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How to cite:
Ciccolini, Francesca; Collins, Tony J.; Sudhoelter, Juliana; Lipp, Peter; Berridge, Michael J. and Bootman, Martin D. (2003). Local and global spontaneous calcium events regulate neurite outgrowth and onset of GABAergic phenotype during neural precursor differentiation. The Journal of Neuroscience, 23(1) pp. 103–11.

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Version: Version of Record

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Local and Global Spontaneous Calcium Events Regulate Neurite Outgrowth and Onset of GABAergic Phenotype during Neural Precursor Differentiation

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Neural stem cells can generate in vitro progenitors of the three main cell lineages found in the CNS. The signaling pathways underlying the acquisition of differentiated phenotypes in these cells are poorly understood. Here we tested the hypothesis that Ca2+ signaling controls differentiation of neural precursors. We found low-frequency global and local Ca2+ transients occurring predominantly during early stages of differentiation. Spontaneous Ca2+ signals in individual precursors were not synchronized with Ca2+ transients in surrounding cells. Experimentally induced changes in the frequency of local Ca2+ signals and global Ca2+ rises correlated positively with neurite outgrowth and the onset of GABAergic neurotransmitter phenotype, respectively. NMDA receptor activity was critical for alterations in neuronal morphology but not for the timing of the acquisition of the neurotransmitter phenotype. Thus, spontaneous Ca2+ signals are an intrinsic property of differentiating neurosphere-derived precursors. Their frequency may specify neuronal morphology and acquisition of neurotransmitter phenotype.

Key words: neural precursors; neuronal differentiation; calcium signaling; neurospheres; NMDA receptors; GABA

Introduction

Neurons and glial cells, the main cell types of the mammalian CNS, arise from a common stem cell (for review, see McKay, 1997). Neural stem cells from embryonic or adult brain proliferate in vitro in response to epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2) to produce clusters of cells termed neurospheres (Reynolds and Weiss, 1992; Ciccolini and Svendsen, 1998; Gritti et al., 1999; Ciccolini, 2002). In such in vitro conditions, neural stem cells self-renew and retain the ability to generate multiple cell types over long periods of time (Reynolds and Weiss, 1996). Thus, neurospheres represent an attractive and simplified system for studying how cell multiplicity and cell specification are achieved during mammalian neural development. In addition, neurosphere-derived precursors retain a stable karyotype, and numerous studies have indicated that cells derived from such cultures survive transplantation and, to some extent, can differentiate and form connections with cells of the host brain (Svendsen and Smith, 1999). Therefore, neurosphere cultures may have a clinical relevance representing a possible alternative to fetal tissue in the therapy of neurodegenerative diseases. However, progress toward the clinical use of such a system has been hampered by the limited yield of mature neurons obtained after transplantation of neurosphere-derived neural precursors. This highlights the importance of investigating the mechanisms responsible for neuronal specification in this system.

Although many growth and neurotrophic factors can influence neuronal differentiation, several lines of evidence suggest that Ca2+ represents a key regulator of this process. Changes in the intracellular Ca2+ concentration as a result of spontaneous or signal-regulated events have been shown to initiate specific cellular programs that are important, for example, in differentiation of skeletal and heart muscle (Berridge et al., 1998). Ca2+ is a versatile intracellular messenger that can mediate divergent local and global responses in the same cell (Bootman et al., 2001). It is established that Ca2+ is a key regulator of cell specification in the developing nervous system. For example, in embryonic Xenopus spinal neurons, two types of spontaneous events have been characterized both in vitro and in vivo: fast-rising global Ca2+ spikes with characteristics of action potentials, and slower Ca2+ transients, termed waves, generated in the growth cone region (Gu et al., 1994). Information encoded in the frequency of Ca2+ spikes controls neurotransmitter expression and potassium channel maturation, whereas Ca2+ waves regulate neurite extension (Gu and Spitzer, 1995; Gomez and Spitzer, 1999). Some of these effects of Ca2+ are mediated by the regulation of the phosphorylation of structural proteins (Lauterwiller and Spitzer, 2000); others, like the acquisition of neurotransmitter phenotype, may require gene transcription (Spitzer et al., 2000).

In the present study, we characterized spontaneous Ca2+ signals in differentiating neurosphere-derived precursors and their effects on specific aspects of neuronal morphology and differentiation. These data, together with the well established functions of spontaneous events identified in other experimental systems, suggest that specific Ca2+ signaling patterns play a key role in the differentiation of neural precursor cells.
**Materials and Methods**

*Culture of primary embryonic striatal cells.* Striatum from embryonic day 14 (E14) CD1 albino mouse embryos (plug day = 1.0) (Charles River, Calco, Italy) were dissected and transferred into ice-cold culture medium consisting of DMEM/F12 (1:1) (Invitrogen, San Diego, CA; ICN Biochemicals, Cleveland, OH), glucose (0.6%), glutamine (2 mM), NaHCO3 (3 mM), HEPEs buffer (5 mM) (all from Sigma, St. Louis, MO), and 2% B27 supplement (Invitrogen) (hereafter referred to as growth medium).

D Systems, Minneapolis, MN) were added at a concentration of 20 ng/ml. EGF (Sigma) and FGF-2 (R & D Systems, Minneapolis, MN) were added at a concentration of 20 ng/ml each. Half of the medium was replaced every 4 d with fresh medium containing the same concentration of growth factors.

**Antibodies.** The following antibodies were used at the indicated dilution: mouse monoclonal antibody to nestin (PharMingen; 1:1000 dilution); mouse monoclonal antibody to β tubulin type III (TuJ1) (Sigma; 1:1000); rabbit polyclonal antibody to glial fibrillary acidic protein (GFAP) (Dako; 1:500); rabbit polyclonal antibody to GABA (Sigma; 1:500); mouse monoclonal antibody to galactocerebroside C (Gal-C) [kind gift of Dr. Neil Scolding (Bristol, UK); 1:10]; mouse monoclonal antibody to bromodeoxyuridine (BrdU) (Amer sham; 1:100).

**Differentiation of neurosphere-derived precursors.** Neurospheres were rinsed in growth medium and mechanically triturated through a fire-polished Pasteur pipette. Cells (2 × 10^5) were plated onto poly-L-lysine-coated coverslips in culture medium containing 1% fetal calf serum and FGF-2 (2 ng/ml) and returned to the incubator. Cells were left in differentiating conditions for up to 10 d. The cell phenotype was determined immunocytochemically at different days after plating (DAP) using antibodies to TuJ1, nestin, and GFAP.

**Immunocytochemistry.** Cells were fixed in 4% paraformaldehyde in PBS containing 4% sucrose for 10 min, rinsed several times in PBS, permeabilized in NP40 (0.5% in PBS) for 5 min, and blocked in goat serum (1.5% in PBS) for 30 min; all were performed at room temperature. After fixation, cells were incubated with primary antibodies overnight at 4°C. GABA and BrdU were detected using biotin-conjugated secondary antibodies and Cy3-conjugated streptavidin (Vector Laboratories, Burlingame, CA). All other primary antibodies were detected using goat FITC-conjugated secondary antibodies (Vector). For BrdU staining, BrdU (10 µM) was added to differentiating neural precursors 1 d after plating. Twenty-four hours later, the cells were washed with culture medium and fixed in 100% ice-cold methanol at −20°C. After an additional 20 min, the cells were washed in PBS once, exposed to HCl (2 M) for 20 min at 37°C, and rinsed twice with Borate buffer (0.1 M). Cells were incubated with BrdU antibody for 2 hr at 37°C and then left overnight at 4°C.

**Neurite measurements and statistical analysis.** At DAP 1, differentiating precursors were exposed to KCl (50 mM), low Ca^2+ conditions, or APV or left untreated (see Results). After 24 hr, cells were fixed, and the proportion of neurons in the culture was analyzed by immunocytochemistry using TuJ1 antibodies. The immunostained neurons were imaged with a Bio-Rad 1024 confocal microscope (Bio-Rad, Hemel Hempsted, UK). Photographs of isolated neurons in culture were taken, and the number of branches and the neurite length were measured using NIH Image. The mean and SEs of at least three independent experiments were calculated, and statistical significance tests (ANOVA with post hoc Newman–Keuls) were performed using a statistical package (Graphpad Prism, San Diego, CA).

Ca^2+ imaging. The cells were loaded with Fluo3 by incubation with 2 µM Fluo3-AM for 30 min at room temperature (20–22°C) in an extracellular medium (EM) containing (in mM): 140 NaCl, 3 KCl, 1 MgCl2, 2 CaCl2, 10 HEPES, 10 glucose. After loading with Fluo3-AM, the cells were washed with EM and incubated for a further 20 min to allow complete deesterification of the dye. Ca^2+ imaging was performed with Noran Oz (Noran, Bicester, UK) and UltraView (PerkinElmer Life Sciences, Cambridge, UK) confocal microscopes, using image sizes of 256 × 256 pixels and acquisition rates of 0.5–7.5 frames per second. Fluo3 was excited with a 488 nm line of an argon ion laser, and the emitted light >505 was detected. Ca^2+ concentration was calculated from the Fluo3 fluorescence emission using a self-ratio equation as described previously (Cheng et al., 1993) assuming a K_d of 810 nm (Thomas et al., 2000). The Ca^2+ imaging data in the present study were obtained at 20–22°C.

**Results**

**Spontaneous Ca^2+ events during neural precursor differentiation: global and localized transients**

Neurosphere cultures were obtained by growing in vitro E14 mouse striatal cells in the presence of both EGF and FGF-2. At the E14 stage, the vast majority of neurosphere-derived cells are undifferentiated (Ciccolini and Svendsen, 1998) and express nestin, a cytoskeleton protein found mainly in neural precursors (Lendahl et al., 1990). During plating onto a substrate, neurosphere-derived precursors differentiate into both glial and neuronal cells. Two days after plating, the majority of the cells expressed both nestin and the astrocytic marker GFAP (Fig. 1), whereas the total number of cells did not change (data not shown). At DAP 5, >90% of the cells classified as neurons stained for TuJ1, consistent with the expression of this marker as the cells matured. Categorizing Ca^2+ transients. Ca^2+ transients were classified as global when the fluorescence increased in the whole cell body. Localized Ca^2+ transients refer to spatially restricted Ca^2+ increases with amplitudes that were three times greater than the SD of the baseline noise. Throughout this study, localized Ca^2+ events were analyzed only in Cells on the basis of morphological criteria (see Material and Methods).
Methods for a detailed description). Spontaneous Ca²⁺ signals were observed in both glial and neuronal cells. Two main types of spontaneous Ca²⁺ events were found in differentiating precursors: global Ca²⁺ transients, which engulfed the whole cell body, and localized events, in which Ca²⁺ increases were limited to a subcellular region, usually a process (Fig. 2). Global events were characterized by a sudden (generally lasting <3 min) and conspicuous increase of the intracellular Ca²⁺ concentration (Fig. 2, regions 4 and 6) or irregular and smaller increases of the intracellular Ca²⁺ concentration (often <200 nM). The local Ca²⁺ signals were of variable amplitude and duration. Although they occurred occasionally in cell bodies (Fig. 2, region 2), they were most often observed in cellular processes (Fig. 2, regions 3 and 5). Similar types of spontaneous Ca²⁺ signals were found when cells were imaged either at room temperature or at 37°C (data not shown).

We next characterized global and local Ca²⁺ events at different days after plating to investigate whether their properties were modified during neural precursor differentiation. Neurosphere-derived precursors were imaged for 10 min at room temperature 3 hr after induction of differentiation (DAP 0) and at DAP 1, 2, and 5 (i.e., 24, 48, and 120 hr after differentiation was induced). For each day, we noted the number of spontaneously active cells in addition to the characteristics of the global and local Ca²⁺ signals. We observed that the percentage of cells displaying global Ca²⁺ signals peaked between DAP 1 and 2 and then declined (Fig. 3A). The frequencies of the global signals reduced (Fig. 3B), and their amplitudes showed a significant increase (Fig. 3C) as the cells differentiated.

The local Ca²⁺ signals were analyzed from DAP 1, by which time the cells had already extended processes. As in the case of global Ca²⁺ signals, we found that the percentage of cells displaying local Ca²⁺ transients and the frequency of such events were greater during the first 2 d of development (Fig. 3A,B). The amplitude of local Ca²⁺ signals did not alter significantly. The overall pattern from these data is that both the global and local spontaneous Ca²⁺ signals were observed more frequently at early stages of neural precursor differentiation.

Throughout the study, the local Ca²⁺ events were analyzed...
only in neuronal cells; however, we did examine the activity of global Ca\(^{2+}\) signals in both neuronal and glial cells. The data presented in Figure 3 showing that global Ca\(^{2+}\) signaling declined in all the cells reflect the pooled activities of the glial and neuronal cells. When global events were analyzed separately in glial and neuronal cells, we found that the spontaneous global Ca\(^{2+}\) signals declined almost in parallel in the two cell populations. The only significant difference between these cell types was observed at DAP 5 when 24.7 ± 8.5% of glial cells and 5 ± 5% of neurons were showing spontaneous global calcium signals.

We examined whether changes in the expression of functional channels and receptors involved in generating Ca\(^{2+}\) signals were responsible for the decline in spontaneous events. The response of neuronal and glial cells to depolarization with KCl (60 mM), caffeine (40 mM), and glutamate (100 \(\mu M\)) was tested at various days after induction of differentiation. Depolarization using KCl evoked acute Ca\(^{2+}\) signals in almost all identified neurons and the majority of glial cells at all stages of differentiation (Fig. 4A). The amplitude of the KCl-induced Ca\(^{2+}\) signals in glial cells did not alter appreciably, whereas the response of the neurons increased substantially from DAP 1 to DAP 8, and then rose dramatically at DAP 10. Caffeine, on the other hand, evoked similar amplitude Ca\(^{2+}\) signals at all stages, with little difference between neurons and glial cells (Fig. 4B). However, caffeine was the most unreliable agonist in that the proportion of responsive cells varied considerably between days, and many cells failed to respond (Fig. 4B, c). The effect of glutamate was also rather variable, but there was a clear trend for an increasing number of neurons to be glutamate responsive with time in culture (Fig. 4C). In summary, the neurons seem to become increasingly competent at Ca\(^{2+}\) signaling as differentiation proceeds. Therefore, the loss of spontaneous Ca\(^{2+}\) signals is not attributable to the neurons lacking the ability to generate Ca\(^{2+}\) signals, but rather results from the incidence of spontaneous events declining as the neurons and glial cells mature.

**Modulating the frequency of spontaneous Ca\(^{2+}\) events**

The observation that the frequency and the number of spontaneous events peaked at early stages of neural precursor differentiation, when neurogenesis occurs, prompted us to investigate whether spontaneous Ca\(^{2+}\) signaling plays a role in neuronal specification. To investigate this hypothesis, we manipulated the culture conditions during neural precursor differentiation to interfere with Ca\(^{2+}\) signaling.

The origin of spontaneous Ca\(^{2+}\) events in differentiating neural precursors is still an object of discussion. In amphibian spinal neurons, it has been proposed that some spontaneous events originate from Ca\(^{2+}\) entry through voltage-dependent Ca\(^{2+}\) channels and that Ca\(^{2+}\) release from intracellular stores contributes to their amplitude (Gu et al., 1994). Therefore, we examined the effect of modulating Ca\(^{2+}\) entry on spontaneous Ca\(^{2+}\) events.

After dissociation, neurosphere-derived cells were plated on poly-L-lysine-coated coverslips in differentiating conditions as described above. On DAP 1, the cells were exposed to three different conditions: low extracellular Ca\(^{2+}\) medium (\(\sim 100 \mu M\)), 50 mM KCl, or untreated (control). During the course of this study, the various treatments were always applied 24 hr after the initial plating on coverslips so that there was no interference with the cells before they were fully adhered.

We found that incubation in low extracellular Ca\(^{2+}\) medium (for \(\geq 24\) hr) completely inhibited the occurrence of all types of spontaneous Ca\(^{2+}\) signal from DAP 2 onward (data not shown). In contrast, the control and KCl-treated cells continued to display spontaneous Ca\(^{2+}\) events. The effect of KCl treatment was to enhance the propensity of the cells to show spontaneous local and global Ca\(^{2+}\) signals at all stages of differentiation. For global signals, this was evident most significantly in the frequency of events (Fig. 5A, a) and the amplitude of the signals (Fig. 5A, b) at various stages. The proportion of active cells at DAP 5 was also enhanced by KCl (Fig. 5A, c), but this was caused primarily by the activity of cells with astrocytic morphology (Fig. 5B, C). Although depolarization increased the frequency and amplitude of global Ca\(^{2+}\) signals, it did not prevent the trend that the neurons within the culture became increasingly quiescent with respect to global Ca\(^{2+}\) signals. The number of active neurons declined even in the presence of KCl (Fig. 5B). The sustained effect of KCl on the frequency of events (Fig. 5A, a) was caused by the activity of glial cells.
The enhancement of local, but not global, Ca\(^{2+}\) signals by KCl was dependent on NMDA receptor activity. In DAP 1 cells, application of KCl increased the occurrence of local Ca\(^{2+}\) signals from control values of 3.9 \pm 0.9 to 12.2 \pm 1.6 events per branch during a 10 min recording (data from at least 15 branches). The NMDA receptor antagonist APV applied together with KCl reduced the incidence of local Ca\(^{2+}\) transients back to control levels (5 \pm 0.9 events per branch; data from 18 branches). APV had no effect on the occurrence of global signals. In addition, APV did not alter the generation of local or global Ca\(^{2+}\) signals when applied in the absence of KCl (data not shown). In control conditions, the majority (>75%) of local Ca\(^{2+}\) signals occurred preferentially at the most distal region of a neurite. When cells were exposed to KCl, the Ca\(^{2+}\) signals still originated preferentially in distal neuritic regions, but they were also detectable in more proximal regions because of a greater spreading of the events (data not shown). Therefore, depolarization not only promoted the frequency and amplitude of local Ca\(^{2+}\) signals, but it also increased their spatial extent. The enhanced spreading of the local Ca\(^{2+}\) signals was also dependent on NMDA receptor activity. In control and KCl + APV conditions, the average spreading of local Ca\(^{2+}\) signals was 3.2 \pm 0.3 and 2.7 \pm 0.2 \(\mu\)m, respectively. In contrast, in the presence of KCl alone, the average length of each event was 5.8 \pm 0.4 \(\mu\)m.

The frequency of spontaneous Ca\(^{2+}\) signals correlates with acquisition of neurotransmitter phenotype and neurite length

By differentiating the precursor cells in the conditions described above, we were able to either inhibit (low Ca\(^{2+}\) medium) or enhance (KCl) the spontaneous Ca\(^{2+}\) signals relative to control conditions. We therefore used these experimental conditions to examine the effect of spontaneous Ca\(^{2+}\) signals on the development of neuronal morphology and neurotransmitter phenotype.

Importantly, 24 hr exposure to these treatments did not cause a change in the rate of neural precursor proliferation or survival. To investigate the effect of the various treatments on cell proliferation, we first analyzed the number of dividing cells in control, KCl, and low calcium-treated cultures. At DAP 2, after 24 hr of differentiation in the presence of BrdU, we found similar BrdU incorporation rates across the different conditions (Table 1), indicating that the modification of neuronal phenotypes observed was not caused by a change in neural progenitor proliferation. To rule out the possibility that

KCl had an even greater effect in enhancing the propensity of the neuronal cells to show local Ca\(^{2+}\) signals. This effect was observed in terms of proportion of active cells (Fig. 6A), frequency of local Ca\(^{2+}\) signals (Fig. 6B), and event amplitude (Fig. 6C). These data indicate that multiple aspects of the local and global Ca\(^{2+}\) signals in the cells acquiring neuronal phenotype could be upregulated using KCl.
Differentiation of cells in the low Ca\(^{2+}\) environment occurs at a lower rate than in control cultures. However, increasing the frequency of the global Ca\(^{2+}\) signals accelerated the acquisition of GABAergic phenotype. When control and KCl-treated cultures were analyzed at DAP 5, we found that most of the neurons were GABA immunoreactive in either condition (Fig. 7). However, at DAP 2 the number of GABA-expressing cells increased significantly after KCl treatment, both in the presence and in the absence of APV, and decreased to almost negligible levels after exposure to low Ca\(^{2+}\) medium (Fig. 8). At DAP 2, there was no significant difference in the proportion of neurons (identified by TuJ1 expression) found in control and treated cultures. These data indicate that enhancing global Ca\(^{2+}\) signaling did not influence the type of neurotransmitter phenotype acquired during neural precursor differentiation. However, increasing the frequency of the global Ca\(^{2+}\) signals accelerated the acquisition of GABAergic phenotype.

We next investigated whether Ca\(^{2+}\) signals affected neuronal morphology. Cultures differentiating in the presence of low Ca\(^{2+}\), KCl, KCl + APV, and control conditions were fixed at DAP 2. The neurons were identified by TuJ1 immunostaining. The number of branch points and the lengths of neurites were measured on neurons found in fields of comparable cell density. Differentiation of cells in the low Ca\(^{2+}\) conditions significantly decreased the neurite length, compared with control cultures (Fig. 9A, B). In contrast, the neuronal processes were significantly longer in KCl-treated cultures than in control (Fig. 9). The KCl-induced change in neuronal morphology was mediated by NMDA receptor activation. As illustrated in Figure 9C, the presence of the NMDA receptor blocker APV abolished the effect of KCl on neurite length. A similar pattern was observed when cells were analyzed after a 4-day exposure to the different treatments. The neurites of KCl-treated DAP 5 cells were 260% longer than those in control (Fig. 9).

### Table 1. Effect of KCl (50 mM) and low Ca\(^{2+}\) treatments on cell proliferation and death in the neuronal precursor cultures

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control</th>
<th>KCl 50 mM</th>
<th>Low Ca(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells staining with TuJ1, i.e., proportion of neurons in the culture (%)</td>
<td>3.7 ± 0.9</td>
<td>2.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Cells that incorporated BrdU (%)</td>
<td>20.5 ± 6.2</td>
<td>15 ± 6.3</td>
<td></td>
</tr>
<tr>
<td>Neurons that incorporated BrdU (%)</td>
<td>3.3 ± 1.1</td>
<td>5.2 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Cells staining with DAPI (%)</td>
<td>7.7 ± 1.6</td>
<td>7.6 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>Cells displaying pyknotic nuclei (%)</td>
<td>3.2 ± 1.1</td>
<td>2.9 ± 1.0</td>
<td></td>
</tr>
</tbody>
</table>

The table shows the percentage of cells staining with the markers described in the left-most column or showing pyknotic nuclei. Neuron- and culture-derived precursors were plated onto poly-lysine-coated coverslips. The indicated treatments and BrdU were added 24 hr after plating. Cells were analyzed after an additional 24 hours. The values represent the means ± SEM. The values in parentheses indicate the number of experiments. For each experiment in at least two fields of cells, each containing an average 80 cells, were analyzed. DAPI and Hoechst concentration were used at concentrations of 1 mg/ml. DAPI was added to live cells 5–10 min before scoring the coverslips. Hoechst was added to fixed cells and left for 40–60 min.

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the applied treatments specifically affected the proliferation of neurons, we analyzed the cell division of neurons during the exposure to the different conditions by examining TuJ1 and BrdU co-staining. These data demonstrate that only neurons, we analyzed the cell division of neurons during the applied treatments specifically affected the proliferation of neuronal population had divided during the 24 hr exposure to 50 mM KCl on the characteristics of local Ca\(^{2+}\) signals at different days after induction of differentiation. Event frequency (A) indicates the number of local Ca\(^{2+}\) signals observed during a 10 min recording. The data shown represent the mean ± SEM of at least three independent experiments in which an average of 76 cells were analyzed for each time point.

To investigate the possibility that the different conditions might affect cell death, we analyzed whether cell viability varied across the different conditions by dye (DAPI) exclusion. Additionally, we investigated the number of pyknotic nuclei found in control cultures and in cultures exposed to KCl or low calcium for 24 hr. Both assays revealed no significant change in cell death across the different conditions (Table 1). Together with the observations that neuronal and total cell numbers are not affected by the various treatments, these data demonstrate that the eventual changes in neuronal phenotype observed after interfering with calcium signals are caused by calcium modulation of neuronal differentiation rather than changes in survival or proliferation rates.

When control and KCl-treated cultures were analyzed at DAP 5, we found that most of the neurons were GABA immunoreactive in either condition (Fig. 7). However, at DAP 2 the number of GABA-expressing cells increased significantly after KCl treatment, both in the presence and in the absence of APV, and decreased to almost negligible levels after exposure to low Ca\(^{2+}\) medium (Fig. 8). At DAP 2, there was no significant difference in the proportion of neurons (identified by TuJ1 expression) found in control and treated cultures. These data indicate that enhancing global Ca\(^{2+}\) signaling did not influence the type of neurotransmitter phenotype acquired during neural precursor differentiation. However, increasing the frequency of the global Ca\(^{2+}\) signals accelerated the acquisition of GABAergic phenotype.

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neurite morphology were rapidly initiated in the differentiating cells and maintained as the cells matured.

These data indicate that the frequency of local Ca\(^{2+}\) signals correlated with neurite extension and neurite branching. Changes in neurite morphology, but not acquisition of neurotransmitter phenotype, required NMDA receptor activation.

### Discussion

In this study, we show that spontaneous Ca\(^{2+}\) signaling is an intrinsic characteristic of differentiating neurosphere-derived precursors. We used two different methods of altering the occurrence of spontaneous Ca\(^{2+}\) signals to examine their effect on neuronal development. Our results indicate that Ca\(^{2+}\) signaling is a mediator of neural precursor differentiation and may regulate the maturation of the neuronal phenotype. Because Ca\(^{2+}\) is a ubiquitous second messenger, these observations may indicate that modulation of the intracellular Ca\(^{2+}\) concentration represents a common target for factors and signals that regulate neural precursor differentiation.

Spontaneous Ca\(^{2+}\) oscillations occur in many different neuronal types and at different stages of maturation (Sorimachi et al., 1990; Yuste et al., 1992; Wong et al., 1995; Owens et al., 2000; Liljelund et al., 2000). They have been implicated in the regulation of several aspects of neuronal differentiation such as migration (Komuro and Rakic, 1996), neuronal wiring (Feller et al., 1996), and neuronal differentiation (Gomez et al., 1995; Gu and Spitzer, 1995; Carey and Matsumoto, 1999). Consistent with a role in the acquisition of neuronal phenotype, we found that in our system spontaneous Ca\(^{2+}\) signals were frequent at early stages of differentiation (i.e., DAP 2) (Figs. 2, 6). In addition, interference with Ca\(^{2+}\) signaling at this early stage correlated with changes in the neuronal morphology and neurotransmitter expression (Figs. 7–9). In developing amphibian neurons, these two particular aspects of neuronal differentiation are regulated by the frequency and the site of generation of spontaneous Ca\(^{2+}\) signals: low-frequency spikes control the development of GABA expression and higher-frequency growth cone-localized Ca\(^{2+}\) waves regulate neurite extension (Gu and Spitzer, 1995). Although our results do not allow a functional discrimination between different types of Ca\(^{2+}\) signals, they show that also in neurosphere-derived precursors, Ca\(^{2+}\) transients can be either localized to a specific cellular region or engulf the whole cell body. In addition to their subcellular location, localized and global signals found in neurosphere-derived precursors show differences in frequency and amplitude that parallel the differences observed between waves and spikes in Xenopus spinal cord neurons. Consistent with results obtained in amphibian spinal cord neurons, our analysis shows a positive correlation between GABA expression and frequency of spontaneous events.

One possible mechanism by which Ca\(^{2+}\) transients could affect GABA receptor expression is the promotion of gene transcription. Several lines of evidence support such a hypothesis. There is a correlation between Ca\(^{2+}\) signaling and the level of glutamic acid decarboxylase transcripts (Watt et al., 2000). In cerebellar granule cells, it has been suggested that Ca\(^{2+}\) ions entering through voltage-gated channels modulate expression of GABA receptor \(\delta\) subunit through activation of CaM kinases (Gault and Siegel, 1997). We also found that the activation of voltage-gated channels by KCl was sufficient to promote GABA expression and that NMDA receptor activity was not required (Fig. 8).

Although our results concur with those from Xenopus spinal cord neurons in terms of a correlation between the frequency of Ca\(^{2+}\) signals and the acquisition of GABA expression, the effect...
of spontaneous Ca\textsuperscript{2+} signals on neurite length appears to be different. In our neurosphere-derived precursors, conditions that abolish Ca\textsuperscript{2+} signals (i.e., low Ca\textsuperscript{2+} medium) do not induce an increase in neurite length. Indeed, neurons from cultures exposed to low Ca\textsuperscript{2+} conditions exhibit shorter neurites than cells in control conditions (Fig. 9). Moreover, membrane depolarization, which increases the frequency of Ca\textsuperscript{2+} signals, increases neurite length and the number of branch points.

This different effect of Ca\textsuperscript{2+} in the two systems may be attributable to several factors other than cell type and species differences. In our experiments, neurons were grown on an astrocytic cell layer that may affect their morphology, for example, by releasing factors into the culture medium. Our results show that the NMDA receptor blocker APV decreases neurite length in KCl-treated cultures but not in control conditions (Fig. 9), indicating that NMDA receptor-mediated neurite extension is operating in depolarizing conditions. It is plausible that KCl-induced NMDA receptor activation by promoting the release of glutamate or growth factors into the medium (Blondel et al., 2000). It is also possible that the molecular machinery underlying neurite extension varies during differentiation, and Ca\textsuperscript{2+} signals may have different effects depending on the particular stage of differentiation. Amphibian neurons exhibit a Ca\textsuperscript{2+}-sensitive period during the 12 hr after plating in which low Ca\textsuperscript{2+} conditions cause an increase in neurite length (Holliday et al., 1991). During this time frame, periodic Ca\textsuperscript{2+} elevations inhibit neurite extension by promoting calcineurin activation and affecting the phosphorylation state of cytoskeletal proteins (Lautermilch and Spitzer, 2000). Because in our system neurons were exposed to low Ca\textsuperscript{2+} 24 hr after plating, it is possible that at this stage different mechanisms preside over neurite extension and are affected differently by Ca\textsuperscript{2+} signals. This suggestion is supported by the observation that the effect of membrane depolarization on neurite length was unaffected by inhibitors of calcineurin (our unpublished observations). Furthermore, it is not always easy to predict the consequence of a rise in Ca\textsuperscript{2+} levels on growth cone behavior. Large rises can cause growth cone collapse, whereas more modest signals can either slow or promote neurite outgrowth (Spitzer et al., 2000).

Besides affecting differentiation of neuronal cells, spontaneous Ca\textsuperscript{2+} signals may play a general role in the differentiation of other precursor cells. Indeed, spontaneous Ca\textsuperscript{2+} oscillations were observed in developing myocytes (Ferrari and Spitzer, 1999), non-neuronal neural crest cells (Carey and Matsumoto, 1999), and endodermal cells (Sauer et al., 1998), indicating that Ca\textsuperscript{2+} transients are a hallmark of developing precursors and not an exclusive property of differentiating neurons. Different types of Ca\textsuperscript{2+} signals are present at different stages of differentiation (Owens and Kriegstein, 1998; Owens et al., 2000), and the molecular machinery dictating Ca\textsuperscript{2+} homeostasis changes during neural precursor development (Maric et al., 2000). These observations suggest that Ca\textsuperscript{2+} transients may participate in different signaling events at different stages of development.

In summary, our data show that complex patterns of spontaneous Ca\textsuperscript{2+} signals can be recorded in differentiating neurosphere-derived precursor cells. Despite their heterogeneity, these events can be grouped loosely into local and global Ca\textsuperscript{2+} signals. These two types of Ca\textsuperscript{2+} signal appear to control different aspects of neuronal precursor development, with the NMDA receptor-dependent local Ca\textsuperscript{2+} signals primarily controlling neurite morphology and the global events regulating acquisition of neurotransmitter phenotype. Although such Ca\textsuperscript{2+} signals persist for many days, they become less frequent as the neurons mature. The Ca\textsuperscript{2+} signals in neuronal cells can be enhanced or inhibited by altering the culture conditions to promote or inhibit Ca\textsuperscript{2+} entry. Such modulation of Ca\textsuperscript{2+} signaling has significant effects on the acquisition of neurotransmitter phenotype and cellular morphology at early (i.e., DAP 2) and late (i.e., DAP 5) stages of neurosphere-derived precursor differentiation.

References


Ciccolini F (2001) Identification of two distinct types of multipotent neural...


