture

ABSTRACT Astrocytes influence neuronal development, synapse formation, and synaptic transmission, partly through affecting neuronal calcium signals. In order to elucidate the extent to which astrocytes modulate neuronal voltage-gated calcium currents, we performed a whole-cell patch clamp analysis of neurons in astrocyte-deplete and astrocyte-enriched conditions. We demonstrate that hippocampal neurons in an astrocyte-enriched environment show augmentation of voltage-gated calcium current at 1–3 days in vitro. Further study in pairs of adjacent neurons showed that the augmentation in calcium current was dependent on direct contact with the astrocyte. Pharmacological analysis demonstrated the augmentation is selective for the N-type calcium current, although immunochemical labeling of the $\alpha 1_B$ subunit of the N-type calcium channel was unchanged. These findings show that astrocytes regulate neuronal voltage-gated calcium currents in a contact-dependent manner. The specificity of the effect for the N-type calcium current at early days in culture has special significance regarding the role of astrocytes in hippocampal synaptogenesis. *GLIA 41:128–136, 2003.*
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INTRODUCTION

Glial cells fulfill many roles in the developing and mature nervous system. During development astrocytes regulate neuronal migration (Gasser and Hatten, 1990; Fishman and Hatten, 1993; Hunter and Hatten, 1995; Thomas et al., 1996), neuronal differentiation (Liu et al., 1998; Blondel et al., 2000), process outgrowth (Hatten et al., 1984; Lieth et al, 1990; Carpenter et al., 1994; Powell and Geller, 1999; Yamada et al., 2000; van den Pol and Spencer, 2000), and synapse formation (Nakanishi et al., 1994; Pfrieger and Barres, 1997; Ullian et al., 2001). In the mature nervous system, glia have been shown to modulate synaptic transmission (Araque et al., 1998a,b; Kang et al., 1998; Newman and Zahs, 1998; Robitaille, 1998), and synaptic plasticity (Tweedle and Hatton, 1984; Muller and Best, 1989). Given the established role of neuronal calcium signaling in these processes (Suarez-Isla et al., 1984; Komuro and Rakic, 1991, 1993, 1996; Basarsky et al., 1994; Gu and Spitzer, 1997; Bahls et al., 1998; Behar et al., 1999; Meberg et al., 1999; Pravettoni et al., 2000; Spitzer et al., 2000; Gomez et al., 2001; Feng et al., 2002; Ramakers et al., 2001), and the demonstrated ability of astrocytes to elevate neuronal calcium (Nedergaard, 1994; Parpura et al., 1994), it seems likely that astrocytes mediate some of their effects on neurons by regulating neuronal calcium levels.

The hypothesis that astrocytes could affect synaptogenesis by regulating voltage-gated calcium channels was recently given credence by the findings of Barras and colleagues (Pfrieger and Barres, 1997; Ullian et al., 2001) which show that astrocytes increase the efficacy and number of synapses formed between retinal gan-

Grant sponsor: National Institutes of Health; Grant number: NS10724; Grant number: NS37585; Grant number: NS37585.

*Correspondence to: Philip G. Haydon, Department of Neuroscience, 215 Stemmler Hall, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6074. E-mail: pghaydon@mail.med.upenn.edu

Received 30 April 2002; Accepted 10 July 2002
DOI 10.1002/glia.10135

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glion neurons in culture and that this increase is associated with an increase in voltage-gated calcium current recorded in the cell body. Astrocyte modulation of voltage-gated calcium current could presumably exert effects at many stages of synapse formation. N-type calcium current is expressed in developing axons preceding synapse formation and the application of an N-type calcium-channel antagonist has been shown to reduce vesicle recycling in these immature axons (Pravettoni et al., 2000). In addition to the presence of N-type calcium channels, the distribution of these channels seems to be important for establishment of functional synapses. Bahls et al., 1998, have shown a shift in N-type calcium-channel distribution from a diffuse to a punctate pattern in neurites of hippocampal neurons between the fourth and eighth days in vitro (DIV); a stage of development associated with rapid synapse formation.

The hypothesis that astrocytes could affect synaptogenesis by regulating voltage-gated channels is also supported by previous findings showing that astrocytes can regulate the appearance of several types of ionic currents in neurons. Wu and Barish (1994) showed that direct contact with astrocytes induces the appearance of transient A-type potassium current, while suppressing a sustained D-type potassium current in mouse hippocampal neurons. Liu et al. (1996, 1997) showed that γ-aminobutyric acid (GABA)-, glycine-, kainite-, and N-methyl-D-aspartate (NMDA)- induced current densities were larger in hippocampal and spinal neurons cultured on astrocytes than those in neurons grown on a poly-D-lysine substrate. In spinal neurons, these effects could not be mimicked by treating neurons with medium conditioned from astrocytes (ACM), indicating a role for direct astrocyte-neuron contact. Furthermore, suppression of spontaneous cytoplasmic calcium elevations in astrocytes by 1,2-bis (2-aminophenoxy)ehane-N,N,N,N-tetraacetic acid (BAPTA) loaded intracellularly, blocked the enhancement of GABA-, glycine-, kainite-, and NMDA- induced current densities in neurons by astrocytes. Contact with glia has also been shown to influence the distribution of sodium and potassium channels in axonal membranes (Bostock et al., 1981; Shrager, 1988; Ritchie et al., 1990: Joe and Angelides 1992; Waxman and Ritchie, 1993). Finally, co-culture with astrocytes can augment the magnitude of voltage-gated calcium currents in retinal ganglion cells (Ullian et al., 2001). Whether all calcium currents are affected remains unclear, and the role for astrocyte-neuron contact in mediating this augmentation is unknown.

The goal of this study was to determine how astrocytes, the predominant form of glial cell of the central nervous system, regulate specific calcium currents. We demonstrate that astrocytes increase the magnitude of the N-type calcium current, and that this augmentation results from local contact between the neuron and the astrocyte. A corresponding increase in the expression of the $\alpha 1_B$-subunit of the N-type calcium channel was not detected, indicating that astrocyte contact augments calcium current through a mechanism other than increased N-typed calcium-channel density.

MATERIALS AND METHODS

Preparation of Cortical Astrocyte Cultures

Primary astrocyte cultures were established from neocortices of 0–3-day postnatal Sprague-Dawley (Harlan) rat pups according to previously established methods (Goslin and Banker, 1991). Animals were deeply anesthetized with halothane before being sacrificed. All procedures for animal use were approved by the IACUC committee of Iowa State University. Macro and micro dissection of brain tissue was carried out in ice-cold Earl's balanced salt solution (EBSS). Cortices were removed and then treated enzymatically with papain (20 U/ml) at 37°C in a 5% CO_2 and 95% air atmosphere for 1 h. The enzymatic reaction was stopped by the addition of trypsin inhibitor (10 mg/ml). Tissue was transferred to modified minimal essential medium (MMEM; MEM (Gibco); L-glutamine, 2 mM; D-glucose, 40 mM; Na-bicarbonate, 14 mM; Na^+-pyruvate, 1%; penicillin, 100 IU/ml, streptomycin, 100 mg/ml; and heat inactivated horse serum, 10%) and dissociated by gentle trituration through a glass serological pipette. The resulting cell suspension was placed into culture flasks (25 cm^2, Falcon).

After 10–14 DIV, a purified astrocyte culture was produced by firmly tapping flasks, then placing them in an orbital shaker (260 rpm at 37°C) for 1.5 h. After medium exchange, flasks were returned to the orbital shaker for an additional 18 h. Afterwards, the cultures were enzymatically treated with trypsin (0.1%), which caused the cells to become detached from the wall of the flask. The enzymatic reaction was stopped with addition of fresh MMEM, and the flasks were again tapped. The resulting cell suspension was transferred to a centrifuge tube, and astrocytes sedimented by centrifugation at 750 rpm for 10 min at room temperature. The resulting pellet was resuspended, gently triturated, and plated onto 12-mm-diameter poly-L-lysine (1 μg/ml; MW 10,000)-coated glass coverslips. All chemicals were purchased from Sigma (St. Louis, MO) unless stated otherwise.

Preparation of Hippocampal Neuronal-Enriched Cultures

Primary hippocampal cultures were established from 0–3 day postnatal Sprague-Dawley (Harlan) rat pups. Hippocampi were dissected from the brain in ice-cold EBSS. After treatment with papain (20 U/ml) at 37°C in a 5% CO_2 and 95% air atmosphere for 1 h, hippocampi were mechanically dissociated in MEM containing: L-glutamine, 2 mM; penicillin, 100 IU/ml, streptomycin 100 mg/ml; mito+ serum extender (Collaborative Biomedical, Bedford MA); and heat-inacti-

vated horse serum, 5%, then plated onto 12-mm glass coverslips coated with poly-L-lysine (1 μg/ml). On the third day in culture (3 DIV), 5-fluoro-2′-deoxyuridine (FUDR, 5 μM) was added to the medium to suppress proliferation of non-neuronal cells. Cultures were fed every four days with fresh MMEM.

Preparation of Astrocyte-Enriched and Astrocyte-Deplete Neuronal Cultures

It should be noted that it is not possible to obtain pure populations of neurons before plating in culture. Therefore in order to study astrocyte-mediated effects on neuronal calcium currents, we used a method in which we could choose hippocampal neurons that differed in their degree of contact with astrocytes. In this method, purified astrocytes (as described above) were plated on only one-half of a 12-mm-diameter coverslip. A small volume of the astrocyte cell suspension (~50 μl) was plated on one side of the coverslip. After attachment to the poly-L-lysine substrate (~3 h) the entire coverslip was covered with MMEM. At 2–3 days later, astrocytes formed a continuous layer that was restricted to one-half of the coverslip. Postnatal hippocampal neurons were dissociated (as described above) and were plated over the entire coverslip, resulting in a coverslip that contained astrocyte-enriched and astrocyte-deplete conditions. Because all cells in this coverslip are exposed to the same culture medium, this culture approach allows us to examine the role of contact, rather than diffusible substances such as cholesterol (Mauch et al., 2001) in mediating glial actions on neuronal calcium currents.

Preparation of Hippocampal Micro-Island Cultures

Coverslips containing hippocampal neurons growing on separated "islands" of cortical astrocytes were established in a manner similar to that described by Furshpan et al. (1976). Briefly, coverslips were coated with 0.15% agarose solution made in sterile water, and allowed to dry for half a day. A solution of 60% poly-D-lysine (1 μg/ml in H_2O), and 40% rat tail collagen type 1 (1 mg/ml in 0.1% acetic acid), was sprayed onto 12-mm coverslips using an atomizer, thus creating small (50–100 μm) patches of substrate. The following day, hippocampal cultures were established as described above. These "micro-island" cultures allowed control over neuron-neuron contact, since an island containing a single neuron, or an island containing a pair of neurons could be chosen for study.

Electrophysiology

Whole-cell patch-clamp recordings were obtained from neurons with an Axopatch-1C amplifier (Axon Institute, CA). The external solution contained (in mM): 1 μM tetrodotoxin, 100 NaCl, 20 TEA Cl, 5 CsCl 2 $MgCl_2$, 20 $CaCl_2$, 10 HEPES, 10 glucose, pH 7.35. Patch pipettes had DC resistance of 5–10 MΩ when filled with internal solution that contained (in mM): 117 TEA Cl, 4.5 $MgCl_2$; 9 EGTA, 4 ATP, 0.3 GTP, 0.1 leupeptin 10 Hepes, pH 7.35. After the whole-cell configuration was established, the membrane potential was held at −70 mV. Macroscopic calcium currents were evoked by depolarizing the membrane to 0 mV for 150–300 ms. Five fractionally scaled hyperpolarizing subpulses were used to subtract leakage current online. Currents were filtered at 1–2 kHz and were sampled at >1 kHz. Peak inward current (pA) evoked by the depolarizing step to 0 mV was divided by the whole-cell membrane capacitance (pF), to obtain a measure of whole-cell peak current density (pA/pF). In addition to measures of current density at a single depolarizing potential, a complete current-voltage (I-V) relation was obtained in each cell by stepping (in +10-mV increments) through a series of depolarizing voltages from a negative holding potential. All recordings were performed at room temperature (20–22°C).

Immunocytochemistry

Cells (1–3 DIV) were fixed in fresh 4% paraformaldehyde for 30 min at room temperature, washed three times in phosphate-buffered saline (PBS) and permeabilized in 0.25% Triton-X for 10 min. Nonspecific antibody binding was blocked by a 30-min incubation in modified PBS containing PBS, goat serum 5%, Triton-X-100, 0.25%, bovine serum albumin (BSA, protease free) 5%, and sodium azide 0.02%. Cells were then incubated in anti-calcium-channel $\alpha 1_B$ subunit antibody (affinity-purified anti-peptide antibody that recognizes α_{1b} subunit in western blots; Sigma), and monoclonal anti-glial fibrillary acidic protein (GFAP) clone G-A-5 (mouse anti-pig) (Sigma). Secondary labeling of the $\alpha 1_B$ subunit was achieved by incubation with Alexa Flour 568 goat anti-rabbit IgG (H+L) conjugate and secondary labeling of GFAP was achieved by incubation with Alexa Flour 488 goat anti-mouse (Molecular Probes, Eugene OR). Fluorescently labeled cells were visualized through a ×60 objective attached to a Nikon 200 inverted microscope. A xenon arc lamp (100 W) was used to illuminate the sample at 480 nm (480DF10, Omega Optical, Brattleboro, VT). Fluorescent emission was collected through a dichroic mirror (510DRLP; Omega Optical) and filtered with a 515EFLP filter (Omega Optical). Images were acquired with a cooled digital camera (ORCA; Hamamatsu, Hamamatsu City, Japan).

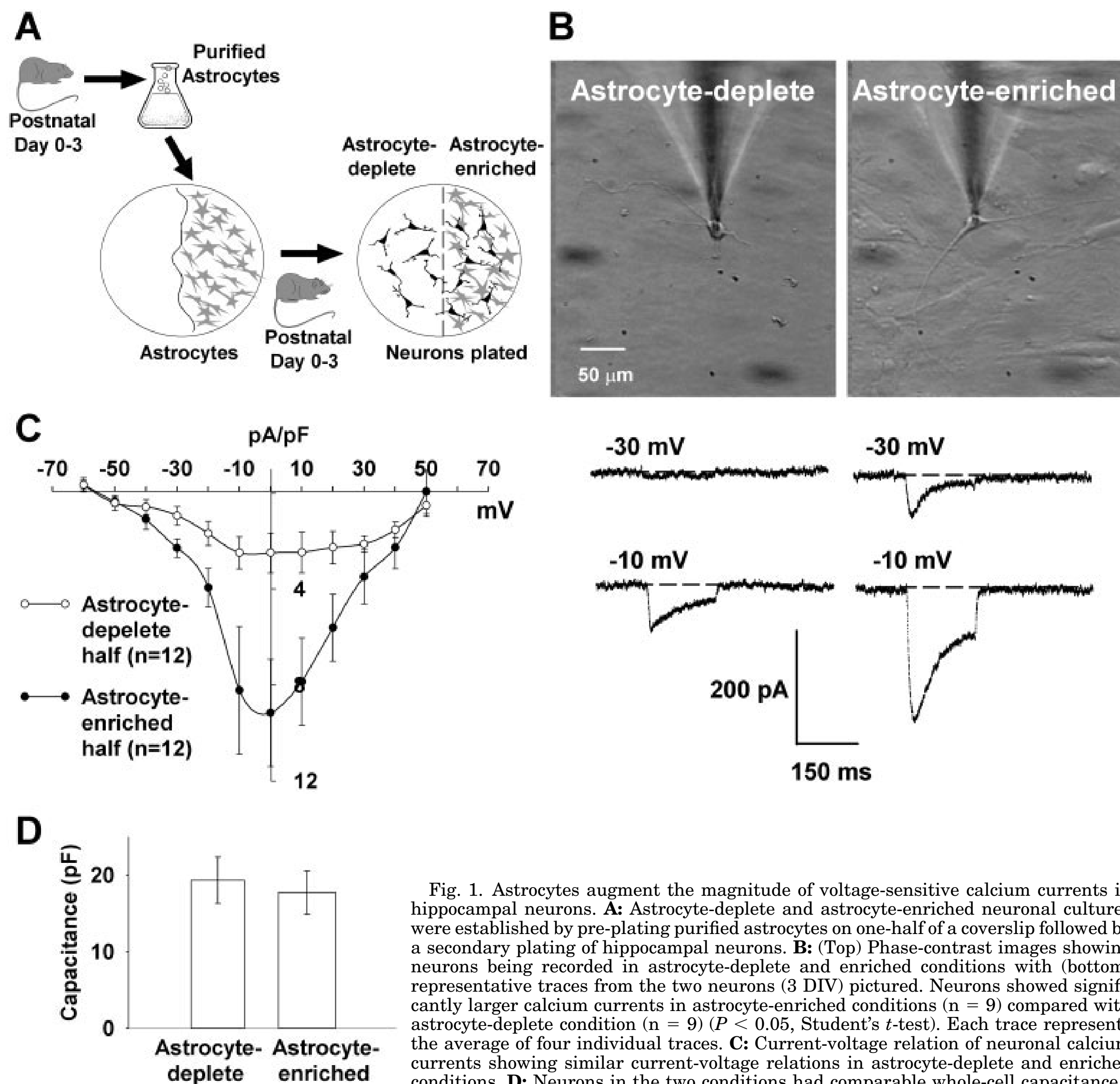

Fig. 1. Astrocytes augment the magnitude of voltage-sensitive calcium currents in hippocampal neurons. **A:** Astrocyte-deplete and astrocyte-enriched neuronal cultures were established by pre-plating purified astrocytes on one-half of a coverslip followed by a secondary plating of hippocampal neurons. **B:** (Top) Phase-contrast images showing neurons being recorded in astrocyte-deplete and enriched conditions with (bottom) representative traces from the two neurons (3 DIV) pictured. Neurons showed significantly larger calcium currents in astrocyte-enriched conditions (n = 9) compared with astrocyte-deplete condition (n = 9) ($P < 0.05$, Student's *t*-test). Each trace represents the average of four individual traces. **C:** Current-voltage relation of neuronal calcium currents showing similar current-voltage relations in astrocyte-deplete and enriched conditions. **D:** Neurons in the two conditions had comparable whole-cell capacitance.

RESULTS

Neuronal Calcium Currents Are Larger in Neurons in an Astrocyte-Enriched Environment

As an initial determination of whether astrocytes regulate the magnitude of neuronal calcium currents we measured macroscopic calcium currents in whole-cell voltage-clamp recordings from hippocampal somata under conditions in which hippocampal neurons were either in contact with, or isolated from astrocytes (Fig. 1A,B). Neurons in astrocyte-enriched conditions displayed significantly larger macroscopic calcium currents than neurons in astrocyte-deplete conditions (Fig. 1C, n = 12, $P < 0.01$) with no affect on voltage-activation threshold or leakage current (astrocyte-enriched 14.5 ± 2.2 pA, n = 8; astrocyte-deplete 17.7 ± 2.8 pA, n = 8). Comparable whole-cell capacitance measures (Fig. 1D) (astrocyte-enriched 17.7 ± 2.8 pF, n = 8; astrocyte-deplete 19.3 ± 3.0 pF, n = 8) indicate that the two groups did not differ with respect to size.

Increased Neuron-Neuron Contact Does Not Account for the Increase in Neuronal Calcium Current Observed in the Astrocyte-Enriched Condition

Because astrocytes augment neurite extension (Hatten et al., 1984; Lieth et al, 1990; Carpenter et al., 1994; Powell and Geller, 1999; Yamada et al., 2000; van den Pol and Spencer, 2000) and because neuron-neuron contact is known to affect the distribution of the

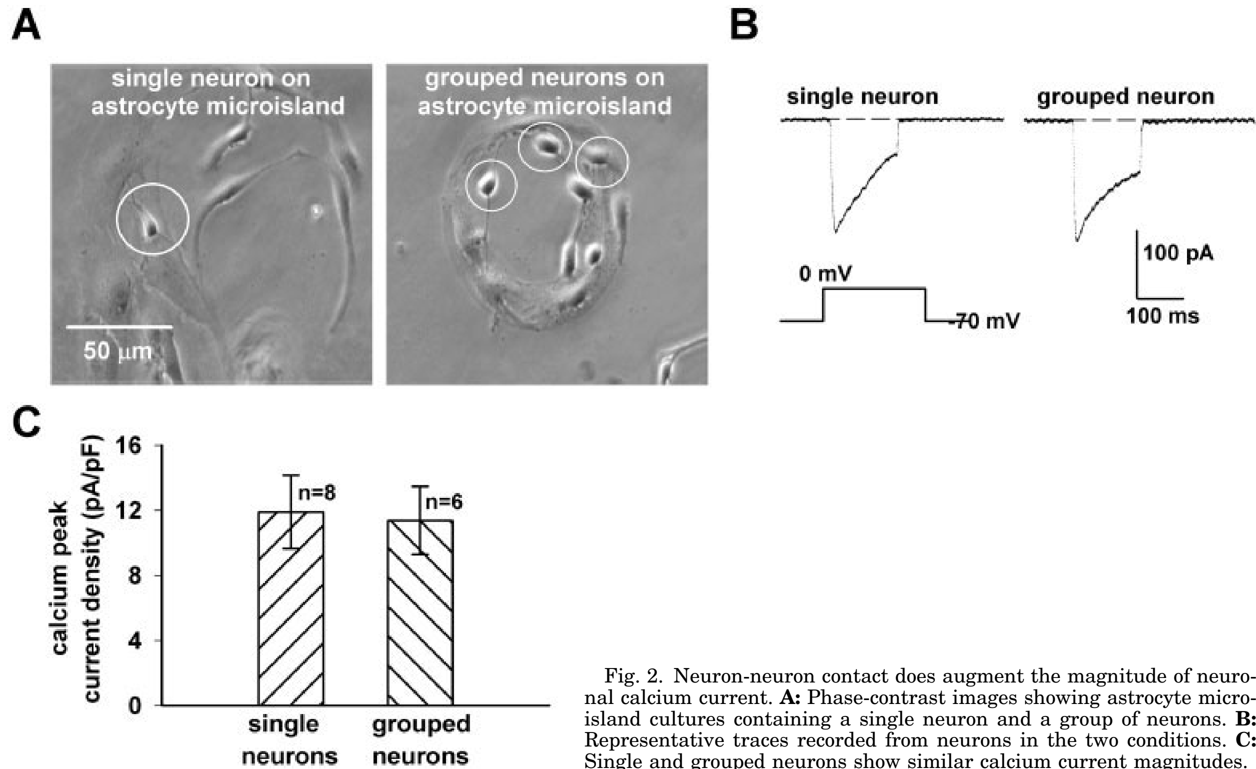

Fig. 2. Neuron-neuron contact does augment the magnitude of neuronal calcium current. **A:** Phase-contrast images showing astrocyte micro-island cultures containing a single neuron and a group of neurons. **B:** Representative traces recorded from neurons in the two conditions. **C:** Single and grouped neurons show similar calcium current magnitudes.

N-type calcium channel (Bahls et al., 1998), it is important to determine whether the astrocyte-induced augmentation of the calcium current is secondary to effects mediated by neuron-neuron contact. In order to control for this possibility neurons were plated in micro-island culture conditions so that we could select either single or paired neurons on astrocyte micro-islands. In these conditions, we detected no difference in the magnitude of the macroscopic calcium current in single or paired neurons (Fig. 2). Although we cannot exclude the possibility that the current is regulated by a neuron making contact with itself, these data suggest that astrocytes directly regulate the appearance of the N-type calcium current.

Augmentation of Neuronal Calcium Current by Astrocytes Is Contact Dependent

The results presented thus far are consistent with other reports of astrocyte augmentation of neuronal current densities (Wu and Barish, 1994; Liu et al., 1996, 1997; Ullian et al., 2001); however, they do not address whether the mechanism of astrocyte action is through released factors or by direct contact. In order to address this question, we plated neurons into relatively low-density conditions so that some would make contact with an astrocyte (ON), while others would be isolated (OFF). We then performed a paired analysis in which calcium current magnitudes were monitored from each of two adjacent neurons, one in the "on-astrocyte" and the other in the "off-astrocyte" configuration (Fig. 3). Neuronal cell bodies that contacted an astrocyte displayed significantly larger calcium currents than did neurons in the off-astrocyte configuration ($P < 0.01$). Because it is likely that neurons in each condition are exposed to similar secreted factors, our results indicate that contact between an astrocyte and neuron is required for the augmentation of the calcium current.

This astrocyte-contact induced augmentation of the calcium current is a transient phenomenon since it is only detected at early times in culture. The astrocyte-induced enhancement of calcium current density was much greater in young (1–3 DIV, 400%) as compared with mature (7, 8, and 9 DIV, 38%) neurons (Fig. 4).

Astrocyte-Neuron Contact Selectively Augments the Magnitude of the N-Type Calcium Current

Hippocampal neurons in culture express both high-voltage activated and low-voltage activated calcium channels. In mature neurons (>7 DIV), the functional coupling of calcium influx to transmitter release at excitatory synapses is mediated through P/Q calcium channels, whereas in immature neurons this function is served mainly by N-type calcium channels (Scholz and Miller, 1995). To assess the functional impact of astrocyte-induced augmentation of neuronal calcium current, we investigated whether astrocytes regulate the magnitude of specific calcium-channel subtypes.

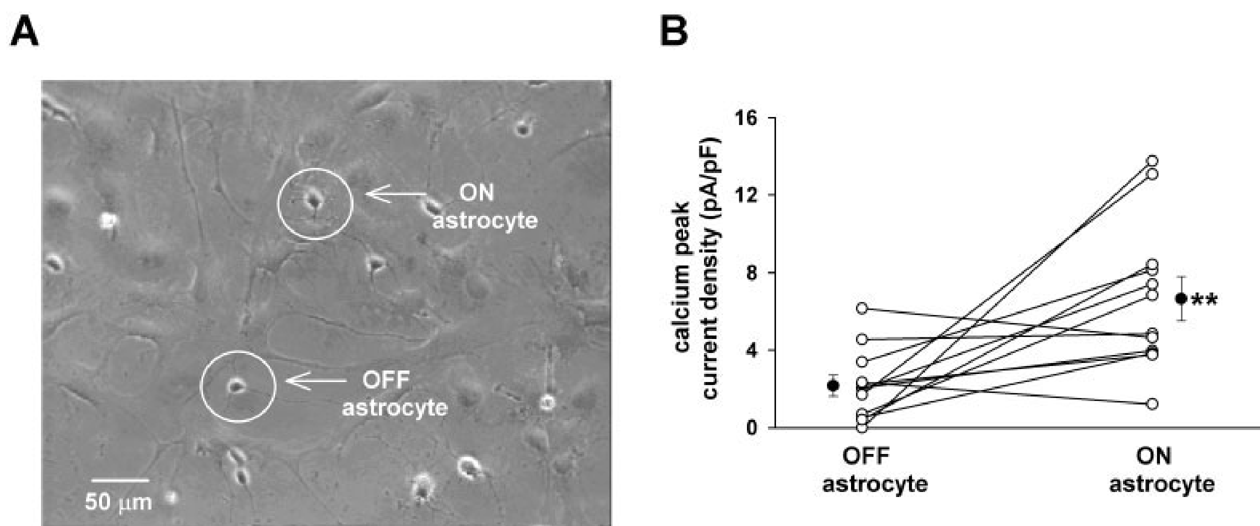

Fig. 3. Astrocyte contact is necessary for enhancement of calcium current. **A:** Phase-contrast image showing a neuronal cell body in contact with an underlying astrocyte (ON astrocyte) and another nearby neuron in which the cell soma does not contact an astrocyte (OFF astrocyte). **B:** The calcium current density was recorded from pairs of neurons in ON (n = 12) and OFF (n = 12) conditions. Group means are represented by single filled circles. Neurons in the ON-astrocyte condition have significantly greater magnitude calcium current density than neurons in the OFF-astrocyte condition ($P < 0.01$, paired *t*-test).

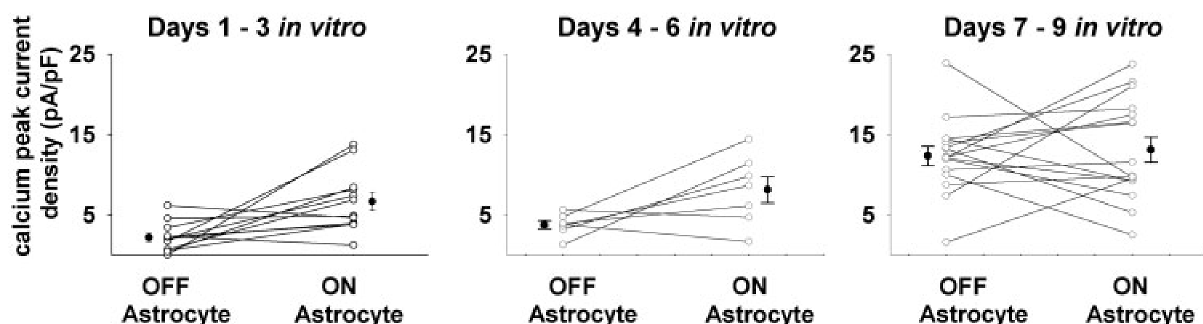

Fig. 4. Paired analysis showing that at 1–6 days, but not after 7–9 days in vitro, neurons contacting astrocytes have larger calcium currents (*$P < 0.01$).

Whole-cell calcium currents were measured in voltage-clamp recordings before and after the addition of one of three calcium-channel antagonists, each acting specifically to block a sub-type of calcium channel as follows; nifedipine (10 μM) for L-type; ω-CgTX GVIA (1 μM) for N-type; and ω-Aga-IVA (500 nM) for P/Q-type. Antagonists were pressure ejected (3 s) from a glass microcapillary tube with an open tip diameter of 3–5 μm. These studies demonstrated that contact with astrocytes selectively augmented the magnitude of the N-type calcium current (Fig. 5), while L-type and P/Q-type currents were unchanged. Immunocytochemistry did not show any obvious change in the density or distribution of the $\alpha 1_B$ subunit of the N-type calcium channel as a result of contact with astrocytes (data not shown). The selective action of astrocytes on the functional expression of the N-type calcium current is particularly exciting in light of the role of this current in mediating transmitter release in developing synapses.

DISCUSSION

The aim of the current study was to determine if astrocytes modulate voltage-gated calcium currents in cultured hippocampal neurons. Our results clearly indicate that neurons in an astrocyte-enriched environment exhibit much larger calcium currents (on the order of 360% larger) than do neurons in an astrocyte-deplete environment. This augmentation of calcium current is dependent on contact between the neuron and astrocyte, and is specific to N-type calcium current. While we interpret this result as being due to a contact-dependent increase in calcium current, it is also remotely possible that a subpopulation of neurons with larger N-type calcium current selectively attach to astrocytes. The augmentation of N-type calcium current shows developmental specificity in that it only occurs at early times in culture (1–3 DIV). At later times, the amplitude of the calcium currents in both culture conditions are of a similar amplitude, suggesting that the

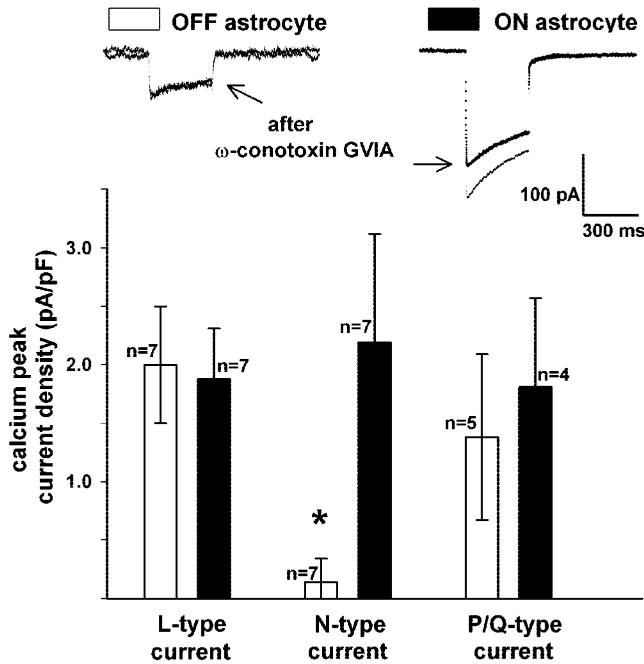

Fig. 5. Astrocytes selectively enhance the magnitude of the N-type calcium current. (Top) Representative traces showing the irreversible reduction of calcium current caused by the application of the N-type calcium-channel antagonist ω-conotoxin GVIA (1 μM). (Bottom) Calcium currents recorded in the presence of nifedipine (10 μM), ω-conotoxin GVIA (1 μM) and ω-agatoxin IVA (500 nM) show that astrocytes selectively augment the N-type calcium current ($P < 0.01$, ANOVA). All recordings were made from neurons cultured at 1–3 days in vitro (DIV).

astrocytes hasten the maturation of the calcium current phenotype.

The specificity of the astrocyte induced augmentation to N-type calcium currents at early times in culture (see also Li et al., 1999) has special significance in regard to the role that astrocytes may play in hippocampal synaptogenesis. Specifically, these results support the hypothesis that by enhancing N-type calcium current, astrocytes would allow for greater transmitter release, which in turn would put this synapse at a competitive advantage over neighbors that do not contact glia. Presumably, those synapses that contact astrocytes would be maintained during the period of synapse elimination that frequently follows a period of exuberant synaptogenesis.

Several lines of evidence support such a hypothesis. An increase in current through voltage-gated calcium channels has been shown to be the initial response upon contact of presumptive pre- and post-synaptic membranes (Feng et al., 2002). It has also been shown that at the time that synapses are forming in culture (Basarsky et al., 1994), N-type calcium current is responsible for initial transmitter release, giving way to predominantly P/Q-type calcium current at later stages in development (Scholz and Miller, 1995; Verderio et al., 1995; Pravettoni et al., 2000). If astrocytes enhance calcium influx through voltage-gated calcium channels in presynaptic terminals, we would predict that astrocyte contact could enhance synaptic transmission. Recent studies show that this is the case. Pfrieger and Barres (1997) have shown that retinal ganglion cells (RGCs) form "inefficient" synapses, characterized by minimal spontaneous synaptic activity and high failure rates during evoked synaptic transmission, when grown on poly-D-lysine as compared with RGCs grown on astrocytes. These studies suggest that astrocytes can induce a sustained modulation of synaptic transmission. Such a sustained enhancement of synaptic transmission has consistently been shown to increase the likelihood that a synapse will form and become stabilized (for review, see Aamodt and Constantine-Paton, 1999). Thus, astrocytes could conceivably contribute to activity-dependent synaptic stabilization (see also Muller, 1993).

Astrocytes are likely to modulate synaptogenesis by both contact-dependent and -independent mechanisms. Astrocytes can release a number of substances (Kettenmann, 1999), including growth factors (Muller et al., 1995; Gomes et al., 2001) and cholesterol (Mauch et al., 2001) that have been shown to influence synaptogenesis. In support of this mechanism is the study by Ullian et al. (2001), showing that the increased synapse formation between neurons co-cultured with astrocytes can be mimicked by simple addition of astrocyte conditioned medium (ACM) to pure neuronal cultures, indicating that soluble factors are responsible for the effect. In other cases, addition of ACM to neuronal cultures fails to replicate fully the effects seen in co-culture suggesting that astrocyte contact is necessary (Nakanishi, et al, 1994; Liu et al., 1996).

Whether contact with an astrocyte regulates only those N-type calcium channels in the local contact vicinity or whether it causes a global "switch-like" activation of channels throughout the whole neuron is likely to be an important issue that may impact the spatial specificity of synaptogenesis. Perhaps chemical factors released from astrocytes "prime" the neuron so that it is prepared for synaptogenesis, and then local, contact-dependent physical interactions regulate spatially discrete aspects of synaptogenesis. The mechanisms underling the contact-dependent augmentation of N current remain to be determined. While many possibilities exist, that we see no change in immunoreactivity for the N-type calcium-channel α subunit suggest local regulation. Perhaps preexisting nonfunctional channels are phosphorylated to lead to new active channels. Alternatively new channels may be locally inserted into the plasma membrane.

In conclusion, our results show that astrocyte-neuron contact enhances N-type neuronal calcium current at a time critical for synapse formation in culture. The resulting enhancement of synaptic transmission between a pre and post-synaptic terminal enwrapped by an astrocyte would then place this tripartite synapse at a competitive advantage, and in this way be critical for the patterning of synaptic networks in the central nervous system.

ACKNOWLEDGMENTS

The authors thank Dr. Don Sakaguchi for comments on an earlier version of this manuscript.

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