1996

Astrocyte volume regulation

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Astrocyte volume regulation

by

Trent Allan Basarsky

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GENERAL INTRODUCTION

Until recently, the prevailing view of neurobiology has been that neurons are the computational elements of the brain while glia function to provide nutritive and mechanical support. It has now become apparent that glial cells may play a much more active role, perhaps by shaping higher brain processes, through a number of dynamic mechanisms. However, in addition to their potential contribution to brain function, abnormal glial processes may lead to deviant brain function. This introduction outlines the key active features of glia, and then discusses potential implications of aberrant glial function.

*Glia possess active properties*

A phylogenetic study of the relative numbers of glia to neurons reveals an increasing glia:neuron ratio as one ascends the evolutionary scale (Reichenbach, 1989). Considering the numerical superiority of glia in higher animals it is tempting to suggest that they play a much more active role than has been previously postulated.

*Glia contain ion channels*

In the 1960's electrophysiological studies of glial cells showed that glia membranes contain primarily large, almost ohmic potassium conductances (Kuffler and Potter, 1964; Kuffler et al., 1966; Orkand et al., 1966) thus suggesting the basis for the large negative resting membrane potentials typical of glia. With the advent of the patch-clamp technique
(Hamill et al., 1981) and the refinement of culture approaches that permit selective growth of
distinct subtypes of glial cells (McCarthy and de Vellis, 1980), a plethora of voltage-
dependent and ligand-gated ion channels have been described in glial cells (Barres, 1991;
Sontheimer, 1992). In fact many glia contain ion channels that are typically thought of as
specific to cells with excitable membranes, such as voltage-dependent $\mathrm{Na}^+$, $\mathrm{K}^+$, and $\mathrm{Ca}^{++}$
channels, as well as $\mathrm{Cl}^-$ and stretch-sensitive channels.

What are the roles of some of the ion channels in glia? Extracellular potassium levels
are thought to be controlled in part by glial potassium channels. Potassium ions enter at sites
of increased external potassium either passively through potassium ion channels, or actively
through the $\mathrm{Na}^+/\mathrm{K}^+$ ATPase. The spatial buffering hypothesis of Orkand et al. (1966)
suggests that once potassium ions enter the cell, a current flow is generated such that efflux
of potassium occurs at distant sites where external potassium is low and an outward driving
force for potassium has developed. The functional coupling of glia through gap junctions is
significant as it increases the extent of this spatial buffer. Potassium channels have also been
implicated in glia proliferation as demonstrated by reduced glia proliferation after potassium
channel blockade (DelBigio et al., 1994; Pappas et al., 1994). Sontheimer et al. (1994)
suggests that the voltage-dependent sodium channel provides a source of sodium for the
sodium-potassium ATPase, rather than functioning in membrane excitability. These voltage-
dependent glial sodium channels are unlikely to be involved in action potential generation for
two reasons: (i) the voltage-dependent steady-state inactivation of glial sodium channels
predicts that most of these channels are unavailable for activation at typical glial resting
membrane potentials (Sontheimer and Waxman, 1992) and (ii) there is a high relative ratio of potassium to sodium channels (Sontheimer et al., 1992). Thus several roles for ion channels in glia are becoming established, such as the regulation of ion homeostasis and cell proliferation.

**Glia respond to neurotransmitters**

In addition to voltage-dependent ion channels, glia also possess ligand-gated ion channels which permit them to respond to neurotransmitters such as glutamate and GABA (Sontheimer, 1992). Binding to these channels can lead to alterations in membrane potential, and subsequent opening or closing of other voltage-dependent channels. A recent report has demonstrated that electrical activity in cultured neurons can cause membrane currents in neighboring astrocytes (Murphy et al., 1993). It was suggested that synaptically released glutamate mediated these currents, though direct demonstration using glutamate antagonists was not possible since the neuronal activity itself was generated through polysynaptic excitatory transmission. Nevertheless, these exciting findings suggest that a rapid, short-range signaling network exists from neurons to astrocytes.

Activation of several glial neurotransmitter and peptide receptors also leads to changes in internal calcium levels (Finkbeiner, 1993). Since calcium is a ubiquitous regulator of cellular function, the sensitivity of glial intracellular calcium levels to external ligands may have functional significance. For example, Dani et al. (1992) demonstrated that neuronal activity in hippocampal slices leads to elevations of intracellular calcium in
astrocytes, and the generation of calcium waves (see below) throughout neighboring astrocytes. Therefore, in addition to the rapid signaling pathway mentioned previously, these results suggest that a slower neuronal to astrocyte signaling circuit also exists.

**Glia contain neurotransmitter transporters**

In addition to responding to extracellular neurotransmitters through the activation of ligand-gated ion channels mentioned above, glia cells also possess a large number of neurotransmitter transporters. These transporters are typically thought to function to regulate extracellular concentrations of neurotransmitter. What is the significance of this regulation? As mentioned in the section entitled *Glia and pathology*, excessive excitatory amino acid can lead to neuronal degeneration through activation of neuronal glutamate receptors. Glutamate uptake into glia cells may reduce this degeneration. Once glutamate is taken up into glia cells it is converted to glutamine by glutamine synthetase, an enzyme localized primarily to glia (Martinez-Hernandez et al., 1977). Glutamine is then released into the extracellular space where it is taken up by neurons and converted back to glutamate (Waniewski and Martin, 1986) or GABA (Battaglioli and Martin, 1991). Thus glia serve as sites to absorb and process glutamate, then return it to neurons in a re-useable form (i.e. glutamine).

Does glia uptake of neurotransmitter affect chemical synaptic transmission? Since chemical synaptic transmission is mediated by the binding of pre-synaptically released neurotransmitter to post-synaptic receptors, it follows that any process that can regulate extracellular levels of neurotransmitter may affect synaptic transmission. It has been
demonstrated (Thompson and Gahwiler, 1992; Isaacson et al., 1993) that action potential evoked inhibitory synaptic currents (IPSC's) were significantly prolonged in the presence of GABA uptake inhibitors, though the time course of miniature IPSC's were unaffected. Isaacson and Nicoll (1993) suggest that GABA uptake regulates the extent of 'spillover' of GABA to adjacent synapses, and that spillover is not significant during miniature synaptic events. In contrast to GABA, inhibition of glutamate uptake has no effect on the time course of non-NMDA- (Isaacson and Nicoll, 1993; Sarantis et al., 1993) or NMDA-receptor (Isaacson and Nicoll, 1993) mediated synaptic transmission, which led the authors to suggest that the diffusion of glutamate from the synaptic cleft, and the kinetics of glutamate-gated ion channels play a dominant role in shaping the post-synaptic current. However, since non-NMDA receptors show rapid desensitization (Patneau and Mayer, 1990), the above experiments were also performed in the presence of cyclothiazide which inhibits receptor desensitization. When desensitization was reduced, uptake inhibition then prolonged the non-NMDA mediated synaptic event (Isaacson and Nicoll, 1993; Mennerick and Zorumski, 1994), suggesting that desensitization may participate in terminating non-NMDA mediated synaptic events. It must be noted that it is likely that these uptake inhibitors were active at both glial and neuronal transporters. Mennerick and Zorumski (1994) also inhibited glial glutamate uptake directly by depolarizing glia cells, which has been shown to reverse the glutamate transporter (Szatkowski et al., 1990), and demonstrated a prolongation of non-NMDA synaptic currents in the presence of cyclothiazide. Therefore glial uptake functions
not only to reduce extracellular levels of neurotransmitter, but may also play a role in neuronal chemical synaptic transmission.

Glia possess the ability for interglial communication

A seminal paper by Cornell-Bell and colleagues (Cornell Bell et al., 1990) demonstrated the existence of propagated calcium waves through cultured astrocytes in response to glutamate. These calcium waves are thought to propagate to neighboring astrocytes by the diffusion of IP3 through gap junctions, resulting in regenerating calcium signals from astrocyte to astrocyte (Charles et al., 1991; Charles et al., 1993). Gap junctions in astrocytes have been studied at a biophysical level (Dermietzel et al., 1991; Giaume et al., 1991), and modulation of these gap junctions by depolarization, glutamate, and calcium has been examined (Enkvist and McCarthy, 1992; Enkvist and McCarthy, 1994). Recently, a common brain derived compound, anandimide, has been shown to inhibit astrocyte gap junctions, and subsequently inhibit interastrocytic calcium wave propagation (Venance et al., 1995). The existence of astrocytic calcium waves and the potential for astrocyte gap junction modulation raises the exciting possibility of long-range extra-neuronal signaling pathways within the brain, whose circuits may be dynamically modulated.

Astrocytes can signal to neurons

Recently a number of reports have demonstrated that elevations in astrocytic calcium levels can lead to elevations in intracellular neuronal calcium concentrations (Charles, 1994;
Nedergaard, 1994; Parpura et al., 1994; Hassinger et al., 1995). There is increasing evidence that glutamate released from astrocytes signals to neighboring neurons. Using HPLC, Parpura et al. (1994) demonstrated that glutamate can be released from astrocytes through a calcium dependent release mechanism in response to the neuroligand bradykinin. In mixed neuronal and astrocyte cultures, elevations of astrocyte calcium were induced through either mechanical stimulation (Charles, 1994; Nedergaard, 1994; Parpura et al., 1994; Hassinger et al., 1995), photostimulation (Parpura et al., 1994), or bradykinin stimulation (Parpura et al., 1994). These increases in astrocyte calcium levels led to subsequent elevations of neuronal calcium (Charles, 1994; Nedergaard, 1994; Parpura et al., 1994; Hassinger et al., 1995), which in some cases were sensitive to glutamate receptor antagonists (Parpura et al., 1994; Hassinger et al., 1995). Using astrocytes from the forebrain, in culture for a longer time period (up to 6 weeks), Nedergaard (1994) demonstrated that the astrocyte induced elevations of neuronal calcium were insensitive to glutamate antagonists, but were abolished by inhibitors of gap junction communication. Regardless of the debate over the mechanism mediating astrocyte-neuron signaling, the presence of a signaling pathway from astrocytes to neurons has been demonstrated. The ability of astrocytes to communicate with neurons suggest that astrocytes possess the potential to significantly shape higher brain function.

**Glia release neurotrophic factors.**

Typically neurotrophic factors are thought to increase neuronal cell viability. Recently a glia derived neurotrophic factor (GDNF) was identified that promotes the
survival of cultured embryonic mesencephalic dopaminergic neurons (Lin et al., 1993).

Subsequently, GDNF was shown to (i) provide protective and reparative effects after MPTP treatment, which causes Parkinsonian symptoms in man (Tomac et al., 1995), (ii) prevention of axotomy induced degeneration (Beck et al., 1995; Oppenheim et al., 1995; Yan et al., 1995) and (iii) prevention of programmed cell death (Oppenheim et al., 1995). In addition to GDNF, glia also are capable of producing other neurotrophic factors. For example, mRNA for brain-derived neurotrophic factor (BDNF) (Zafra et al., 1992; Moretto et al., 1994), NGF (Moretto et al., 1994) and NT-3 (Moretto et al., 1994) is present in glial cells. Stimulation of astrocytic adenylate cyclase with forskolin, significantly increases mRNA levels of both BDNF and NGF (Zafra et al., 1992). Furthermore, it has been demonstrated that neurotransmitters such as norepinephrine and dopamine also increase BDNF mRNA expression in astrocytes (Zafra et al., 1992). Additionally, astrocytes are also capable of producing ciliary-neurotrophic factor (CNTF) which has been shown to increase neuronal survival, and induce neurite outgrowth (Richardson, 1994). It must be mentioned that with the exception of GDNF and CNTF, the predominant source of these neurotrophins under physiological conditions are neurons. However, Zafra (1992) has suggested that astrocytic release of neurotrophins under pathophysiological conditions may serve a significant role.

Recent evidence suggests that several neurotrophic factors affect synaptic function. For example, enhancement of synaptic strength has been induced by application of BDNF, neurotrophin-3 (NT-3), or CNTF (Lohof et al., 1993; Kang and Schuman, 1995; Stoop and Poo, 1995), and BDNF knockout mice exhibit impaired long-term potentiation (Korte et al.,
There is no evidence to date examining the effect of GDNF on synaptic function. Given that neurotrophins can regulate not only neuronal viability, but also modulate synaptic transmission, it is possible that glia may participate in determining neuronal synaptic connectivity through the release of neurotrophins. The first paper in this dissertation deals with the characterization of synapse formation of neurons co-cultured with astrocytes. It establishes the time course of appearance of several vital components of the synaptic machinery, such as calcium channels, presynaptic proteins, and the functional appearance of synaptic transmission. In addition to providing information on synaptic components that may normally regulate synapse formation, this paper establishes a baseline for future studies aimed at determining the effect of astrocytes on promoting synaptogenesis.

**Glia and pathology**

The active signaling properties of astrocytes outlined above suggest that a perturbation to astrocytes could have profound effects on neuronal function. Astrocytic swelling is observed under a variety of pathological states such as ischemia (Garcia et al., 1977; Jenkins et al., 1982), brain trauma (Castejon, 1980), hypoglycemia and status epilepticus (Siesjo, 1981), prolonged hypoxia (Yu et al., 1972), acute hypoxia with hypercapnia (Bakay and Lee, 1968) hepatic encephalopathy (Norenberg, 1981) traumatic brain edema (Gerschenfeld et al., 1959), experimental (Barron et al., 1988) and closed head injury (Bullock et al., 1991). Since astrocyte swelling has been shown to cause the release of several excitatory amino acids (EAA) including glutamate, aspartate, and taurine (Kimelberg
et al., 1990) and elevated levels of EAA are thought to partially mediate neuronal
degeneration (Olney, 1969; Simon et al., 1984; Wieloch, 1985; Choi, 1988; Faden et al.,
1989), it has been suggested astrocytes may serve as one of the sources of neurotoxic EAAs
(Kimelberg et al., 1990). In further support of this hypothesis, inhibition of swelling-induced
EAA release by the anion transport inhibitor L644,711 has been shown to significantly
improve recovery in an experimental closed head injury (Cragoe et al., 1986; Kimelberg et
al., 1987). Additionally, an elegant study by Hochman et al (1995) recently demonstrated the
blockade of epilepsy using furosemide, a chloride cotransport inhibitor. The authors suggest
that furosemide action was related to astrocyte volume regulation, and the subsequent effect
on the extracellular space. It is worthwhile noting that furosemide also inhibits astrocyte-neuron signaling (Parpura et al., 1994), suggesting that another potential mechanism of
epilepsy blockade could be through the inhibition of the astrocyte-neuron signaling pathway
described earlier.

In addition to releasing EAAs, swollen astrocytes also release other neurotoxins such
as quinolinic acid which could contribute to excitotoxin induced injuries (Whetsell et al.,
1988). Quinolinic acid has been shown to cause neuronal excitation through an interaction
with the NMDA receptor. The rate limiting enzyme in quinolinic acid synthesis is 3-
hydroxyanthranilate oxygenase (3-HAO), and has been shown to be localized primarily in
astrocytes, and elevated in brain regions that show neuronal degeneration in Huntington’s
disease (Schwarcz et al., 1988). Additionally, the enzyme that degrades quinolinic acid,
quinolinic acid phosphoribosyltransferase (QPRT), is present primarily in astrocytes, but is
also found in neurons (Du et al., 1990). Thus astrocytes contain the necessary enzymes to regulate quinolinic acid concentrations, and astrocytic swelling could lead to NMDA-dependent neuronal degeneration through the inappropriate release of quinolinic acid.

Astrocytes are capable of maintaining neurotransmitter uptake mechanisms and pH regulation processes due to the presence of a strongly hyperpolarized membrane potential, and a large inward sodium gradient providing an energy source to drive the appropriate pumps. Astrocyte swelling leads to membrane depolarization and the collapse of this sodium gradient. This collapse could result in the failure or compromise of many basal astrocyte homeostatic mechanisms, such as maintenance of extracellular levels of ions and neurotransmitters, which may lead ultimately to aberrant neuronal function.

**Physiology of astrocytes during swelling**

Given the potential significance of astrocyte swelling in pathological states, a detailed understanding of the cellular events that occur during volume changes in astrocytes is necessary. In the presence of reduced osmolarity solutions astrocytes swell, and then recover their volume to near normal levels in the continued presence of anisosmotic conditions by a process known as regulatory volume decrease (RVD). The following section outlines some of the known events that occur during astrocyte swelling and the subsequent RVD.
Astrocyte swelling alters membrane conductances

In response to hyposmotic saline astrocytes depolarize, which is thought to occur due to the opening of stretch activated non-specific cation channels (Kimelberg and O'Connor, 1988). Recently, mechanosensitive ion channels that are sensitive to stretch (stretch-activated) and channels that are stretch and membrane curvature sensitive (curvature-sensitive stretch-activated) were characterized in rat astrocytes (Bowman et al., 1992). Additionally, large conductance anion channels (~ 300 pS), with low ion selectivity and capable of passing amino acids such as aspartate and glutamate, have also been shown to open during swelling (Jalonen, 1993; Pasantes-Morales et al., 1994; Pasantes-Morales et al., 1994). The opening of such channels subsequently permits calcium influx either through these channels directly or through the resulting depolarization leading to the activation of voltage-activated calcium channels. The rise in intracellular calcium subsequently triggers calcium-activated potassium and perhaps calcium-activated chloride channels. It has been proposed that it is the net efflux of potassium and chloride and osmotically obligated water that permits the return of astrocytes to near initial volumes (O'Connor and Kimelberg, 1993). In contrast, release of amino acids is not thought to be involved in RVD, since removal of extracellular calcium inhibits RVD, but does not affect amino acid efflux. Therefore it has been suggested that potassium and chloride efflux are the main determinants of RVD, while amino acid efflux is merely a consequence of hyposmotic-induced swelling (O'Connor and Kimelberg, 1993).
It is interesting to note that the anion transport inhibitor L-644,711 which was mentioned previously as an effective therapeutic tool which improved the outcome of subjects exposed to experimental head trauma (Cragoe et al., 1986; Kimelberg et al., 1987; Barron et al., 1988), also inhibits the large conductance anion channel (Jalonen, 1993) and blocks the hyposmotic-induced release of glutamate and taurine (Kimelberg et al., 1990). This suggests that perhaps the hyposmotic-induced release of glutamate is mediated through this large conductance anion channel. It is also worthwhile to note that L-644,711 inhibits astrocyte swelling in the in vivo model of brain trauma (Barron et al., 1988), yet has no effect on hyposmotic-induced cell swelling in cultured monolayers of astrocytes (Kimelberg et al., 1990), raising the possibility that the improved outcome in the in vivo model may also have been due to a reduction in swelling, in addition to an inhibition of glutamate release.

**Cytoskeleton involvement**

Given that swelling typically induces morphological changes, it is reasonable to suggest that the cytoskeleton may play a role in volume regulation of astrocytes. Altered volume regulation in a number of cell lines has been shown after the F-actin cytoskeleton has been disrupted using such agents as cytochalasin B. For example, the extent of hyposmotic-induced swelling following cytoskeletal disruption can be unaffected in epithelial or Ehrlich ascites tumor cells (Foskett and Spring, 1985; Cornet et al., 1993), potentiated in HSG cells (Fatherazi et al., 1994), or inhibited in PC12 cells (Cornet et al., 1993). Furthermore, RVD is sensitive to cytoskeletal disruption (Foskett and Spring, 1985; Cornet et al., 1993; Cornet et
al., 1993; Fatherazi et al., 1994), though there are some instances where RVD is insensitive to cytochalasin (Edmonds and Koenig, 1990).

How might the cytoskeleton regulate volume dynamics? One possibility is that cell volume changes are normally inhibited by cytoskeletal components creating a physical barrier to volume increases. In support of this hypothesis a transient breakdown of the actin network has been observed during hyposmotic stress (Mills et al., 1994). A second possibility is that membrane proteins such as ion channels and transporters are linked to cytoskeletal elements (Mills et al., 1994). Thus during swelling these channels may be activated by virtue of their cytoskeletal tether due to changes in membrane tension. One must realize, however, that increases in membrane tension are not necessarily coincident with increases in cell volume, since many cells possess the ability to unfold membrane invaginations to prevent cell lysis (Hoffmann and Kolb, 1991). A discussion of the elastic properties of membranes suggest that membranes undergo lysis if membrane area increases of greater than 2 to 4 % are achieved through stretching (Hoffmann and Kolb, 1991). Despite the wealth of knowledge regarding the involvement of the cytoskeleton in volume regulation in other cell lines, the role of the cytoskeleton in astrocyte volume dynamics is ill-defined. The second paper in this dissertation addresses the role of the F-actin cytoskeleton in regulating volume responses of astrocytes.
Membrane trafficking

In addition to the membrane dynamics discussed in the previous section, endo- and exocytotic membrane trafficking also plays a pivotal role in the volume regulation of a number of cells. The regulated exocytotic insertion and endocytotic removal of specific transport proteins provides an additional mechanism to control solute flow across plasma membranes. For example, a number of transporters and pumps such as GLUT-4, the Na\(^+\)-H\(^+\) antiporter, K\(^+\) -dependent H\(^+\) pump, and a number of channels such as chloride, calcium, and the water channel have all been shown to be regulated through alterations in membrane trafficking (Bradbury and Bridges, 1994). Furthermore, the cytoskeleton has been implicated as a regulatory element that may prevent the fusion of vesicles with the plasma membrane upon stimulation. For example, the insertion of the ADH sensitive water channel has been shown to be associated with cytoskeletal rearrangement. A decrease in F-actin was observed upon stimulation of the ADH-sensitive water channel, and washout of ADH led to the subsequent return to control levels (Ding et al., 1991). Furthermore, stabilization of F-actin inhibited ADH stimulated water transport. Finally, confocal microscopy revealed a dissolution of F-actin at the water transporting apical, but not lateral, membranes of these bladder epithelial cells (Holmgren et al., 1992).

Another consequence of membrane trafficking is alterations in plasma membrane area. As mentioned previously, most bilayers will lyse if their area is forced to increase by greater than 2 to 4%. Therefore regulated membrane trafficking may provide a route to supply additional membrane to the plasma membrane under conditions such as cell swelling,
where cell lysis may occur. Most of the information regarding membrane traffic regulated solute transport stems from cells that face extreme osmotic conditions such as bladder epithelia. It is not known to what extent this type of transport regulation is used in other cells such as astrocytes. The second paper in this dissertation addresses the involvement of membrane trafficking processes in the volume regulation of astrocytes.

The elucidation of the cellular events during volume regulation of astrocytes will provide valuable insight into physiological and pathophysiological conditions. Since disrupted volume regulation may underly several disease states and their complications, a detailed understanding of volume regulation in astrocytes may provide new therapeutic options for conditions of brain trauma.

Dissertation organization

This dissertation is composed of two papers. A general introduction which reviews the entire body of work precedes the papers, and a general summary is given after both papers. A separate general reference section lists the references cited in the general introduction and general summary. Paper 1, authored by T.A. Basarsky, V. Parpura, and P.G. Haydon has been published in *The Journal of Neuroscience* and was performed in equal collaboration with Vladimir Parpura. Paper 2 has been submitted by T.A. Basarsky, and P.G. Haydon to *The Journal of Neuroscience*. I performed all experiments included in this paper.
HIPPOCAMPAL SYNAPOGENESIS IN CELL CULTURE:
DEVELOPMENTAL TIME COURSE OF SYNAPSE FORMATION,
CALCIUM INFLUX, AND SYNAPTIC PROTEIN DISTRIBUTION


Abstract

The formation of chemical synapses between hippocampal neurons in primary cell culture was studied using electrophysiology, calcium imaging and immunocytochemical approaches. Inhibitory and excitatory synapses formed within 12 days in cell culture (DIC) that were sensitive to the N-type calcium channel blocker, omega-conotoxin GVIA (ω-CgTx). At 4 DIC, immature connections were present in which spontaneous, but rarely evoked, synaptic currents were detected. At both 4 and 12 DIC, the synaptic proteins rab3a, synapsin I, and synaptotagmin were present in hippocampal neurons, but the subcellular distribution changed from one in which immunoreactivity was initially distributed within soma and neurites to a punctate varicose appearance. Correlated with the transformation from immature to mature synaptic states, was the onset of ω-CgTx-sensitive calcium influx. Taken together these data suggest that the expression of functional ω-CgTx-sensitive calcium influx is temporally coincident with synapse formation, and that during the maturation of the synapse there is a redistribution of synaptic proteins.
Introduction

Much of our knowledge about synaptogenesis has been gained from studies of neuromuscular development where agrin and aria, synthesized in motoneurons, regulates the expression and aggregation of postsynaptic acetylcholine receptors (Falls et al., 1990; McMahan, 1990). For the development of fast synaptic transmission it is also necessary that presynaptic macromolecules are synthesized, transported to the presynaptic terminal and are arranged with the appropriate mutual spatial relation. For example, it is necessary for the calcium channel to become localized in close proximity to the secretory vesicle to supply calcium in a sufficiently high concentration to stimulate secretion (Simon and Llinas, 1985; Zucker and Fogelson, 1986). It is also unclear whether different calcium channels are subject to the same regulation during synaptogenesis. For example, are L-type channels that do not stimulate transmitter release subject to similar regulation as N-type channels that do supply calcium to stimulate synaptic transmission (Takahashi and Momiyama, 1993)? Little information is available concerning the regulation of these presynaptic developmental events.

Studies of nerve-muscle synapse formation have shown that the synaptic target supplies retrograde signals which control the appropriate development of presynaptic machinery. Muscle cells manipulated into contact with growth cones of neurons derived from the *Xenopus* neural tube cause a local increase in resting calcium level (Dai and Peng, 1993), a rapid induction of secretion (Xie and Poo, 1986), and a local reorganization of the growth cone such that the quantal content of evoked synaptic transmission is augmented (Sun and Poo, 1987). In *Helisoma*, muscle fibers activate presynaptic protein kinase A which causes an elevation of the resting calcium level of neuronal growth cones, and an enhancement of the presynaptic calcium influx during action potentials (Funte and Haydon, 1993; Zoran et al., 1993). After many hours of contact, the calcium-sensitivity of the
secretory apparatus is then increased (Zoran et al., 1991). These regulatory events lead to the effective coupling of presynaptic action potentials to secretion.

In contrast to studies of neuromuscular synapse formation, there is a paucity of studies of the regulation of synaptogenesis between central neurons. Central neurons can contain many types of calcium channels, yet only a restricted sub-set stimulates transmitter release (Takahashi and Momiyama, 1993). A priori one would expect a differential regulation of these different sub-sets of calcium channels during synaptogenesis. Furthermore, it is unclear when specific calcium channels develop in relation to the onset of synapse formation, when and where synaptic associated proteins are expressed and when excitation becomes coupled to secretion. Using dissociated hippocampal cell cultures, the goal of this study is to elucidate the temporal relation between the appearance of functional synaptic transmission, L- & N-type calcium channels, and synaptic protein distribution. This study demonstrates that while many components of the presynaptic neuron are expressed early in cell culture the development of ω-CgTx-sensitive N-type, but not nifedipine sensitive L-type calcium influx is temporally correlated with the delayed detection of evoked synaptic transmission. Some of this data has appeared in preliminary form (Basarsky et al., 1992).

Methods

Cell Culture

Hippocampi were dissected from 1-4 day-old Sprague-Dawley rats. Tissue was incubated for 1 hour at 37 °C in Ca\(^{2+}\)- and Mg\(^{2+}\)-free Earle's balanced salt solution (EBSS, pH 7.35; Gibco) containing papain (20 U/ml; Sigma), Heps (10 mM), L-cysteine (0.2 mg/ml), glucose (20 mM), penicillin (100 U/ml) and streptomycin (100 mg/ml). Tissue was washed once with fresh EBSS and then placed in EBSS (pH 7.35) containing Heps (10 mM)
and trypsin inhibitor (10 mg/ml, type II-O; Sigma) for 5 minutes. After being rinsed, hippocampi were mechanically dispersed by triturating through a fire-polished glass pipette. Cells were plated into poly-L-lysine (1 mg/ml, MW 100,000; Sigma) -coated glass-bottomed dishes. Cultures were maintained at 37 °C in a humidified 5% CO2/95% air atmosphere. Culture medium consisted of Eagle's minimum essential medium (Earle's salts; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma) and containing 40 mM glucose, 2 mM L-glutamine, 1 mM pyruvate, 14 mM NaHCO3, penicillin 100 U/ml and streptomycin 100 mg/ml (pH 7.35). The dissociation procedure was modified from previously described procedures (Leifer et al., 1984; Huttner and Baughman, 1986; Mattson and Kater, 1989). To suppress proliferation of non-neuronal cells arabinosylcytosine (ARA-C, 5 µM) was added after 2-3 days in culture. Cultures were fed once a week by exchanging 30% of the medium with fresh medium.

**Electrophysiology**

Conventional dual whole-cell recording techniques (Hamill et al., 1981) were employed to stimulate and record synaptic currents from cultured hippocampal neurons that had been grown in cell culture for 1 to 21 days. All experiments were performed at room temperature (22-24 °C). Normal external saline contained (in mM): NaCl 140, HEPES 10, KCl 5, CaCl2 2, MgCl2 2, pH 7.35 with NaOH. Pipette solutions contained (in mM): K gluconate 140, EGTA 10, MgATP 4, GTP 0.1, HEPES 10 pH 7.35 KOH. The osmolarity of the external saline was adjusted with sucrose to be 10 mOsm higher than the internal pipette solution. Pipettes fabricated from 1.5 mm O.D. borosilicate glass had d.c. resistances of 3 - 7 MΩ. Postsynaptic cells were voltage clamped at a range of -80 to -30 mV to test for the presence of excitatory and inhibitory synaptic transmission. Presynaptic cells were held in current clamp at -60 mV. The presence of spontaneous synaptic events was assayed at
holding potentials of -70 and -40 mV in the first 7 minutes after whole-cell access. Evoked connections between neuronal pairs was measured by current injection to induce action potentials in the putative presynaptic cell and monitoring postsynaptic responses in voltage clamped postsynaptic neurons. Evoked connections were considered monosynaptic if the synaptic delay was less than 5 ms from the peak of the presynaptic action potential to the onset of the postsynaptic response and the synaptic latency remained unchanged with repetitive stimulation. This was confirmed in some experiments by elevating external divalent cations by 2 mM to reduce neuronal excitability. All drugs were administered via bath application.

Pharmacology test protocols consisted of 20 action potentials stimulated at 2 second intervals. To reduce variability in synaptic responses, the first response was discarded, and the remaining nineteen episodes were averaged.

Data was acquired from 2 Axopatch 1-C amplifiers using pCLAMP software (v 5.51, Axon Instruments) and Labmaster hardware (TL-1, Scientific Solutions) after filtering at 1 kHz. Records were also digitized and stored on VCR tape (Vetter PCM/VCR).

CNQX (6-Cyano-7-nitroquinoxaline-2,3-dione) was purchased from Tocris Neuramin, ω-CgTx GVIA, picrotoxin and TTX (tetradotoxin) were from Sigma.

Immunocytochemistry

Presence of synapse specific proteins was determined by indirect immunocytochemistry procedures. Primary monoclonal antibodies directed against synaptotagmin (CL 41.1) and rab3a (CL 42.2) were generously provided by R. Jahn and a polyclonal antibody (CG-454/455) directed against synapsin I was generously supplied by A. Czernik. The antibodies were used at the following dilutions: synaptotagmin (1:250), rab3a (1:50, and 1:500), and synapsin I (1:250). Cells were fixed with 4% paraformaldehyde in
PBS at room temperature for 30 minutes. After permeabilization with Triton-X-100 and incubation with BSA (5%) and goat serum (5%) to prevent non-specific binding, primary antibodies were added and the cells were incubated overnight at 4 °C. After washout of the primary antibodies, goat anti-mouse and goat anti-rabbit rhodamine conjugated antibodies (1:200, Fisher) were added and the preparation was incubated for 1 hour at room temperature. In some preparations, after immunochemical staining, the cells were washed with PBS containing 0.1 % NaBH₄ for 30 minutes at room temperature to reduce autofluorescence (Beisker et al., 1987).

Visualization was accomplished using two separate imaging systems. For most experiments, conventional epifluorescence microscopy was utilized using a silicon intensified target (SIT) camera driven by Image-1/AT software (v. 4.0, Universal Imaging Corporation).

In some cases, to gain enhanced spatial resolution, a Noran Odyssey real-time laser scanning confocal microscope equipped with Image-1/AT software (v. 4.0) was used. Optical sections were collected through a 60 X plan-apochromatic objective which gave a single section optical thickness of 0.7 μm (full-width at half-maximum, FWHM). The initial and final optical planes were specified to ensure that all immunoreactive areas were captured.

**Calcium Imaging**

Calcium levels were estimated using fura-2/AM and ratiometric imaging techniques (Gryniewicz et al., 1985). All measurements were made from somatic regions. Cells were loaded with fura-2/AM (2 μM) for 40 minutes at 37°C. One μl of 25%(w/w) of Pluronic F-127 was mixed per 1 ml of fura-2/AM loading solution (2 μM) to aid solubilization of the ester into aqueous medium. After washing, fura-2/AM was de-esterified for 40 minutes at 37°C. All experiments took place at 22-24°C. Normal external saline was (in mM) NaCl
140, KCl 5, MgCl$_2$ 2, CaCl$_2$ 2 and HEPES 10 (pH 7.35). In zero calcium saline, Ca$^{2+}$ was replaced with Mg$^{2+}$ and 2 mM EGTA was added.

To test for the presence of voltage-sensitive calcium influx, neurons were incubated in TTX (1 μM) to prevent spontaneous action potentials, and were depolarized using elevated potassium saline that was applied by a 30 second pressure ejection pulse from a puffer pipette (~ 2-3 μm opening; 5-8 psi). In this saline, KCl (50 mM) replaced NaCl.

Image processing and analysis was performed using either QFM ratiometric software and a QX-7 processor (Quantex Corp.) or Image1 / Fluor (v 1.63g, Universal Imaging Corporation, CA). Background subtracted ratio images (340/380 nm or 350/380) were used to calculate the [Ca$^{2+}$]$_i$ according to Equation 5 of (Grynkiewicz et al., 1985).

Calibration of fura-2 was performed in situ (Thomas and Delaville, 1991). Briefly, cells were permeabilized with the calcium ionophore 4-bromo-A23187 (10 μM, Molecular Probes) in the presence of 4 mM calcium to obtain R$_{max}$. or 0 Ca$^{2+}$/10 mM EGTA to obtain R$_{min}$. Fura-2 fluorescence was then quenched with Mn$^{2+}$ (20 mM) to acquire background fluorescence levels for R$_{min}$ and R$_{max}$. In some cases, at the end of an experiment, digitonin (40 μM) was added to measure the extent of compartmentalization of Fura-2. In all cases where this was done, compartmentalization was not significant. R$_{min}$ was 0.17 to 0.21, R$_{max}$ was 2.86 to 3.90, and Fo/Fs was 3.44 to 10.57. Similar calibration values were obtained when parallel calibrations were performed using fura-2 penta-potassium salt in vitro. There were no differences in the calibration values between cells at different times in culture. A K$_d$ of 224 nM as previously reported (Grynkiewicz et al., 1985) was used. For experiments where osmolarity was increased by the addition of 300 mM sucrose, a linear relationship between ionic strength and K$_d$ was assumed. The K$_d$ was adjusted to 990 nM, based on previously reported values (Grynkiewicz et al., 1985).
Cells were kept for further analysis if the calcium accumulation due to the first application of high [K⁺] saline exceeded 50% of the resting calcium level. Using this criteria 97% (583 of 600 tested) of neurons were responsive.

For experiments involving application of calcium channel antagonists, DMSO, or zero calcium, high [K⁺] applications were 12 minutes apart. DMSO (0.1%), nifedipine (5 μM) or zero calcium saline were uniformly bath applied 5 minutes prior to the second application of high [K⁺] while ω-CgTx (100 nM) and cadmium (100 μM) were bath applied at 10 and 1-2 minutes prior to the second high [K⁺] application.

Only cultures which remained viable for at least two weeks, and in which evoked connections were detected in at least 50% of all pairs assayed electrophysiologically on day 12, were included in electrophysiology, calcium imaging, and immunocytochemistry analysis.

**Results**

*Pharmacology of Synaptic Currents*

As an initial step in characterizing synapse formation between cultured hippocampal neurons, the pharmacological properties of action potential evoked and spontaneous synaptic currents were examined in neurons cultured from 1 to 14 days. In normal saline spontaneous inhibitory and excitatory synaptic currents were present. While some of these spontaneous synaptic events likely arise from the generation of action potentials in synaptically connected neurons, the majority of the events are due to spontaneous release of neurotransmitter since they were still detected in the presence of TTX (1 μM). Bath application of 100 μM picrotoxin completely and selectively abolished spontaneous and action potential evoked synaptic inhibitory currents (Fig. 1 A), while bath application of 10 μM CNQX completely inhibited both action potential evoked and spontaneous excitatory synaptic currents (Fig. 1B).
Figure 1. Evoked and spontaneous synaptic connections in hippocampal neurons 12-13 days after plating into culture.

(A) *Upper trace, left.* Recording of spontaneous inhibitory and excitatory synaptic events. *Right,* Inhibitory spontaneous synaptic events are blocked by bath application of 100 μM picrotoxin. *Lower trace* Evoked inhibitory synaptic connections in normal saline (1) or with bath application of 100 μM picrotoxin (2).

(B) *Upper trace, left.* Spontaneous inhibitory and excitatory spontaneous synaptic events in a different cell than A. *Right,* Excitatory spontaneous synaptic events are blocked by bath application of 10 μM CNQX. *Lower trace,* Evoked excitatory connections in normal saline (1) are abolished in the presence of CNQX (2). Evoked responses shown are the average of 19 responses evoked at 2 second intervals. Current and voltage traces are graphically offset for clarity.
This pharmacological profile is consistent with GABA_A mediated inhibitory synaptic responses and CNQX sensitive non-NMDA mediated excitatory synaptic events. The absence of an NMDA component may be attributed to the lack of glycine and the presence of 2 mM MgCl_2 in the bath saline.

**Pharmacology of Presynaptic Calcium Channels**

Since action potentials must admit calcium to trigger transmitter release (Katz, 1969; Mulkey and Zucker, 1991) we examined which calcium channel subtypes mediate calcium influx necessary for neurotransmitter release in 12-13 day old hippocampal neurons. The specific N-type calcium channel antagonist, ω-CgTx, was used to determine the contribution of calcium influx through N-type channels in mediating transmitter release. Bath application of 100 nM ω-CgTx differentially reduced excitatory and inhibitory synaptic currents. In the presence of ω-CgTx, excitatory synaptic currents were inhibited 58 ± 7% (SEM, n=6), while inhibitory synaptic currents were reduced by 87 ± 3% (SEM, n=15) of their initial amplitude (Fig. 2 B, C). Thus N-type calcium channels are required for inhibitory synaptic transmission and contribute calcium for excitatory synaptic transmission. In contrast, the L-type calcium channel antagonist nifedipine (5 μM) did not inhibit either excitatory (-36 ± 21%; n=6) or inhibitory (-9 ± 6%; n=14) synaptic connections (Fig. 2 A). The incomplete inhibition of excitatory synaptic transmission supports other observations that multiple sub-types of calcium channels supply calcium to stimulate transmitter release. (Takahashi and Momiyama, 1993)

**Time Course of Synapse Development**

Having characterized the properties of inhibitory and excitatory synaptic transmission in culture, we examined the temporal pattern of synapse development. Initially, the
Figure 2. Evoked synaptic responses in 12 DIC hippocampal neurons are not reduced by the L-type calcium channel antagonist nifedipine, but are differentially sensitive to the N-type calcium channel antagonist ω-CgTx.

The averaged response at 10 minutes post antagonist addition was compared against the initial averaged response. Postsynaptic excitatory (left) and inhibitory (right) responses in the absence (1) or presence (2) of 5 μM nifedipine (A) or 100 nM ω-CgTx (B) are shown. Current and voltage traces are graphically offset for clarity. Cumulative effect of ω-CgTx data from 6 excitatory and 15 inhibitory synaptic pairs is shown in (C).
development of functional hippocampal synapses was examined using electrophysiological approaches. Cultures were assayed for synaptic events at 2 day intervals ranging from days 2 through 14 after plating into culture (Fig. 3). Spontaneous synaptic events were detected as early as day 2 in culture. By day 4, spontaneous synaptic events were detected in 32 ± 13 % of all cells assayed, while evoked connections were only detected in 11 ± 4 % of all synaptic pairs assayed. On day 12, spontaneous events were detected in 86 ± 17 % of all cells assayed, and evoked connections were detected in 75 ± 22 % of all cell pairs assayed. The temporal pattern of development shows that the appearance of evoked synaptic transmission was delayed in comparison to spontaneous synaptic events (Fig. 3)

Based on this developmental profile we used day 4 and day 12 cultures to represent immature and mature synaptic states. We chose day 4 as it was a time when spontaneous synaptic events were substantially present but evoked connections were still sparse. In contrast, day 12 cultures represented a more synaptically mature state, as determined by the increased number of cells with spontaneous and evoked connections.

Availability of Releasable Neurotransmitter

Considering the paucity of spontaneous synaptic events at day 4 in culture, we determined if synaptic events could be induced in cells that were initially synaptically silent ("silent cells") by the application of high osmolarity saline. Application of high osmolarity saline has been shown to cause an increase in spontaneous transmitter release (Fatt and Katz, 1952; Hubbard et al., 1968; Malgaroli and Tsien, 1992; Manabe et al., 1992). Imaging of intracellular calcium levels during the application of high osmolarity saline did not reveal a significant difference in the emission ratio of Fura-2 due to excitation at 350 and 380 nm (n=23, p > 0.7 Student t-test). However, if the K_d of fura-2 is adjusted for the change in ionic strength that occurs due to the shrinkage of the presynaptic terminal in the presence of
Hippocampal cultures were assayed for the presence of spontaneous and evoked synaptic currents at various times after plating. The percentage of cells where spontaneous or evoked connections could be detected are shown as a function of days in culture. The horizontal bars arbitrarily represent the developmentally immature state at 4 DIC and the more synaptically mature state at 12 DIC. Each bar represents the mean ± SEM from a minimum of 33 cells or 30 synaptic pairs at each time point for spontaneous or evoked synaptic events respectively.
high osmolarity saline (Delaney et al., 1991) a calcium accumulation of 292 ± 27 nM was observed.

In 12 day old cultures, application of high osmolarity saline in the presence of TTX reliably produced a large increase in spontaneous synaptic events (Fig. 4 A). Both inhibitory and excitatory spontaneous events were induced at 12 DIC. In 4 day old cultures, spontaneous synaptic events were absent in 27 out of 38 cells examined. Application of high osmolarity saline to these 27 cells without spontaneous synaptic events resulted in the detection of spontaneous transmitter release in 7 of these cells (Fig. 4 B) with inhibitory (n=3) and excitatory (n=4) events induced. Thus, even in 26% of preparations in which no synaptic events were initially detected, postsynaptic receptors and presynaptic release apparatus were present.

Development of Synapse Specific Proteins

In the functional synapse, the release of neurotransmitter is dependent on the coupling of calcium channels with a calcium responsive secretory apparatus. As a first step in understanding the rate limiting steps in the development of functional synapses we performed immunocytochemistry to ask whether synaptic proteins are expressed in a temporal pattern similar to that of functional synapse formation. A multitude of synapse specific proteins implicated in neurotransmitter release have been characterized (for review see (Sudhof and Jahn, 1991; Jessell and Kandel, 1993)). We determined if the pattern of expression of the synaptic vesicle protein synaptotagmin (p65), and the vesicle associated proteins synapsin Ia, and rab3a were developmentally regulated. In immature cultures, synaptotagmin immunoreactivity was localized primarily to the soma with some staining throughout neuritic processes (Fig. 5 A). In contrast, in mature cultures the staining profile was strikingly different, with the appearance of intense punctate staining along neuritic processes
Figure 4. Application of high osmolarity saline to synaptically mature and immature neurons causes neurotransmitter release.

A. Application of saline enriched with 300 mM sucrose and 1 μM TTX reliably produced a large increase in spontaneous synaptic events in day 12 neurons. A typical increase in excitatory events is shown. B. Left panel shows the absence of spontaneous synaptic events in 71% of 4 day old cells examined. Right panel. Application of high osmolarity saline to these silent cells produced synaptic events in 26% of these cells. An increase in spontaneous inhibitory events is shown, but both excitatory and inhibitory events were induced (see text). High osmolarity saline was delivered by picospritzer driven pressure ejection from a patch pipette for 30 seconds directly onto the neuronal somata. Constant bath perfusion of normal saline with 1 μM TTX was maintained throughout.
Figure 5. Confocal microscopy of synaptotagmin immunoreactivity.

(A) Top view of a composite image reconstructed from 40 optical sections taken at 0.25 μm vertical increments from a 4 day old culture. Intense golgi and diffuse neuritic staining is apparent. (B) A single optical section from the middle of the stack of sections used to construct the topview shown in (A) demonstrating that somatic staining is intracellular. (C) Top view of a composite image reconstructed from 40 optical sections taken at 0.35 μm vertical increments from a 12 day old culture. Distinct punctate staining is present on neurites and the soma. (D) Single optical section from the middle of the stack of sections used to construct the topview in (C) reveals that the somatic staining is not intracellular and is likely due to neurite contact on the soma surface. Calibration bar is 10 μm.
This punctate staining is characteristic of synaptic release sites in hippocampal neurons (Bekkers and Stevens, 1989; Fletcher et al., 1991). The appearance of distinct puncta with maturation was also evident for synapsin I, & rab3a (Fig. 6).

To further investigate the cellular localization of these synaptic proteins, confocal microscopy was utilized. Optical sectioning revealed the presence of synaptotagmin immunoreactivity in the soma in a pattern consistent with its presence in the golgi apparatus and throughout the neurites in immature cultures (73%, n=74) (Fig. 5 B). In contrast, in mature cultures synaptotagmin immunoreactivity was observed only on the surface of the somatic region, consistent with neuritic-somatic contact sites (100%, n=79) (Fig. 5 D). Synaptotagmin immunoreactivity in golgi was not detected in these mature cultures. Neuritic staining was exclusively localized to neuritic contact sites. A similar pattern of staining was seen for rab3a and synapsin I. However, in immature cultures these proteins did not show golgi accumulation, but instead a diffuse pattern throughout the soma and neurites was seen (Fig. 6).

These patterns of immunoreactivity are consistent with previous studies (Fletcher et al., 1991; Matteoli et al., 1991), and in addition we have shown that the immature immunoreactivity profile is temporally correlated with a functionally immature synaptic state of the neurons.

**Development of Voltage Dependent Calcium Influx**

Since the ω-CgTx, but not the nifedipine sensitive calcium channel is involved in evoked synaptic transmission, we have determined the temporal pattern of expression of these two channel types in relation to the formation of functional synaptic connections. As an initial step, the development of high [K+] evoked calcium influx was examined using
Figure 6. Conventional epifluorescent immunoreactivity profiles of rab3a and Synapsin I.

Distribution of rab3a (A) and Synapsin I (B) from a 4 day old culture. Somatic and neuritic staining is evident. Punctate varicose staining of rab3a (C) and Synapsin I (D) is apparent in 12 day old cultures. Calibration bar is 10 μm.
conventional calcium imaging techniques. 50 mM K+ saline was applied to neurons from a
puffer pipette while monitoring the calcium levels of neuronal somata. At all times in
culture, high [K+] caused an enhancement of neuronal calcium levels which required the
presence of calcium in the bathing saline. After greater than 6 days in culture high [K+]
caused a greater change in the calcium level of neurons. For example, at 4 days in culture
voltage-dependent calcium accumulation due to application of high [K+] was 224 ± 10 nM
while by 12 days in culture it was 466 ± 28 nM (Fig. 7).

To further examine the relation between the development of calcium influx and
synaptogenesis we studied the contribution of N-type and L-type calcium channels to calcium
influx. Using synaptically immature (4 DIC) and mature cultures (12 DIC) we determined
the contribution of each channel type to voltage-dependent calcium accumulation (Fig. 8).
Neurons were depolarized with two applications of elevated [K+] separated in time by ten
minutes. Repeated applications of [K+] reliably elevated calcium levels that are due to
calcium influx since omission of calcium from the bathing medium reduced calcium
accumulation (98 ± 1 %, n=30) or cadmium (100 μM), a general calcium channel antagonist,
reduced calcium accumulation by 72 ± 3 %, n=27, p < .01.

In synaptically immature neurons, 76 ± 2 % (n=36) of the calcium influx is mediated
by L-type nifedipine sensitive channels (Fig. 8). The nifedipine carrier DMSO (0.1 %) did
not attenuate calcium influx (n=33). In contrast to nifedipine, addition of α-CgTx at this
same stage in culture did not affect the voltage-dependent calcium accumulation (4 ± 3 %
inhibition, n=29) indicating that L- but not N-type calcium channels are expressed in
neuronal somata in synaptically immature neurons. In synaptically mature neurons when the
calcium accumulation has increased from 224 to 466 nM, nifedipine still attenuated the
calcium accumulation causing a 67 ± 2 % (n=34) reduction in high [K+] evoked calcium
Figure 7. Potassium evoked calcium accumulation increases in a pattern parallel to that of synapse formation.

A statistically significant increase in calcium accumulation was first observed at 10 DIC as compared to 4 DIC. Points represent mean ± SEM. Calcium accumulation was determined from a minimum of 39 cells at each time period. Statistical significance (*) was established at p<0.01 using one-way ANOVA with post hoc Scheffe's test.
100 nM ω-CgTx or 5 μM nifedipine was bath applied to synaptically immature 4 day cultures or synaptically mature 12 day cultures. A, B. Calcium levels in response to high [K⁺] application measured from single 4 or 12 day old cells in the presence or absence of antagonist. C. Summary of inhibition of high [K⁺] evoked calcium accumulation. Inhibition was calculated as the percent inhibition of calcium accumulation during the second application (ΔCa₂) of high [K⁺] compared to the first (ΔCa₁). (Inhibition = 100 x (1-[ΔCa₂/ΔCa₁]) where ΔCa = peak [Ca²⁺]ᵢ subtracted from resting [Ca²⁺]ᵢ). Each culture dish was exposed to only one treatment. The significance of the effects on high [K⁺] evoked calcium accumulation was established at p<0.01(*) using one way ANOVA with post hoc Scheffee's test. Comparison of nifedipine and ω-CgTx-sensitive components at 4 and 12 DIC revealed a significant (p<0.01 φ) increase of the ω-CgTx-sensitive component from 4 DIC to 12 DIC. Bars represent means ± SEM.
accumulation. Additionally, at this time, an \( \omega \)-CgTx-sensitive component to calcium influx has developed. Addition of 100 nM \( \omega \)-CgTx attenuated the \( [K^+]_{\text{evoked}} \) calcium accumulation by 43 ± 4 % (n=39). This indicates that both L- and N-type calcium channels are being functionally expressed in the somata of synaptically mature neurons. Since evoked synaptic connections are sensitive to the N-type calcium channel antagonist \( \omega \)-CgTx, the timing of the development of functional synaptic transmission may reflect the appearance of functional N-type calcium channels.

**Discussion**

The formation of chemical synapses in the nervous system is the result of the complex interplay between a series of developmental events. Neuronal growth cones are responsible for guiding the extension of the axon to the target field where local interactions with potential synaptic targets regulate the development of presynaptic apparatus and the formation of the functional synapse. The interactions between future synaptic partners that subsequently lead to synapse formation are ill defined.

Much of our understanding of presynaptic development has arisen from a few model systems. For example, chick ciliary ganglion neurons and dissociated neurons from *Xenopus* spinal cord can release transmitter before contacting synaptic targets (Young and Poo, 1983). This suggests that presynaptic neurons contain much of the machinery necessary for synaptic transmission in the absence of signals supplied by target cells. Studies on identified neurons of *Helisoma* have similarly shown that one presynaptic neuron, B5, is promiscuous and forms novel synapses with all synaptic targets tested (Haydon and Kater, 1988; Haydon and Zoran, 1989). When contact is made between such cells, functional synaptic interactions are detected within seconds of target contact, indicating that presynaptic machinery has been pre-
synthesized in preparation for synapse formation. In contrast, another identified neuron of *Helisoma*, B19, is selective in synapse formation (Haydon and Kater, 1988; Haydon and Zoran, 1989; Zoran et al., 1989; Zoran et al., 1990; Zoran et al., 1991). Many hours of contact, and protein synthesis are necessary for the presynaptic neuron B19 to gain the ability to couple presynaptic action potentials with neurotransmitter release. Thus, different neurons may use different strategies during synapse formation. While some pre-synthesize presynaptic apparatus others require instructive cues from target cells to complete the developmental expression of presynaptic ion channels and synaptic proteins.

In hippocampal neurons there are several days of neuron-neuron contact before functional synaptic transmission is detected. At 4 days in culture only 11% of neuron pairs have evoked synaptic transmission while by day 12 it has increased to 75%. Correlated with this change in detectability of functional synapses are several changes in the cellular properties of the presynaptic cell. Synaptically immature preparations do express synaptic proteins rab3a, synapsin 1a and synaptotagmin. However, immunocytochemistry indicates that the distribution of these synaptic markers changes from one in which there is significant somatic immunostaining to punctate staining restricted to varicose boutons in mature synaptic cultures. It is likely that this change in distribution of synaptic proteins, and associated vesicles, accounts in part for the slow acquisition of functional synaptic transmission.

Addition of high osmolarity medium to neurons in 4 DIC cultures stimulates the release of transmitter. Because transmission can be detected in response to high osmolarity medium at early times in culture, the development of action potential evoked transmitter release is likely to be limited by the acquisition of presynaptic properties rather than by the presence of postsynaptic receptors. In support of this possibility (Craig et al., 1993) have shown that AMPA sensitive channels are expressed in hippocampal neurons in the absence of
contact by presynaptic axons at early times in culture. The ability to release transmitter, as detected by SSCs and high osmolarity medium indicates that much of the presynaptic apparatus is functional. Even though much of the release apparatus is present, action potentials rarely evoke transmitter release. A number of possibilities may account for these observations. Perhaps a limited pool of vesicles contributes to this low probability of detecting synapses. Additionally the calcium-secretion coupling mechanism or calcium influx through the appropriate calcium channels may not be fully developed at this stage in culture. An increase in neurite outgrowth could also increase the number of putative contact sites, resulting in an increase in the detectability of evoked secretion. Finally, axonal vs dendritic differentiation may be changing in parallel with synapse formation.

Inhibitory and excitatory synapses are sensitive to ω-CgTx, but not nifedipine. While other calcium channel sub-types may also be required for synaptic transmission, we chose to compare the development of one calcium channel involved directly in transmitter release (N-type) with one channel that does not stimulate release (L-type). An examination of the development of nifedipine-sensitive and ω-CgTx-sensitive calcium influx demonstrated that L- and N-type calcium channels are functionally expressed at different times in relation to synaptogenesis. L-type channels, which do not stimulate transmitter release, develop prior to the onset of functional transmission, whereas N-type channels which contribute calcium in transmitter release, appear at a later time in development when synaptic transmission has been established.
References


HYPOSMOTIC-INDUCED SWELLING OF ASTROCYTES:
CYTOSKELETON INVOLVEMENT AND MEMBRANE DYNAMICS

A paper submitted to the *Journal of Neuroscience* by T.A. Basarsky and P.G. Haydon.

Abstract

Astrocyte swelling can lead to the release of neurotransmitters and has been documented during several pathological conditions (reviewed in Kimelberg, 1991). While astrocytes are known to actively regulate cell volume, the roles of the F-actin cytoskeleton and membrane cycling in this process are ill-defined. A combination of atomic force microscopy (AFM) and a fluorescent dye dilution assay were utilized to examine the dynamics of hyposmotic-induced volume changes of astrocytes. Treatment with cytochalasin B, that disrupts the F-actin cytoskeleton, enhanced the hyposmotic-induced volume increase, but the rate of the compensatory regulatory volume decrease (RVD) was unaffected. To determine whether exo- or endocytosis plays a role in cell swelling and RVD we employed the endocytotic tracer FM 1-43, and whole-cell capacitance measurements. Hyposmotic saline neither enhanced cell surface membrane area nor caused altered membrane trafficking consistent with the hypothesis hyposmotic saline causes plasma membrane unfolding followed by membrane reconvolution during RVD. Furthermore, the F-actin based cytoskeleton may function to retard peak volume changes, presumably by restricting
membrane unfolding, but does not function in controlling RVD. In addition to hyposmotic saline, α-latrotoxin stimulates glutamate release from astrocytes (Parpura et al., 1995). We demonstrate that α-latrotoxin does not significantly increase astrocyte volume, indicating that α-latrotoxin does not act through a swelling-activated pathway.

Introduction

Astrocytes represent a significant portion of brain cell volume, and swelling of astrocytes has been shown to occur in a number of pathological conditions such as experimental and closed head injury, acute hypoxia with hypercapnia, prolonged hypoxia, ischemia, hepatic encephalopathy, hypoglycemia and status epilepticus (reviewed in Kimelberg, 1991). Swelling of astrocytes induces the release of glutamate, aspartate, and taurine (Kimelberg et al., 1990). Since elevated extracellular levels of excitatory amino acids (EAA) are thought to partially mediate brain damage in some of these conditions due to the death of neurons through activation of EAA receptors, (Olney, 1969; Simon et al., 1984; Wieloch, 1985; Choi, 1988) an understanding of the regulation of astrocyte swelling is critical.

Given the potential significance of astrocyte swelling in brain pathophysiology, we utilized a number of techniques to examine changes in astrocyte membrane dynamics in response to hyposmotic-induced swelling. In addition to swelling in response to hyposmotic saline, astrocytes also possess the ability to restore cell volume towards initial levels in the face of continuous hyposmotic conditions (Kimelberg and Frangakis, 1985; Olson et al., 1986). This process, termed regulatory volume decrease (RVD), is regulated in part by ion channels and transporters, and has been demonstrated in a number of cell lines (reviewed in Hoffmann and Simonsen, 1989; Hoffmann and Kolb, 1991; Sarkadi and Parker, 1991). With
the significant shape changes that occur during swelling, it is likely that the cytoskeleton also
plays a role in volume regulation. RVD can be slowed or eliminated in a number of cell lines
by disruption of the F-actin cytoskeleton with cytochalasin (Foskett and Spring, 1985; Cornet
et al., 1993; Cornet et al., 1993; Fatherazi et al., 1994), although there are some instances
where RVD is insensitive to cytoskeletal disruption (Edmonds and Koenig, 1990). We have
examined the effect of cytoskeletal disruption on the magnitude of astrocyte volume changes
as well as the rate and extent of RVD during hyposmotic-induced swelling.

To prevent a cell from bursting during swelling, there can either be an increase in
plasma membrane area, or an unfolding of pre-existing plasma membrane that is not fully
distended. In some cell lines, vesicular fusion is induced during swelling as a mechanism to
insert osmo-regulatory transporters into the plasma membrane (Lewis and de Moura, 1982;
Lienhard, 1983; Harris et al., 1986; Brown, 1989a; Brown, 1989b; Bradbury and Bridges,
1994). Therefore we have used a combination of endocytotic tracers and electrophysiology to
monitor membrane trafficking during hyposmotic-induced swelling to determine the role for
membrane trafficking as a control pathway for regulation of volume changes.

Methods

Cell Culture

Enriched type-1 astrocytes were prepared from 1-3 day old Sprague-Dawley rat
cortices as described previously (Parpura et al., 1995). Briefly, cortices were dissected, then
enzymatically (Papain, 20 IU/ml; 60 minutes at 37°C) and mechanically dissociated. Cells
were plated in culture flasks (Corning, #3013) and maintained at 37°C in a 5% CO2/95% air
humidified incubator. Cells were grown to confluency (typically 10-20 days), and then
shaken for 1.5 hours, rinsed, and shaken again for an additional 18 hours in an orbital shaker
at 260 rpm, 37°C. The remaining adherent cells were detached with mild trypsin treatment (0.1 %) for 1-3 minutes. This suspension was spun at 100 x g for 10 minutes, and the pellet resuspended. This suspension was plated onto either untreated 35 mm plastic culture dishes (Falcon, #3001) for electrophysiology, AFM, and some photomultiplier tube (PMT) experiments, or onto poly-L-lysine coated (1 mg/ml, 100,000 MW) square coverslips (22 mm) for the remaining imaging experiments.

Fluorescence Microscopy

BCECF Volume Measurements

Cells were incubated in BCECF/AM (5 μM, Molecular Probes) and 0.02 % pluronic acid for 60 minutes at 37°C in culture media. Cells were rinsed with saline several times and maintained at 37°C to permit dye de-esterification. All experiments were performed at room temperature (22-24 °C). BCECF was excited at the pH-insensitive isosbestic wavelength of 440 nm (440 DF10, Omega Optical) and emission was collected at greater than 520 nm (DM510 dichroic equipped with a BF520 barrier filter, Omega Optical). For camera based acquisitions, images from a Hamamatsu SIT camera were collected by a Matrox-MVP/AT frame grabber board driven by Image-1/AT software (v 4.0, Universal Imaging, PA.). Images were analyzed using Meta-Morph software (Universal Imaging). For all camera based image acquisitions, a Nikon 40x Fluor/Ph objective was used (numerical aperture, n.a.; 1.3). For photometric based acquisitions, Clampex software (v 6.0.2. Axon Instruments, CA) drove a Digidata 1200A interface at 111 kHz to collect PMT emissions from a Thorn EMI PMT, and to trigger a Uniblitz shutter (Vincent and Associates, NY). Data was processed using either Fetchex (v 6.0.2) or AxoScope (v 1.0) software. A combination of microscope objectives were used in the PMT experiments (see Results). For the simultaneous electrophysiology and fluorescence experiments using a PMT, attempts were made to achieve
maximum recording stability by imaging astrocytes grown directly on plastic dishes. Despite the significant reduction of optical efficiency by imaging through a plastic dish, a signal to noise ratio of ~ 10:1 was still typical. To reduce photobleaching, shutter open times were limited to 30 ms for PMT experiments, and camera based image acquisitions were limited to 2 frame averages. Due to the slower response time of SIT camera based systems over PMTs, an additional 80 ms delay was added after the shutter opened before image acquisition began. Images were acquired every 30 s for 20 minutes, and hyposmotic saline was applied for 10 minutes after acquiring a 5 minute baseline. Images were background subtracted before intensity measurements were made. Any cell that demonstrated a drift in fluorescent intensity of greater than 5 % during the baseline period was excluded from all analysis.

The rate of RVD was computed as the change in normalized fluorescence from the peak (t=6.5 min) to the end of hyposmotic treatment (t=15 min) divided by the time (8.5 min). The extent of RVD was computed as the percent decrease in fluorescence intensity between the peak and end of hyposmotic treatment.

**Actin Visualization**

Conventional fluorescence microscopy approaches were used to visualize actin localization using rhodamine-conjugated phalloidin (Molecular Probes). Briefly, cells were treated with cytochalasin B (40 μM, Sigma) for either 0, 20, 40, or 60 minutes, and all cells were fixed with 4 % paraformaldehyde in PBS. Cells were rinsed three times with PBS (5 minutes each) and then incubated with rhodamine phalloidin (1:50) in PBS/Triton (0.25% Triton X-100) for 60 minutes. Following 3 more rinses with PBS, the cells were imaged immediately using the previously described imaging system (Nikon 40x Fluor/Ph objective) with conventional rhodamine optics.
FM 1-43

Astrocytes were exposed to 10 μM FM 1-43 (Molecular Probes) for the various times indicated, then rinsed for 10 minutes in normal saline before viewing. Thirty-two frame averages were acquired from the previously mentioned imaging system using conventional fluorescein optics and a Nikon 40x Fluor/Ph objective.

Electrophysiology

Electrophysiological measurements were made from single astrocytes using conventional patch-clamp recording techniques. Pipettes were fabricated from 1.5 mm O.D. borosilicate glass tubing (F.H. Hare Co.) using a Narishige PP-83 dual stage pipette puller and typically had resistances of 3 - 6 MΩ using the normal internal solution (see Solutions). To reduce electrode capacitance pipettes were coated with dental wax by rotation of the pipette on a strip of dental wax under a dissecting microscope. Data was collected at 167 kHz from an Axopatch 1-C amplifier through a Digidata 1200A interface using pCLAMP (v 6.0.2) and Axoscope (v. 1.0) software (Axon Instruments, CA). Membrane capacitance was computed from the integral of the current transient in response to a 10 mV hyperpolarizing voltage command. The steady state current component of each response was used to measure input resistance, and then subtracted before integrating for the capacitance measurements. Typically, conventional whole-cell recording techniques were used. In some cases, perforated patch techniques were employed using the amphotericin B method (Rae et al., 1991). Upon forming a GΩ seal, perforation was continuously monitored by applying a 10 mV hyperpolarizing voltage pulse every 30 seconds for 15 minutes to ensure that stable access had been achieved. In some cases, at the conclusion of the experiment, gentle suction was applied to achieve the whole-cell configuration and verify the integrity of the perforated patch during the experiment.
In some cases, dual electrophysiology and PMT based dye dilution experiments were performed. This approach was problematic since the perforated patch recordings often ruptured into whole-cell mode upon hyposmotic treatment. This problem was restricted to only the dual electrophysiology and BCECF dye dilution experiments. Perforated patch experiments alone were stable for several solution changes. It appears as if a combination of imaging and perforated patch electrophysiology destabilizes the membrane sufficiently that rupturing into whole-cell mode was very routine upon hyposmotic treatment.

Atomic Force Microscopy

All atomic force microscopy was performed using a TopoMetrix LifeSciences Explorer AFM equipped with a 150 x 150 μm X-Y scanner with a 12 μm Z liquid scan head. Standard A-framed silicon nitride cantilevers with a manufacturers reported spring constant of 0.03 N/m were used for all imaging. During line scan imaging, the set point voltage was continuously adjusted at 2 minute intervals to ensure that minimal forces were being applied to the cell. This was accomplished by adjusting the set point current to the minimum allowable current of our instrumentation, which resulted in a retraction of the tip. Then the photodetector current was measured and the set point was readjusted to a constant value (4 - 5 nA) above the photodetector current. This ensured that a constant force was being applied to the cell. During solution changes the AFM tip was retracted using the above technique and additionally the feedback laser was powered down. This prevented any accidental false contacts onto the astrocyte, which were shown in initial experiments to be extremely damaging to individual cells. For each line scan experiment, a square region of 150 μm was initially sampled, and a single line that intersected the nucleus, cytoplasm and a significant portion of the substrate was selected. Although it was routinely quite easy to distinguish between the substrate and the astrocyte, force-distance curves were performed at the
conclusion of the experiment on nuclear, cytoplasmic, and substrate regions. In all cases, the substrate region demonstrated the typically sharp inflection at the point of tip-substrate contact for a hard substrate, while force curves on cellular regions demonstrated much smoother inflection profiles (Fritz et al., 1994). The substrate region was used to compensate for any tilt in the recording chamber by computing a linear regression of the substrate region for every line and this baseline was subtracted from each line before height measurements were made. All experiments were performed at 22-24°C. We estimate that forces of 1 - 5 nN were applied to the cells during imaging. It has been reported previously that these cells can withstand forces of up to 60 nN without any detrimental effects (Parpura et al., 1993).

Solutions

In all imaging experiments the normal external saline contained (in mM) NaCl, 90; KCl, 5; CaCl₂, 4 MgCl₂ 4; sucrose, 100; HEPES, 10; (pH 7.35). Hyposmotic saline was identical, with the exception that the 100 mM sucrose was removed. For electrophysiology, the internal patch pipette solution typically contained (in mM) K-gluconate, 140; MgATP, 4; Tris-GTP, 0.1; EGTA, 10; HEPES, 10; (pH 7.35). For most perforated patch experiments the internal solution was (in mM) KCl, 55; K₂SO₄, 75; MgCl₂, 8; HEPES, 10; amphotericin B, (pH 7.35). In some cases a cesium based internal solution was used which contained (in mM) Cs-gluconate 117.5; NaCl, 10; MgCl₂, 4; MgATP, 2; Tris-GTP, 0.2; EGTA, 5; HEPES, 15; amphotericin B (pH 7.2). To prepare amphotericin B, a solution containing 6 mg amphotericin B (Sigma) in 100 ml of dimethyl sulfoxide (Sigma) solution was freshly prepared every 4 - 6 hours, and 4 µl of this solution was added to 996 µl of recording solution and vigorously mixed. All salines were filtered using 0.22 µm filters (Corning, #25970).
Statistics

Unless otherwise stated, significance was established at p < 0.05 using a two-way ANOVA followed by a Tukey's post-hoc comparison, and values are given as means ± S.E.M. All statistical tests were performed using GB-STAT / Win (v. 5.3 Dynamic Microsystems, MD).

Results

Fluorescence measurements of astrocyte swelling.

We used a recently developed fluorescence microscopy dye dilution assay (Tauc et al., 1990; Eriksson et al., 1992; Parpura et al., 1995) to monitor astrocyte volume. In this technique the fluorescent emission of a mobile dye loaded into the cellular cytoplasm is measured. When the cell volume increases, the dye is diluted and the fluorescent emission decreases. By exciting the fluorescent dye BCECF at the pH-insensitive isosbestic point of 440 nm, complications due to changes in intracellular pH are removed. When astrocytes are exposed to hyposmotic saline there is a decrease in BCECF emission in the region overlying the nucleus (-9.2 ± 0.48 %, n=54), but an increase in the surrounding cytoplasm (11.7 ± 1.0, n=50). (Fig. 1. A, C, Fig. 5). This is particularly evident when ratios of the BCECF intensity are displayed (Fig. 1, B).

Atomic Force Microscopy of astrocyte swelling

We were concerned that the apparent volume increase of the cytoplasm may be due to an artifact of the dye dilution assay. To directly detect cell volume changes we used AFM to measure astrocyte height. To increase the temporal resolution of AFM, a single line scanning technique was employed. With this approach the atomic force microscope tip is repeatedly
Figure 1. Effect of hyposmotic saline on BCECF fluorescence emission in cultured astrocytes.

(A) Time course of BCECF intensity during hyposmotic saline application. 3 images were selected from a 20 minute time course at the times indicated. Hyposmotic saline was added immediately after the second panel. Note the decrease in fluorescence intensity of the nuclear region and the increase in intensity of the cytoplasmic region in the third panel (t=6.5 min, corresponding to 1.5 minutes in hyposmotic saline). (B) Ratio images of normal saline or hyposmotic saline treated cells. A decrease in fluorescence intensity upon hyposmotic treatment is revealed as a decrease in the ratio (nucleus), while an increase in intensity produces an increase in the ratio (cytoplasm). (C) Average time course of fluorescence changes in a single cell. Scale bar 40 μm.
scanned over the cell nucleus, cytoplasm, and substrate. As shown in Figure 2 A, AFM is capable of detecting changes in astrocyte height in response to hyposmotic saline. Additionally, the height profile of a single astrocyte shows that there is a consistent increase in the height of both the nuclear and cytoplasmic regions of an astrocyte (Fig. 2 B), with little evidence of lateral expansion or retraction. In 5 of 5 cells, hyposmotic saline caused an increase in astrocyte height of 405 ± 52 nm measured over the nuclear region and 236 ± 13 nm measured over the cytoplasmic region.

To address whether swelling preferentially occurs over the nucleus or cytoplasm, we examined height changes in the nuclear or cytoplasmic regions using AFM. A linear regression of the relative height change in response to hyposmotic saline as a function of height in normal saline did not reveal any significant correlation. (m = -0.0167, r = 0.36). This low correlation likely arises due to the high variability on the location of swelling (Fig. 3. A, B). Note that in one cell swelling seemed to occur primarily over the cytoplasmic region (Fig 3 A), but in another cell swelling was much more uniform (Fig 3 B). Therefore this data indicates that both the nuclear and cytoplasmic regions of astrocytes reliably swell in response to hyposmotic saline, and that in general there is not a preferential location of swelling. Furthermore the data demonstrate that the dye dilution assay can be used to measure cell volume changes if the measurements are restricted to the nuclear region of the cell.

_Evaluation of fluorescence volume measurements_

Why is there a paradoxical increase in cytoplasmic fluorescent intensity of BCECF when the cell is in fact swelling? The dye dilution assay relies on the ability to collect fluorescence emissions from an optical section that is thin, relative to the region that is being
scanned over the cell nucleus, cytoplasm, and substrate. As shown in Figure 2 A, AFM is a capable of detecting changes in astrocyte height in response to hyposmotic saline. Additionally, the height profile of a single astrocyte shows that there is a consistent increase in the height of both the nuclear and cytoplasmic regions of an astrocyte (Fig. 2 B), with little evidence of lateral expansion or retraction. In 5 of 5 cells, hyposmotic saline caused an increase in astrocyte height of $405 \pm 52$ nm measured over the nuclear region and $236 \pm 13$ nm measured over the cytoplasmic region.

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Figure 2. Atomic force microscopy of astrocytes during swelling.

(A) Successive linescans over time during hyposmotic application. An increase in height is apparent after hyposmotic treatment. The 3 thick bands are due to solution changes or rinses. Since the laser was powered down for these exchanges, no height measurements were recorded. The alternating thin dark bands at ~2 minute intervals represent the continual adjustment of the cantilever setpoint to compensate for any drift in the system and to ensure that minimal forces were applied to the astrocyte. Note that the Y-axis is time, and the X-axis is distance. (B) Single line scan height profiles before, during, and after hyposmotic treatment.
Figure 3. Localization of height changes during hyposmotic saline treatment measured with the AFM.

(A) and (B) show height measurements from two different cells. The AFM tip was scanned from a region overlying the nucleus (left) to the membrane overlying the cytoplasm (center) and on to the plastic substrate (right). Two height measurements are shown for each cell, one in normal saline, and one in hyposmotic saline. In addition to the height profiles, the percent change in height for each cell is also shown. Note that in (A), there is a large percent change primarily in the cytoplasm, while the height change appears more uniform in (B).
viewed (Fig. 4). If the optical section is too thick relative to the sample, for example at the cytoplasm, then the measured fluorescence is representative of the entire cellular fluorescence, since all of the emitting dye is in the optical section. Consequently cytoplasmic dilution of the dye is undetectable since light is being collected from all of the dye molecules. Thus if the cell swells, but still remains thinner than the optical section, there will not be a change in the amount of dye in the optical section, resulting in no change in fluorescent intensity. Taken further, if the optical section is focused slightly above the region of interest, such as the cytoplasm, one may not be collecting emission from all of the cell when in normal saline. When the cell swells, more of the cytoplasmic region (and more dye) enters the optical section resulting in an increase in fluorescent intensity, despite an overall dilution of dye. This could explain the increase in fluorescence intensity observed in the cytoplasm in response to hyposmotic saline. In contrast, the nuclear region is thicker than the optical section, and therefore there is not any change in the volume that is being optically sampled (Fig 4.). When swelling results in dilution of the dye, fluorescent emission is reduced. In our initial experiments using the dye dilution assay either a Nikon 20x Ph (n.a., 0.4), or a Nikon 20x Fluor (n.a., 0.75) objective were used. When using these objectives, which have relatively thick optical sections, minimal fluorescence changes over the nuclear region were detected. Robust nuclear changes were seen only with either a Nikon 40x Fluor/Ph (n.a., 1.3) or with a Nikon 40x Ph (n.a., 0.55) (data not shown). This supports the idea that a thin optical section and a relatively thick region of interest are necessary for accurate volume measurements using the dye dilution technique.

Prior to using the dye dilution assay to evaluate the role of the cytoskeleton in volume regulation, we determined whether astrocytes exhibit a stereotypical volume response to multiple applications of hyposmotic saline. All measurements were made over the nuclear region. Hyposmotic saline was applied twice to the same dish at a 10 - 15 minute interval
Figure 4. Interpretation of the BCECF fluorescence dilution technique.

The thickness of the cytoplasmic region (left) is less than the thickness of the optical section, thus fluorescence from all of the dye molecules, regardless of concentration, is being collected. Thus there is no net decrease in fluorescence upon cell swelling. When examining the nuclear region (right), the optical section is thinner than the cell. Upon swelling, the concentration of the dye is reduced, but the effective volume that is being optically sampled remains constant, resulting in a decrease in fluorescence intensity.
with a different group of cells imaged with each application. There was not a significant
difference between the peak volume changes in the first and second applications (Fig. 5)
\(n=54, n=27, p > 0.05\). In addition to examining peak volume responses, two parameters of
RVD were determined. There was a significant decrease in the rate (first, \(0.89 \pm 0.08 \% / \) 
min, \(n=54\); second, \(0.50 \pm 0.10 \% / \) min, \(n=27, p < 0.05\)) and extent (first, \(86 \pm 7.4 \%\), \(n=54\); 
second, \(49 \pm 9.7 \%\), \(n=27, p < 0.01\)) of RVD between first and second applications. Since
there is not a uniform response in both applications, we restricted our analysis of cytoskeletal
involvement to the first application of hyposmotic saline.

**Effect of cytoskeletal disruption on astrocyte volume regulation**

With any cellular volume change there are associated changes in cellular shape, and it
follows that the cytoskeleton could play a role in volume regulation. We have used
cytochalasin B to disrupt the astrocyte F-actin cytoskeleton to determine its involvement in
astrocyte volume responses. A time course of actin disruption (Fig. 6 A), demonstrates that
significant disruption of the cytoskeleton does not occur until after 40 minutes of incubation
with 40 \(\mu\)m cytochalasin B. After treating cells with cytochalasin B (60 min) volume
responses of astrocytes to hyposmotic saline were measured using the dye dilution technique.
The average time course of the fluorescence response of control cells (\(n=54\)) and cells treated
with cytochalasin B (\(n=26\)) is shown in Fig. 6 B. Following cytoskeletal disruption
hyposmotic saline caused a significantly greater volume change in astrocytes (Fig 6 C) \(p < 
0.01\).

The cytoskeleton is also important in the regulatory volume decrease that occurs in
response to hyposmotic stress. A comparison of the rate of RVD during the first application
of hyposmotic saline measured at the nucleus reveals no significant difference between
control (\(n=54\)) and cytochalasin B (\(n=26\)) treated cells (Fig. 6 D) \(p > 0.05\). The extent of
Figure 5. Summary of fluorescence changes during multiple applications of hyposmotic saline.

(A) Average time course of nuclear region fluorescence changes during the first or second application of hyposmotic saline. (B) Average peak fluorescence changes in response to hyposmotic saline. Peak fluorescence changes were computed from the difference between intensities at \( t = 6.5 \) min and \( t = 5.0 \) min. Note that hyposmotic saline was added at \( t = 5.0 \) minutes and therefore images at \( t = 6.5 \) minutes correspond to 1.5 minutes in hyposmotic saline. The time interval between first and second applications was 10 - 15 minutes.
Figure 6. Effect of cytochalasin B on astrocyte volume regulation.

(A) Time course of actin disruption with cytochalasin B. Cultures were treated with cytochalasin B (40 μM) for the indicated time periods, and actin localization was visualized with rhodamine phalloidin. Each image is from different dishes and is representative of the majority of the cells within a given dish. (B) Average time course of fluorescence changes with hyposmotic saline in cytochalasin B treated or control cells. (C) Peak fluorescence changes for cytochalasin B and control cells (as described in Figure 5). In some cells, there were focus shifts in the middle of the hyposmotic application. These cells were excluded from the average time course, but were included in all other analysis since measurements at the necessary times were still valid. This explains why the peak measurements in (C) are slightly lower than those presented in (B). (D) The rate of RVD is unaffected by cytochalasin B treatment. (E) The extent of RVD is significantly reduced upon cytochalasin B treatment. See Methods for description of computation. Scale bar 40 μm.
RVD during the first hyposmotic application was, however, significantly reduced after treatment with cytochalasin B (Fig. 6 E) (n=54 in control, n=26 in cytochalasin B, p < 0.05).

Membrane cycling during swelling

Several studies have shown exo- and endocytotic membrane trafficking during hyposmotic-induced volume changes (Lewis and de Moura, 1982; Lienhard, 1983; Harris et al., 1986; Brown, 1989a; Brown, 1989b). We have used a combination of fluorescence microscopy and electrophysiology to examine the dynamics of membrane trafficking in astrocytes during hyposmotic-induced swelling. Betz and colleagues (Betz and Bewick, 1992) have developed a fluorescent probe, FM 1-43, to monitor exo- and endocytosis. We have employed this probe to determine if there are changes in membrane trafficking in response to hyposmotic-induced swelling by examining the uptake of FM 1-43 when cells are treated with hyposmotic saline. Figure 7 demonstrates that there is a high level of basal endocytotic uptake of FM 1-43, even in the absence of hyposmotic saline. If astrocytes undergo significant exocytosis during swelling, one would predict a major phase of endocytosis during RVD. Co-application of hyposmotic saline and FM 1-43 did not reveal any significant change in the uptake of FM 1-43 as compared to basal loading (n=5) (Fig. 7 A). Distinct punctate staining was seen after basal loading without a noticeable increase in the number of punctae following hyposmotic saline loading. There was a slight increase in non-specific plasma membrane and background staining which is due to multiple exposures to FM 1-43. We also examined membrane cycling when hyposmotically swollen astrocytes were returned to normal saline. Astrocytes were exposed to hyposmotic saline for 9 minutes, and then FM 1-43 was applied for a total of two minutes (final 1 minute of hyposmotic saline and 1 minute in the subsequent normal saline rinse) (n=5). As shown in Figure 7 B, there is not a noticeable change in the extent or pattern of staining between basal loading or
Figure 7. Basal and hyposmotic-induced loading of astrocytes with the endocytotic tracer FM 1-43.

(A) FM 1-43 was applied for a 12 minute period in normal saline (left) to reveal basal loading. Subsequently this cell was exposed to FM 1-43 during 10 minutes of hyposmotic saline and 2 minutes of the subsequent normal saline rinse (right). (B) Application of FM 1-43 for 2 minutes in normal saline (left) to reveal basal loading. Subsequently, following a 9 minute exposure to hyposmotic saline, FM 1-43 was applied for 2 minutes (1 minute of hyposmotic saline and 1 minute in the subsequent normal saline rinse) (right). Note the lack of a significant difference in the punctate staining pattern between basal (left) and hyposmotic (right) conditions. Scale bar 20 μm.
hyposmotic-induced loading. The lack of any significant change in FM 1-43 uptake in either protocol suggests that there is little membrane cycling induced by hyposmotic saline.

**Electrophysiological measurements during astrocyte volume changes**

As a second independent method to monitor membrane traffic, perforated patch clamp techniques were utilized to determine if changes in membrane capacitance were induced by hyposmotic treatments. We were concerned that if membrane trafficking was occurring, conventional whole-cell recording techniques may have washed out a necessary component for exocytotic and endocytotic activity and therefore we utilized perforated patch clamp techniques. Hyposmotic saline induced characteristic inward currents (-119 ± 25 pA) and a decrease in input resistance (46 ± 17 %) (n=6) (Kimelberg and O'Connor, 1988). A slight decrease in apparent membrane capacitance (-12 ± 3.8 %, p < 0.05, Wilcoxon Signed-Rank) was also observed. All measurements were made at the peak response during hyposmotic application. We were concerned that the reduction in input resistance decreased the effective length constant of the astrocyte resulting in an artifactual decrease in membrane capacitance due to inadequate space clamp. Therefore experiments were performed with a cesium based internal pipette solution to block potassium channels and increase the effective length constant (n=4). The average input resistance of astrocytes increased from 0.88 ± 0.35 GΩ when using regular internal saline to 3.2 ± 1.3 GΩ when using the cesium internal saline. In this configuration, hyposmotic saline caused a subsequent decrease in input resistance of 33 ± 11%, with little change in membrane current (7.6 ± 3.2 pA), and no significant change in membrane capacitance (5 ± 2.6 %, p > 0.05, Wilcoxon Signed-Rank). Dual perforated patch electrophysiology and fluorescence imaging were performed to verify that astrocytes actually underwent swelling when a patch-pipette was attached to the cell. In all cases where dual recordings were successful (n=3), robust volume changes occurred (data not shown). These
data indicate that hyposmotic saline-induced characteristic cell swelling and conductance changes in astrocytes, with no change in membrane surface area.

**Does an astrocyte neurotransmitter secretagogue induce volume changes?**

After evaluating the utility of the BCECF fluorescence dilution technique to measure astrocyte volume changes, we used this technique to ask whether a known neuronal neurotransmitter secretagogue causes a volume change in astrocytes. Parpura and colleagues (Parpura et al., 1995) recently demonstrated that astrocytes release the neurotransmitter glutamate when perfused with $\alpha$-latrotoxin, the active component of black widow spider venom that induces neurotransmitter release at conventional neuronal synapses (Ceccarelli and Hurlbut, 1980; Hurlbut et al., 1990). We used the fluorescence dilution technique to determine if $\alpha$-latrotoxin exerts its secretagogue effect through a swelling-mediated mechanism. There was no significant change in cell volume when astrocytes were exposed to puffer application of 30 nM $\alpha$-latrotoxin for 15 minutes (- 0.1 ± 1.9 %, n=18, p > 0.05). Additional experiments performed on neuronal cultures confirmed that puffer application of 30 nM $\alpha$-latrotoxin was effective in promoting transmitter release at neuronal synapses, as evident by a large increase in baseline synaptic events (data not shown). This data suggests that $\alpha$-latrotoxin is not exerting its secretagogue effect through a swelling-activated pathway in astrocytes.

**Discussion**

**Fluorescence volume measurements**

Application of hyposmotic saline to cultured astrocytes results in a characteristic volume response composed of an initial rapid volume increase, followed by a subsequent
regulatory volume decrease (RVD). Recently Tauc et al. (1990) developed a fluorescence
dilution technique capable of measuring volume changes in individual cultured cells. This
approach has more recently been applied to cultured astrocytes (Eriksson et al., 1992; Parpura
et al., 1995; Parpura et al., 1995). While we have shown that the BCECF fluorescence
dilution technique is a sensitive assay to measure the cellular volume of single cells, it is
apparent that a sufficiently thick sample is required. While this technique is able to detect
fluorescent changes at both the nuclear and cytoplasmic regions of astrocytes, height
measurements using AFM demonstrate that the apparent volume decrease in the cytoplasm is
an artifact of the fluorescence imaging technique. Presumably, one could detect volume
changes in the cytoplasm if a sufficiently thin optical section could be obtained. Given these
restraints in quantifying localized volume changes based on measurements of BCECF
fluorescence intensity we used the AFM to study height changes in different cellular regions.
AFM indicated swelling in all regions of the astrocyte, and variability between cells in the
locations of maximum height changes, suggesting no preferential location of swelling.

Cytoskeletal involvement in astrocyte volume dynamics

The cytoskeleton has been suggested to be directly involved in volume regulation in
some cell types such as epithelia (Foskett and Spring, 1985), Ehrlich ascites tumor (Cornet et
al., 1993), PC12 (Cornet et al., 1993) and HSG cells (Fatherazi et al., 1994), although its role
in astrocytes has been undefined. Our results demonstrate that the peak volume change
around the nucleus is potentiated by treatment with cytochalasin B, which is comparable to
results in HSG cells (Fatherazi et al., 1994). However, there is evidence in other systems
that cytochalasin B either attenuates (Cornet et al., 1993) or does not alter the hyposmotic-
induced peak volume change (Foskett and Spring, 1985; Cornet et al., 1993). Our results
suggest that one component of the cytoskeleton is involved in regulating the extent of swelling, perhaps by creating a physical limitation to excessive volume changes.

A critical involvement of the actin network in RVD has been demonstrated in some systems (Foskett and Spring, 1985; Cornet et al., 1993; Cornet et al., 1993; Fatherazi et al., 1994), though there are some instances where RVD is insensitive to cytochalasin (Edmonds and Koenig, 1990). However, in astrocytes the role of actin in RVD is ill-defined. Disruption of the F-actin cytoskeleton with cytochalasin B did not affect the rate of RVD, indicating that astrocyte RVD is not regulated by the cytoskeleton. O'Connor and Kimelberg (1993) suggest that swelling of astrocytes in response to hyposmotic media activates non-specific cation swelling-activated channels. They suggest that the subsequent depolarization leads to calcium influx and the activation of calcium-activated potassium and chloride channels resulting in a net efflux of KCl, and a return to isotonic conditions (RVD). Furthermore, the appearance of nonspecific cation currents upon exposure to hyposmotic saline (Kimelberg et al., 1990) or membrane stretch (Bowman et al., 1992) has been demonstrated in astrocytes, though the effect of cytoskeletal disruption on these currents was not explored. Since the rate of RVD was unaffected by cytoskeletal disruption in our experiments, it is unlikely that swelling-activated or stretch-sensitive non-specific cation channels are linked to the F-actin cytoskeleton.

The extent of hyposmotic-induced volume increases might be due to a counterbalance of a volume increase due to swelling, and a volume decrease due to RVD. Therefore the potentiation of the peak volume increase, as observed with cytochalasin B, may actually result from a reduction in the rate of RVD, rather than an enhancement of swelling. This is unlikely because the rate of RVD was unaffected by cytochalasin B. The simplest interpretation of our data is that cytochalasin B removes a cytoskeletal physical limitation that normally retards volume increases.
Membrane Trafficking

There are a number of cell systems where alterations in membrane trafficking occur during swelling. In some cases vesicle insertion into the plasma membrane adds transporters which are activated to regain isosmotic conditions (Lienhard, 1983; Brown, 1989a; Brown, 1989b; Bradbury and Bridges, 1994). Additionally, it is possible that vesicles are inserted to provide additional membrane necessary for cellular expansion, thus preventing the cell from bursting. Using two separate approaches we were unable to detect a change in membrane trafficking induced by hyposmotic saline. This is in agreement with Parsons et al. (1989) who demonstrated a lack of any detectable change in membrane area during hyposmotic-induced swelling of astrocytes based on serial section measurements. They suggest that an unfolding of pre-existing membrane accounts for the additional membrane necessary for swelling, with subsequent reconvolution of this membrane during RVD, rather than an active membrane insertion and retrieval mechanism. Given that some cells show extensive actin breakdown during swelling (Kimelberg, 1991; Cornet et al., 1993; Mills et al., 1994), and that hyposmotic-induced swelling is potentiated after treatment with cytochalasin B, we suggest that the F-actin cytoskeleton regulates this ability to unfold the plasma membrane.

Involvement of swelling in α-latrotoxin induced neurotransmitter release

It has been shown that astrocytes release several transmitters such as glutamate, aspartate, and taurine in response to hyposmotic stress (Kimelberg et al., 1990). Since α-latrotoxin induces the release of glutamate from astrocyte cultures (Parpura et al., 1995), we determined if α-latrotoxin exerted its secretagogue effect through a swelling-induced or volume increase mechanism. We found no evidence for an increase in astrocyte cell volume upon stimulation with α-latrotoxin, supporting the idea that the mode of action of α-
latrotoxin in astrocytes is not initiated through a swelling-activated mechanism. This is in agreement with previous studies (Parpura et al., 1995) where the neuroligand bradykinin induces glutamate release from astrocytes without a concomitant volume increase. Thus while swelling is a stimulus for neurotransmitter release from astrocytes, other stimuli such as α-latrotoxin and bradykinin can induce neurotransmitter release through separate release pathways.

In conclusion, during hyposmotic-induced swelling, increases in cell volume are permitted by the unfolding of plasma membrane, rather than the insertion and retrieval of new membrane through exo- and endocytosis. In astrocytes the F-actin cytoskeleton restrains the swelling of a cell during hyposmotic treatment but does not contribute to active regulatory processes.
References


GENERAL SUMMARY

This dissertation deals with the regulation of synapse formation between cultured hippocampal neurons, and the volume dynamics of astrocytes during hyposmotic-induced swelling.

The developmental time course of synapse formation in cultured hippocampal neurons was examined. After 12 days in culture (DIC) inhibitory and excitatory synapses formed which were sensitive to the N-type calcium channel antagonist ω-conotoxin GVIA (ω-CgTx) while, at 4 DIC, immature connections were present in which spontaneous, but rarely evoked, synaptic currents were detected. A comparison of 4 and 12 DIC neurons revealed the presence of the synaptic proteins rab3a, synapsin I, and synaptotagmin, but the subcellular distribution changed from one in which immunoreactivity was initially distributed within the soma and neurites to a punctate varicose appearance. Correlated with these changes from immature to mature synaptic states was the development of ω-CgTx-sensitive calcium influx. These data suggest that the expression of functional ω-CgTx-sensitive calcium influx is temporally coincident with synapse formation, and that during the maturation of the synapse there is a redistribution of synaptic proteins.

While astrocytes are known to actively regulate cell volume, the roles of the F-actin cytoskeleton and membrane cycling in this process are ill-defined. A combination of scanning probe microscopy and a fluorescent dye dilution assay were utilized to dynamically examine hyposmotic-induced volume changes. Treatment of astrocytes with cytochalasin B to disrupt the F-actin cytoskeleton enhanced the hyposmotic-induced volume increase, but the rate of the compensatory regulatory volume decrease (RVD) was unaffected. Hyposmotic saline neither enhanced cell surface area nor caused altered membrane trafficking. These data are consistent with the hypothesis that astrocytes unfold plasma membrane upon
treatment with hyposmotic saline, and reconvolute membrane during RVD. Furthermore, the F-actin based cytoskeleton may function to retard peak volume changes, presumably by restricting membrane unfolding, but does not function in controlling RVD. In addition to hyposmotic saline, glutamate release from astrocytes can also be stimulated by α-latrotoxin. Application of α-latrotoxin in the presence or absence of external calcium does not cause a significant increase in astrocyte volume, indicating that α-latrotoxin is not exerting its secretagogue effect through a swelling-activated pathway.
GENERAL REFERENCES


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