Calcium Transient Activity and Neuronal Differentiation of the Neural Crest

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A Dissertation

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CERTIFICATE OF APPROVAL

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TABLE OF CONTENTS

List of figures and tables

Chapter III

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ABSTRACT

I have used the developing neural crest to investigate a mechanism that regulates the neuronal differentiation of multipotent progenitor cells. During development, neural crest stem cells undergo lineage restriction and give rise to a unipotential precursor. These precursors will then differentiate into their determined phenotype, when the appropriate signals are present. My thesis first establishes the validity of studying neuronal differentiation in culture by comparing the phenotype of neural crest neurons differentiated in vitro with the in vivo neuronal fates of the truncal neural crest: dorsal root ganglion and superior cervical ganglion neurons. Once established, I then examine the role of calcium transient activity in regulating neuronal differentiation of cultured neural crest cells.

I first ascertained if neurons differentiated in culture exhibited identical voltage-dependent calcium current (VDCC) profiles to neurons differentiated in vivo. Using whole-cell recordings, I determined the VDCC profiles of acutely dissociated dorsal root ganglion and superior cervical ganglion neurons. I compared these VDCC profiles with those exhibited by neurons differentiated in culture. Depending on the growth conditions, neurons differentiated in culture exhibited VDCC profiles that were identical to either sensory or sympathetic neurons. Once these currents were established, I found that they were not modifiable by the growth factors used in this study. To further support the hypothesis that VDCC profiles are indicative of a specific neuronal phenotype, I showed that growth conditions that supported sensory VDCC profiles also induced the expression of other sensory markers: capsaicin-sensitivity and Brn 3.0-immunoreactivity. The results from this study suggested that sensory and sympathetic neuronal lineages could differentiate in culture.

Next, I determined if calcium transient activity plays a significant role in regulating neuronal differentiation of cultured neural crest cells. First, I monitored intracellular calcium levels in cultured neural crest cells and found that these cells exhibited calcium transient activity during the period of neurogenesis. I then correlated calcium transient activity with several neuronal features and found that only young neurons and a subpopulation of neural crest derived cells exhibited this calcium transient activity. To determine if calcium transient activity played a role in neuronal development, I blocked calcium transient activity in culture and observed a reduction in the number of neurons differentiated. Using lineage-tracing, I found that cells exhibiting calcium transients could give rise to neurons, while inactive cells did not. Based on these results, I concluded that calcium transient activity was required for neuronal differentiation of cultured neural crest.

Finally, I determined the mechanism by which calcium transients are generated. Using the superfusion of various calcium channel blockers while monitoring intracellular calcium levels, I found that the IP_3R was responsible for the regulated release of intracellular calcium during calcium transient events. Extracellular calcium modulated calcium transient activity. Excitable ion channels located in the plasma membrane were not involved, however. Variations in the intracellular IP_3 and Ca^{2+} levels also are factors in regulating calcium transient activity. I concluded from this study that the IP_3R is responsible for the increase in intracellular calcium levels during a calcium transient event and that the pattern of activity is governed by the excitable properties of the receptor.

The results of my thesis support a model that calcium transient activity, in conjunction with extrinsic factors, regulates the neuronal differentiation of cultured neural crest cells.

Introduction

Overview of developmental themes: specification of multipotent cells

During embryogenesis, one cell (the egg) will give rise to a diverse population of cells that make up an organism. In many tissues, this process involves the initial generation of stem cells, which are a self-renewing population of cells that retain the ability to produce all types of progeny in a particular region at any subsequent time during development (Lillien, 1998; Orkin, 1995). For example, the hematopoietic stem cell can give rise to the red blood cells, white blood cells and the immunocompetent cells of the circulatory and immune systems (Gilbert, 1991). Similarly, the central nervous system (CNS) contains stem cells that give rise to neurons, oligodendrocytes, astrocytes, while the neural crest stem cells give rise to the neurons and glia of the peripheral nervous system (PNS) (Gage, 1998; Mujtaba *et al.*, 1998; Stemple and Anderson, 1992; Temple and Alvarez-Buylla, 1999).

Stem cells become restricted in their developmental potential prior to giving rise to a single fate (Lillien, 1998). The general agreement is that stem cells do not commit directly to a single fate, but rather produce multipotent progenitors that are restricted in their developmental potential to a subset of potential phenotypes (Fig. 1). These lineage-restricted progenitor cells will then become specified to a single fate, giving rise to a unipotent precursor cell that can differentiate into the resultant phenotype of that lineage (Fig. 1).

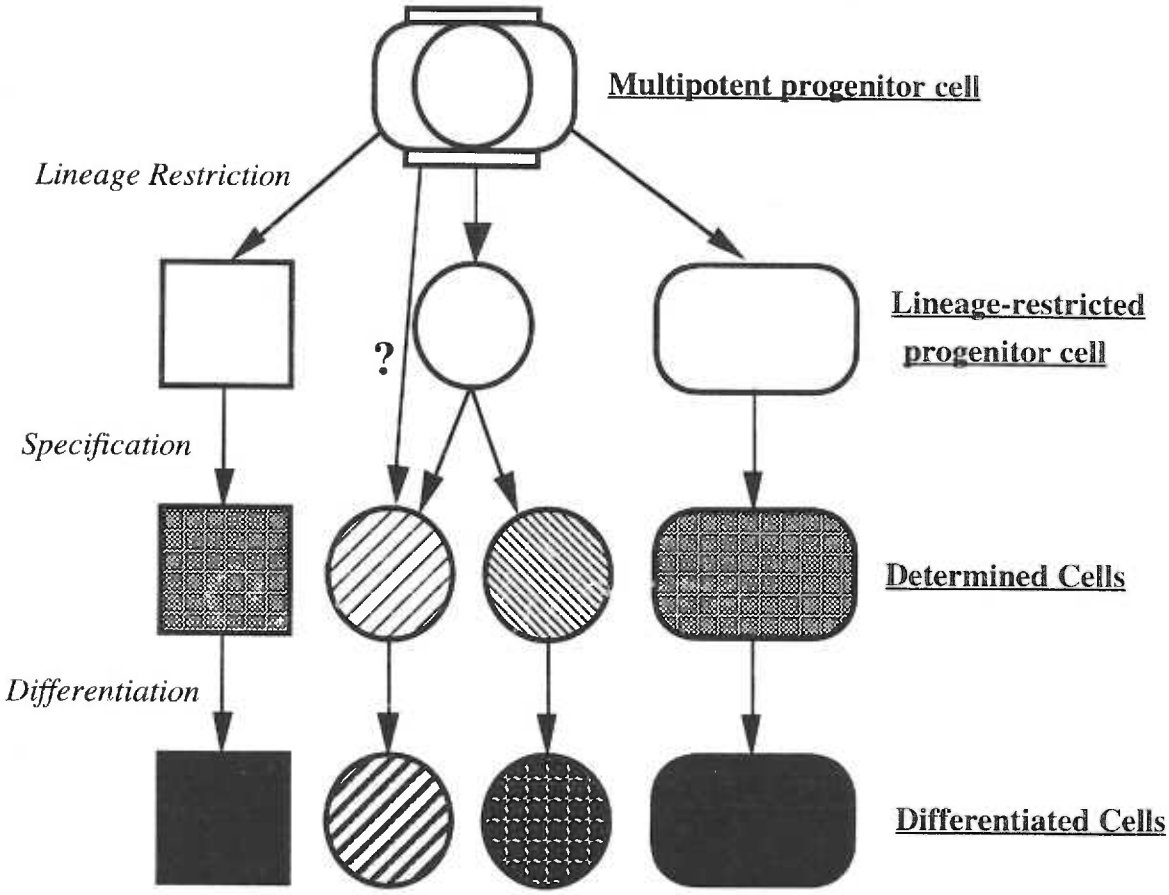

Fig. 1. The possible relationships of multipotent progenitor cells, lineage-restricted cells, and determined cells. The '?' indicates the questionable nature of that relationship. This model is modified from a review by Lillien (1998).

Although the mechanisms for lineage restriction are not well understood, factors that regulate stem cell fate may be either extrinsic or intrinsic. Extrinsic factors either cause stem cells to commit to a particular lineage or selectively promote the proliferation or survival of committed cells (Lillien, 1998). For example, the locally restricted appearance of glial-restricted progenitor cells in the ventral spinal cord is due to the release of Sonic Hedgehog by the notochord (Pringle *et al.*, 1996). Intrinsic factors, cell autonomous processes that determine the differentiative potential of a cell, may also regulate lineage restriction of stem cells. For example, in the nematode, *C. elegans*, the determination of blastomere identity is regulated by the asymmetric distribution of receptors and cellular components (Bowerman, 1995; Sulston *et al.*, 1983). During development, both extrinsic and intrinsic factors are required for the complex determination of a cell's fate. The extent to which both contribute to the fate of a stem cell is not known.

Specification and differentiation of the neural crest

The neural crest is a classic model system of stem cell specification and differentiation (Horstadius, 1950; Le Douarin, 1982; Weston, 1970). This transient population of pluripotent stem cells arises from the dorsal aspect of the neural tube at the border between the prospective epidermis and prospective neural plate of the early embryo (Selleck and Bronner-Fraser, 1995). As the neural tube closes, neural crest cells migrate away from the neural tube along characteristic pathways (Bronner-Fraser *et al.*, 1991; Serbedzija *et al.*, 1990). Neural crest cells migrating from different regions of the neural tube give rise to distinct phenotypes (for review, (LaBonne and Bronner-Fraser, 1998; Selleck *et al.*, 1993)). The cranial neural crest cells contribute to the connective tissue and periocular skeleton of the face, glia, and cranial sensory ganglia. Vagal neural crest

cells give rise to the enteric nervous system and smooth muscle. Truncal neural crest cells form melanocytes, peripheral sensory and sympathetic ganglia, Schwann cells, and chromaffin cells. Finally, the sacral neural crest cells contribute to the post-umbilical levels of the enteric nervous system.

The fate of a neural crest cell in vivo is a subset of that cell's developmental potential (LaBonne and Bronner-Fraser, 1998; Le Douarin, 1982; Le Douarin and Dupin, 1993). Quail-chick chimeric studies have shown that chick neural tube taken from one region and inserted in a quail embryo in a different region will produce neural crest cells capable of giving rise to the developmental fates of the new region (Le Douarin, 1982; Le Douarin *et al.*, 1993). For example, vagal neural crest transplanted caudally can give rise to sympathetic ganglia, while rostrally transplanted truncal neural crest can give rise to enteric neurons. While it is clear that neural crest cells are multipotent, they are not totipotent. Ectomesenchymal derivatives, for example, are specific to the cranial NC. Neural tubes from lower axial levels, when heterotopically transplanted to the cranial region, are incapable of producing ectomesenchymal derivatives; Transplantation of cranial neural crest to lower axial levels produces ectopic cartilage (Le Douarin, 1982).

When do neural crest cells become restricted? Evidence indicates that some restrictions occur prior to migration (i.e., cranial crest development). In other cases, it has been shown that post-migratory neural crest cells maintain a degree of multipotency. Back-transplanting chick embryonic dorsal root ganglia (DRG) into quail embryos prior to the onset of neural crest migration has shown that embryonic DRG contain both autonomic and sensory progenitors (Le Douarin, 1982; Le Douarin and Dupin, 1993; Le Douarin *et al.*, 1993). It was unclear, however, whether the autonomic neurons arising from the back-transplanted chick DRG came from a neuron that switched identity or from an undifferentiated cell. To address this, chick/quail chimeric nodose ganglion (chick –

neural crest-derived, quail – placode-derived) were back transplanted into quail. In these experiments it was found that all neurons from the donor ganglion (placode-derived) died while the neural crest-derived nonneuronal cells differentiated into neurons (Ayer-Le Lievre and Le Douarin, 1982). The neurons were generated from a progenitor population that was quiescent or fated to become glia in the cranial sensory ganglia. From studies like this, it was concluded that phenotypic fate was plastic, and that the target tissue stringently selected for the differentiation of specific developmental fates.

Heterochronic transplantation studies have been used to determine the developmental potential of neural crests that emigrate from the neural tube at different times. Normally, the first truncal neural crest cells migrate ventrally and give rise to the sympathoadrenal lineage. Neural crest cells migrating from the same region at a later stage form the dorsal root ganglia and then the melanocytes of the skin (Selleck *et al.*, 1993). When young neural tubes were back transplanted into embryos at the advanced stages of neural crest migration, the transplanted neural crest cells only gave rise to dorsal derivatives, while older neural tubes back transplanted into younger embryos gave rise to both dorsal and ventral derivatives (Weston and Bulter, 1966). The results from these experiments suggested that environmental factors, rather than the age of the cell, influenced the fate of a neural crest cell. To support this, subsequent studies have shown that back transplanted neural tubes can give rise to both dorsal and ventral derivatives in older embryos, when the early migrating neural crest cells are ablated (Baker *et al.*, 1997). These heterochronic and the preceding heterotopic experiments showed that the neural crest, as a population, is multipotent.

Lineage analysis has determined that individual neural crest cells are multipotent. In vivo studies, using injected fluorescent dextrans, have found that single neural crest cells could produce diverse derivatives, including dorsal root ganglia, autonomic ganglia, and

melanocytes (Baker *et al.*, 1997; Bronner-Fraser *et al.*, 1980; Collazo *et al.*, 1993; Serbedzija *et al.*, 1989). Clonal studies done in vitro have shown that neural crest cells are heterogeneous with respect to their developmental potential with 80% of the clones containing 2-6 distinct cells types while the rest are restricted to a single fate (Baroffio *et al.*, 1988; Bronner-Fraser *et al.*, 1980; Henion and Weston, 1997; Ito *et al.*, 1993; Sieber-Blum and Cohen, 1980). Based on these studies, the neural crest is comprised of a heterogeneous population of multipotent progenitor cells.

This heterogeneity in the developmental potential of neural crest cells has led investigators to suggest that neural crest cells are a stem cell population that undergoes lineage restriction prior to commitment to a specific lineage (LaBonne and Bronner-Fraser, 1998; Stemple and Anderson, 1992; Weston, 1991). Several studies have shown that the neural crest exhibits characteristics of a stem cell population (Lillien, 1998; Rao and Anderson, 1997; Stemple and Anderson, 1992). Furthermore, there are isolated populations of neural crest stem cells that persist into the late gestation of the embryo (Morrison *et al.*, 1999). However, most neural crest cells become restricted in their developmental potential, losing the more ventral fates first (Henion and Weston, 1997; Weston, 1991). Several studies have shown that neurogenic potential is lost with time in vivo and in vitro (Henion and Weston, 1997; Marusich, 1993; Vogel *et al.*, 1992). It is unclear, if neuronal progenitors die or they have become nonresponsive to differentiative signals.

Neuronal development: determination

The transition from an undifferentiated cell to a fully differentiated neuron comprises two steps: determination and differentiation. During the process of determination, an

undifferentiated cell will commit to a specific neuronal lineage. Once committed, the undifferentiated cell becomes a neuronal precursor. This precursor will then differentiate into a neuron.

The first stage in neuronal development is the establishment of a neuronal fate. Several genes have been discovered in Drosophila that endow cells with the competence to adopt a neuronal fate. These genes are designated proneural (Brunet and Ghysen, 1999; Chitnis, 1999; Jan and Jan, 1994). Two such genes are the achaete-scute complex and atonal, each of which specifies separate neuronal fates. Homologues of these genes have been identified in mammals. For the achaete-scute complex, there is a mammalian achaete-scute homologue, Mash-1 (Lo *et al.*, 1991). For atonal, there are the neurogenins (Sommer *et al.*, 1996). Mash-1 is a marker for autonomic neuronal progenitors and is required for their development (Anderson, 1994; Guillemot *et al.*, 1993). In Mash-1 knockout experiments, these populations are absent. Furthermore, Mash-1 is required for the regulation of transcription factors responsible for subsequent autonomic differentiation (Hirsch *et al.*, 1998; Itoh *et al.*, 1997; Torii *et al.*, 1999). Many of these downstream transcription factors must be expressed in the presence of Mash-1 for proper neuronal differentiation to occur. For example, forced expression of Phox-2a in the absence of Mash-1 does not result in a neuronal phenotype (Hirsch *et al.*, 1998; Lo *et al.*, 1998). However, in neural crest, stem cells can be induced to differentiate into autonomic neurons in the absence of Mash-1 by forcing the expression of Phox-2a and elevating cAMP and/or intracellular calcium levels (Lo *et al.*, 1999). The neurogenins are required for the development of the cranial sensory ganglia and are expressed in the sensory neurons of the peripheral nervous system (Blader *et al.*, 1997; Fode *et al.*, 1998; Ma *et al.*, 1999; Ma *et al.*, 1996; Ma *et al.*, 1998; Perez *et al.*, 1999; Sommer *et al.*, 1996).

The expression of a proneural gene, however, is not sufficient for a cell to differentiate into a neuron. Other signals, extrinsic and intrinsic, are required for neuronal differentiation to begin. For example, the activity of Mash-1 requires the presence of a growth factor, bone morphogenic protein-2 (BMP-2). BMP-2 induces the onset of Mash-1 expression (Shah *et al.*, 1996). Subsequent expression of Mash-1 makes the cell competent to respond to further BMP-2 signaling, inducing autonomic neuronal differentiation (Lo *et al.*, 1997). Exposure to BMP-2 alone is insufficient to initiate overt neuronal differentiation. In addition to Mash-1 and BMP-2, other conditions must be met before sympathetic neurogenesis occurs. Several studies, for example, have shown that neural crest cells need to be cultured at high cellular density for sympathetic neuronal differentiation to occur (Adler and Black, 1985; Anderson *et al.*, 1997; Freidin *et al.*, 1993; Morrison *et al.*, 1999). It is not clear what cell density is doing. One possible effect of culturing neural crest cells at high density is that it raises intracellular calcium levels. Elevated intracellular calcium levels have been shown to effect the expression of several autonomic characteristics, including tyrosine hydroxylase, dopamine beta hydroxylase expression and cellular morphology (Kim *et al.*, 1995; Lo *et al.*, 1999; Wakade *et al.*, 1995). Therefore, it has been suggested that both extrinsic signalling (BMP-2) and intrinsic signalling (intracellular calcium) are required for autonomic neuronal differentiation (Lo *et al.*, 1999).

Calcium signalling in development

Intracellular calcium plays a variety of important roles in regulating embryonic development. During fertilization, for example, calcium release in response to sperm entry regulates the release of cortical granules, which create the physical barrier to polyspermy. (Sardet *et al.*, 1998; Whitaker and Swann, 1993). Calcium oscillations are

required for progression through the cell cycle (Berridge, 1995; Poenie *et al.*, 1985; Sardet *et al.*, 1998; Whitaker and Patel, 1990). In zebrafish, the outer embryonic layer, beginning at the 32-cell stage, exhibits synchronized calcium transient activity as it differentiates into the enveloping layer (Reinhard *et al.*, 1995). Understanding how this molecule is regulated will provide insight as to the role of calcium in development.

Calcium homeostasis

Ca^{2+} is a ubiquitous 2nd messenger, responsible for regulating a wide range of cellular processes. However, prolonged elevations in intracellular Ca^{2+} will induce irreversible damage to all cells (Berridge, 1997b). Therefore, cells maintain their resting calcium concentration below 100 nM, much lower than extracellular levels (mM) (Carafoli, 1987). This large electrochemical gradient creates a driving force such that calcium entry into the cell is rapid. When both the intracellular calcium concentration ($[Ca^{2+}]_i$) and the activation of Ca^{2+}-dependent effectors are at low levels, a cell can respond quickly to swings in $[Ca^{2+}]_i$ (Pozzan *et al.*, 1994).

A cell exerts tight control over intracellular Ca^{2+} levels through a complex regulation of calcium influx and efflux parameters across select cellular membranes (Berridge, 1997b). Calcium channels located in the plasma membrane regulate calcium exchange between the cytoplasm and the extracellular space. The cell also sequesters calcium intracellularly in the endoplasmic reticulum (ER) and the mitochondria. The ER exhibits regulative control over calcium crossing its membrane, while the mitochondria is a high-capacity calcium sink that passively sequesters calcium in response to a robust rise in intracellular calcium levels. The cytosol also has a significant calcium buffering capacity (up to 99% of the free Ca^{2+}), which affects both the rate at which Ca^{2+} rises and the diffusion of intracellular Ca^{2+} (Pozzan *et al.*, 1994).

The cell utilizes several proteins to regulate calcium influx and efflux across the plasma membrane. Calcium efflux is primarily regulated by the plasma membrane Ca^{2+} ATPase and Na^{+}/Ca^{2+} exchanger, both which are activated by elevated intracellular calcium levels to maintain resting calcium levels (Carafoli and Stauffer, 1994). Ca^{2+} influx across the plasma membrane is mediated by voltage-dependent, ligand-gated, and store-operated calcium channels. Voltage-dependent calcium channels (VDCC) regulate calcium influx by opening in response to a membrane depolarization. Although very fast, the characteristic rise and fall in intracellular calcium in response to VDCC activation depends on the calcium channel type expressed. Ligand-gated calcium channels (LGCC) open in response to a ligand, and therefore are comparatively slower than the VDCC. Store-operated calcium channels (SOCC) are a special class of LGCC that open in response to a ligand released by a calcium-depleted ER.

The ER is the primary source for regulated release of intracellular calcium for the cell. The ER sequesters Ca^{2+} through the action of a Ca^{2+} ATPase that is different from the one located on the plasma membrane (Carafoli, 1987). Two classes of receptor-operated channels regulate the release of ER intracellular calcium stores: inositol 1,4,5-triphosphate receptor (IP_3R) and ryanodine (RyR) (Ehrlich and Bezprozvanny, 1994). Three isoforms of the RyR have been discovered, and they are expressed in skeletal muscle and Purkinje cells of the cerebellum (RyR1); cardiac muscle (RyR2); and in the CNS (RyR3) (McPherson and Campbell, 1993; Ogawa, 1994; Sorrentino and Volpe, 1993). Calcium and cADP-ribose are agonists for this receptor (Sitsapesan *et al.*, 1995). IP_3R family is comprised of three isoforms that are selectively distributed to all cell types: neuronal (type 1 and 3) and the rest (type 2) (Danoff and Ross, 1994; Nakanishi *et al.*, 1996; Sharp *et al.*, 1993; Sharp *et al.*, 1999). Cells may express more than one receptor subtype and even form heterotetrameric receptor-operated channels comprised

of two IP_3R isoforms (Miyakawa *et al.*, 1999; Monkawa *et al.*, 1995). Unlike the activation of the RyR, the IP_3R has two co-agonists, calcium and IP_3. Furthermore, IP_3R activity is modulated endogenously by a complex arrangement of kinase activities (Iino, 1996; Joseph, 1996).

Types of calcium signals and transduction specificity

Ca^{2+} signals can be either elementary or global events. Elementary events are highly localized increases in intracellular Ca^{2+} levels that contribute to the resting $[Ca^{2+}]_i$, local activation of Ca^{2+}-dependent processes, and the activation of global events (Bootman and Berridge, 1995). Because of the degree of localization, transient increases in $[Ca^{2+}]_i$ can have opposing effects within the same cell. For example, Ca^{2+} entry can relax a coronary smooth muscle by activating a Ca^{2+}-dependent K^+ channel to hyperpolarize the membrane or it can contract the muscle by releasing Ca^{2+} from the sarcoplasmic reticulum (Berridge, 1997b).

Global calcium events are initiated by the coordinated activation of elementary events (Berridge, 1997b). These global events are usually in the form of a baseline spike or wave (Thomas *et al.*, 1996). Baseline spikes are characterized by transient increases in $[Ca^{2+}]_i$ that rise rapidly from the baseline $[Ca^{2+}]_i$ and recover over a slower time course. Calcium waves are symmetrical oscillations that generally occur at a higher frequency than the baseline spikes. For both events, release from ER calcium stores is responsible for the primary rise in intracellular calcium levels. Little is known about how calcium waves are regulated (Gu *et al.*, 1994). For calcium spikes, however, studies have shown that both the IP_3R and RyR can contribute to the generation of the global event. Agonist availability and receptor modulation determines the frequency of the calcium spikes. Several studies have shown the IP_3R capable of generating complex patterns of calcium

spike events. Factors known to regulate IP_3R activity are intracellular calcium and IP_3 levels; phosphorylation state; and IP_3R isoform expression (Cardy *et al.*, 1997; Hagar *et al.*, 1998; Joseph, 1996; Lievremont *et al.*, 1996; Toescu, 1995; Wojcikiewicz and Luo, 1998).

Global calcium events can regulate distinct cellular processes. Calcium waves have been shown to regulate the cytoskeletal architecture in growth cones and early phases of fertilization (Callamaras *et al.*, 1998; Gu *et al.*, 1994; Sardet *et al.*, 1998). Calcium spikes can differentially activate gene expression based on the amplitude, duration and frequency of the calcium spike, as well as subcellular localization of the calcium transient event (Bading *et al.*, 1997; Dolmetsch *et al.*, 1997; Fields *et al.*, 1997; Gallin and Greenberg, 1995; Ginty, 1997; Hardingham *et al.*, 1998). For example, in neurons, elevations in cytoplasmic calcium activated genes associated with the serum-response element, while an increase in nuclear calcium levels activated genes associated with the cAMP response element (Hardingham *et al.*, 1997). In DRG neurons, the frequency of calcium spikes regulated the activation of CREB and MAPK to regulate gene expression (Fieber and Adams, 1991; Fields *et al.*, 1997). The mechanism by which calcium spikes are decoded by the cell is unclear. Recent studies have looked at the role of calmodulin dependent-protein kinases in decoding calcium signals (De Koninck and Schulman, 1998; Schulman and Hanson, 1993; Schulman *et al.*, 1992). These large calcium-binding protein complexes are regulated by the phosphorylation state of the complex, which is established by the intracellular calcium levels. The kinetics of autophosphorylation and dephosphorylation may give a cell a short-term "calcium" memory by which to base the regulation of downstream transcriptional events (Dolmetsch *et al.*, 1997; Dolmetsch *et al.*, 1998).

Neuronal development: differentiation

Neuronal differentiation results in a change in cellular morphology, proliferative state, and expression of various cellular proteins. Almost all neurons become postmitotic prior to the expression of neuronal markers. The exception is sympathetic neurons, which express several neuronal characteristics prior to becoming postmitotic (Rohrer and Thonen, 1987). Once postmitotic, neurons begin to express features that are unique to their particular phenotype, e.g., ion channels and neurotransmitters, as well as features that are common to all, i.e., panneuronal characteristics.

The process by which a neuronal precursor differentiates into a specific neuronal phenotype is unclear. One model is that neuronal precursors are not determined at birth, and that environmental influences specify neuronal identity as the neuron differentiates. Another model is that neuronal precursors are committed to a particular lineage. As with most models, it is a combination of both that explains how neuronal precursors give rise to specific neuronal phenotypes. Nonmodifiable traits are thought to be established early in neurogenesis, predetermined by the expression of specific proneural genes. Modifiable traits are acquired during the differentiation process and may be regulated by transcription factors activated after the onset of neuronal differentiation. For example, Mash1 expression regulates the differentiation of the panneuronal markers in differentiating autonomic neurons, while the transcription factor Phox2A regulates the expression of several sympathetic traits, e.g., tyrosine hydroxylase, dopamine β hydroxylase and c-RET (Groves *et al.*, 1995; Hirsch *et al.*, 1998; Lo *et al.*, 1999; Lo *et al.*, 1998).

Although studies have shown that proneural genes regulate several panneuronal characteristics, e.g., NCAM and neurofilament expression, proneural regulation of phenotype-specific characteristics has not been established. A phenotypic trait that may result from the commitment to a specific neuronal phenotype is the establishment of ion channels. Ion channels are acquired early in neurogenesis, and the maturation of these conductances is a cell autonomous process (Henderson and Spitzer, 1986). Neural crest-derived neurons acquire cation currents within the first 24 hours after the onset of neurogenesis with little change in the current density as they mature (Bader *et al.*, 1983; Nerbonne and Gurney, 1989; Spitzer, 1994a; Spitzer, 1994b). Furthermore, neurons acquire ion channels in a phenotype-specific manner. For example, chick sensory and autonomic neurons express ion channels in a temporal pattern that is unique for the particular class of neuron (Gottmann *et al.*, 1988). At maturity, peripheral sensory and autonomic neurons exhibit an array of ion channels that are significantly different from each other (Mintz *et al.*, 1992). Experiments need to be done to determine if neurons establish distinct arrays of ion channels early in development, and if this property is plastic.

Questions addressed in this thesis

The overall theme of my thesis is to understand the process of neuronal differentiation in the neural crest. I have focused on two areas: commitment to a neuronal phenotype and the requirements for neurogenesis. We know that the trunk neural crest gives rise to peripheral sensory and sympathetic neurons, both of which express distinct ion channel repertoires. Do neural crest neurons, differentiated in culture, express ion channel repertoires similar to neurons differentiating in vivo? If so, do ion channel repertoires indicate a commitment to a specific neuronal phenotype? Answers to these questions

will help in understanding when commitment to a specific neuronal phenotype occurs and confirm the validity of studying neural crest neuronal differentiation in culture. I have also focused on the role of calcium transient activity in regulating neuronal differentiation. There is evidence that calcium transients play significant roles during the development of a variety of cell types. Do neural crest cells exhibit calcium transients in culture? Are these calcium transients required for neuronal differentiation? What is the underlying mechanism responsible for the generation of these calcium transients? Many studies have identified extrinsic factors that regulate neuronal differentiation. Few studies, however, have explored intrinsic processes.

Materials and Methods

Cell culture

Neural crest cultures

Details of the culture system have been described elsewhere (Matsumoto, 1994a). Briefly, neural tubes from somite level 10 to the more posterior unsegmented somitic mesoderm were dissected from embryonic day 9.5-10 Swiss-Webster mouse embryos. Cleaned neural tubes were placed onto an air-dried collagen substrate. Crest cells were allowed to migrate from the neural tubes for 18-24 hours. The tubes were then removed using a sharpened surgical knife. The cultures were washed twice and fed 1.5 mls of our standard culture medium (see below). The cultures were fed every 3-4 days.

The culture medium consisted of L-15 medium (Flow) modified for a 5% CO_2 atmosphere (Mains and Patterson, 1973). For the first 24 hours of plating, this basal medium was supplemented with 2.5% (v/v) rat serum (Harlan), penicillin/streptomycin (100 U/ml; 100 µg/ml; Gibco), and 2 mM glutamine (Gibco) and 1% rat embryo extract. 24 hours after plating, the rat embryo extract was replaced with one or more of the following growth factors: human recombinant leukemia inhibitory factor (R & D systems), mouse recombinant leukemia inhibitory factor (R & D systems), nerve growth factor (Sigma), rat recombinant ciliary neurotrophic factor (a gift from Rae Nishi, OHSU), glial derived neurotrophic factor (Amgen), neurotrophin-3 (R & D), and bone

morphogenic protein-2 (Genetics Institute). The final concentration for all growth factors was 10 ng/ml, except for nerve growth factor, which was used at 100 ng/ml.

Dorsal root ganglia and superior cervical ganglia cultures

DRG and SCG were removed from newborn mice. The ganglia were cleaned and incubated at 37 °C for 20 minutes in calcium- and magnesium-free Hanks balanced salt solution (HBSS; Gibco) with collagenase (1 mg/ml; Worthington) and dispase (4 mg/ml; Boehringer Mannheim). The cells were triturated 25 times using a fire-polished pasteur pipette. L-15 with 10% fetal calf serum (Gibco) was added to the cell-enzyme suspension to inactivate the enzymes. The cells were pelleted by centrifugation, resuspended in the same culture medium as neural crest cultures (see above), and then plated at a density of approximately 1000 neurons/cm^2 on a collagen matrix. For cultures used within 24-48 hours, the medium contained 100 ng/ml NGF, while long-term cultures (10-14 days) were supplemented with either hLIF or mLIF.

Embryonic dorsal root ganglia cultures

DRG were removed from embryonic day 12 mouse embryos. The ganglia were incubated at 37 °C for 5 minutes in trypsin/EDTA (ICN). L-15 with 10% fetal calf serum was added to the cell-enzyme suspension to inactivate the trypsin. The ganglia were pelleted, then resuspended in the same culture medium as the neural crest cultures. The neurons were dissociated by gentle trituration (approx. 25x) in growth medium. The dissociated cells were plated at an approximate density of 1000 neurons/ cm^2 on a collagen matrix.

Physiology

Electrophysiological recordings

Whole-cell recordings were used to analyze the calcium currents as described by (Hamill *et al.*, 1981). Cultured neurons were placed into a recording chamber containing a bath solution of (in mM; Sigma): 140 NaCl, 5.4 KCl, 0.18 $MgCl_2$, and 2.93 $CaCl_2$, 2.6 HCO_3, 0.56 H_2PO_4, adjusted to pH 7.3 with 150 NaOH (Furshpan *et al.*, 1986). The internal solution was composed of (in mM; Sigma): 140 Cesium-methanesulfonate, 4.5 $MgCl_2$, 9 HEPES, and 9 EGTA (adjusted to pH 7.3 using 140 CsOH). To promote stability of the currents, 4 mM Mg-ATP (Sigma) and 0.3 mM GTP (Sigma) was added as well as an ATP-regenerating system which included 14 mM phosphocreatine (Sigma) and 50 U/ml creatine phosphokinase (Sigma) (Mintz et al, 1992). The patch pipettes were made from 100 µl micropipettes (VWR) using a two-step puller (Narishige) with resistances of 2-4 Mohms. Whole-cell recordings were performed using an Axopatch 1-D patch clamp amplifier (Axon Instruments). Series resistance compensation was accomplished using a series resistance compensation circuit. Data were discarded if the current trace showed signs of series resistance problems such as a slow tail current. Compensations were also made for linear leak and capacitive currents by subtraction of an appropriately scaled current elicited by a 10 mV hyperpolarization. Corrections were made to the command potential for the liquid junction potential between the internal solution and the bath solution, in which the pipette current was zeroed before seal formation. pCLAMP software (Axon instruments) was used to generate voltage clamp protocols. IGOR Pro (Wavemetrics) was used for data analysis. Following attainment of the whole-cell configuration, the external bath solution was changed to a recording solution containing (in mM): 130 NaCl, 2.5 KCl, 1 $MgCl_2$, 10 HEPES, 8 glucose, 5 $BaCl_2$, 50 TEA-Cl

(Sigma), and 500 nM TTX (Sigma) (adjusted to pH 7.3 with 150 NaOH). Neurons were clamped at a holding potential of -90 mV and then stepped to 0 mV, once every 10 seconds. The currents elicited were filtered at 5 kHz (low-pass Bessel filter), digitally sampled at 1 ms intervals (pCLAMP), and stored on a laboratory computer (Unisoft). Once a stable recording was attained, the bath solution was switched to the same solution plus 10 μM nimodopine (Sigma), to block L-type currents; this block was reversed by washing in recording solution. To block N-type currents, recording solution plus 5 μM ω–conotoxin GVIA (Sigma) was administered via a puffer pipette. The ω-conotoxin GVIA puffer was then removed and replaced by a puffer pipette containing recording solution plus 50 nM ω-Agatoxin-IVA (a gift from Pfizer), to block P-type current. We then followed this with 3 mM $CoCl_2$ (Sigma) to determine total calcium current. The toxins were suspended in recording solution containing 1 mg/ml cytochrome C (Boehringer Mannheim). All experiments were performed at room temperature. L-type currents were determined as the difference in the peak deflections of currents recorded in recording solution and currents recorded in the presence of nimodopine. N-type currents were determined as the difference in the peak deflections of currents recorded in recording solution (following reversal from nimodopine wash) and currents recorded in the presence of ω-conotoxin GVIA. P-type currents were determined as the difference in the peak deflections of currents recorded in the presence of ω-conotoxin GVIA and currents recorded in the presence of ω-agatoxin-IVA.

Calcium imaging

Neural crest cultures were loaded in the dark with the Ca^{2+} indicator dye, Oregon-Green 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid - 1 (OGB; Molecular Probes). To do this, neural crest cultures were incubated (room temperature) for at least 30 minutes in our basic calcium recording solution (CaRec) plus 2 μM of the

acetoxymethyl ester form (membrane permeable) of OGB (OGB-AM). The cultures were then washed with CaRec for 10 minutes prior to imaging. The composition of CaRec is as follows (in mM): 160 NaCl, 3 KCl, 10 Dextrose, 10 Hepes, 0.8 $MgCl_2$ and 5 $CaCl_2$. OGB-AM was dissolved in DMSO plus 0.2% pluronic to make 2 mM aliquots. The OGB-AM stock solution was diluted 1:1000 in CaRec for loading neural crest cultures.

To image intracellular calcium in cultured neural crest cells, the OGB-loaded culture was placed on the stage of an inverted light microscope (Nikon Diaphot 200) and continuously perfused with CaRec at room temperature (21-25 °C). Illumination was provided with a Hg Lamp. A dichroic cube in the path of the light provided an excitation wavelength of 488 nm and collected emissions of wavelengths longer than 515 nm. The illumination intensity was reduced using a series of neutral density filters (ND4, ND2, and either ND 0.3 or 0.6) to reduce photobleaching and photodynamic damage to the cells. Images were acquired using an intensified (Hamamatsu) CCD camera (Cohu) controlled by a PowerPC 7100 (Macintosh) computer running Cytos image acquisition software (ASI, Eugene). Images were acquired every 3 seconds with each image consisting of an average of 8 frames. The exposures were computer-regulated by rotating a filter wheel (Lambda 10, Sutter instruments) from a closed position to a second position containing either an ND 0.3 or 0.6 neural density filter and then back to the closed position. The fluorescence micrographs were digitized, and relative changes in $[Ca^{2+}]_i$ were determined for selected cells using data analysis software (IGOR, Wavemetrics) on a Macintosh computer. The data are expressed as the % change in fluorescence (fluorescence(t)-baseline fluorescence) over baseline fluorescence (% $\Delta F/F_0$). The baseline fluorescence was defined as the average of five minimum-level images for each trial. Events were counted if transient elevations in $[Ca^{2+}]_i$ exceeded 50% of baseline fluorescence. Calcium transient duration was estimated by measuring the time from the

initial deviation from baseline to return. Unless specified, calcium spiking frequency is expressed as events/hour. Average values are expressed as mean ± SEM.

To determine the effects of pharmacological blockers on calcium transient activity, we first monitored calcium transient activity in normal CaRec for 30 minutes to establish a baseline of activity. Next, we monitored calcium transient activity for 30 minutes in CaRec plus blocker. The percentage of active cells and the extent of calcium spiking frequency in these cells were compared using Student t-test to test for significance. In selected experiments, we washed out the blocker with normal CaRec and observed the degree of reversibility. Unless otherwise noted, all chemicals used in this thesis were purchased from Sigma. In most cases, preparation of a superfusion medium containing a chemical was done so by dissolving the chemical directly into normal CaRec. In cases where chemical solubility was a concern, we dissolved the chemical first in DMSO, followed by a 1:1000 dilution into normal CaRec. In some experiments, the concentration of the chemical used would have significantly increased the osmolarity of the recording medium if dissolved directly. For those experiments, the medium was prepared by exchanging equimolar concentrations of the chemical used for NaCl.

Histochemistry

Antibodies

Rabbit anti-Brn 3.0 was a generous gift from Dr. Eric Turner. Mouse anti-Hu (mAB 16A11) was obtained from the Monoclonal Antibody Facility at the University of Oregon. Rabbit anti-neuron specific enolase was purchased from Incstar.

Immunocytochemistry

Standard immunocytochemical protocols used were described previously (Matsumoto, 1994a). Briefly, cultures were washed in phosphate buffered saline (PBS) and fixed for 20 minutes at room temperature in 4% (w/v) paraformaldehyde (Sigma). After fixation, the cells were washed in PBS. Following a 1 hr incubation with a blocking buffer (PBS, 0.1% triton X (Sigma), 5% goat serum (Gibco)), the cultures were incubated with a primary antibody at room temperature for 24 hours. Unbound primary antibody was removed by washing 4 times for 5-minute periods in blocking buffer. Primary antibodies were visualized using either fluorescent or biotinylated secondary antibodies. For fluorescence microscopy, the primary-labeled culture was incubated with the secondary antibody (Molecular Probes) for 40 minutes at 37 °C. Biotinylated secondary antisera were applied for 1 hour and then visualized following the instructions supplied by the Vectastain ABC kit (Vector laboratories). The cultures were viewed on an inverted microscope (Nikon Diaphot) with appropriate filters. Cells were scored as positive if the signal exceeded background. Incubation with normal serum was used as a control.

For correlating calcium transient activity and immunocytochemistry, calcium-imaged cultures were fixed and immunolabeled on grid dishes with the grid coordinates captured for each recorded field. Following immunocytochemistry, labeled cultures were oriented to the captured grid coordinates and comparisons made between immunolabeled cells and cells that previously exhibited calcium transient activity.

Capsaicin assay

The capsaicin assay was performed as described in Matsumoto (1994b). This assay is based on the differential uptake of Co^{2+} into capsaicin-sensitive and insensitive cells. The

plasma membrane, normally impermeable to Co^{2+}, will allow the ion to enter the cell upon activation of the capsaicin receptor. The selectively sequestered Co^{2+} can be visualized using an intensification protocol. Briefly, cultures were washed 5 times in a saline solution (assay buffer) consisting of (mM): 5.8 NaCl, 5.0 KCl, 2.0 $MgCl_2$, 12.0 glucose, 137 sucrose, 0.75 $CaCl_2$, 10.0 HEPES, pH 7.4 (KOH). They were then placed in a cobalt solution (5.0 mM $CoCl_2$ in assay buffer) containing 10 mM capsaicin (Sigma). Controls had assay buffer plus cobalt without capsaicin. After 10 minutes, the cultures were washed 4 times in assay buffer followed by 5 minute incubation in assay buffer that had been saturated with hydrogen sulfide (Sigma). The cultures were then washed 4 more times in assay buffer and then fixed for 20 minutes in 4% (w/v) paraformaldehyde at room temperature. The cultures were than washed 4 times in PBS and processed for silver intensification. The intensification procedure was based on TIMM's intensification protocol (Bacon and Altman, 1977). The following steps were done at 60 °C. The cultures were equilibrated in distilled water then transferred to a solution consisting of (mM; Sigma): 3% (w/v) gum arabic, 15 hydroquinone, 280 sucrose, pH 2.3 (adjusted with citric acid) for 20 minutes. The solution was then replaced with the same solution plus silver nitrate (Sigma) and was changed to a fresh silver nitrate solution after 20 minutes to reduce background staining. After 40 minutes, the cultures were rinsed once in distilled water and stored in PBS. The neurons were counted in the cultures at 400x using an inverted microscope (Diaphot, Nikon) in an area measuring roughly 500 mm^2 that was etched in the center of the dish. In the capsaicin assays, cells stained brown/black scored throughout the cytoplasm were scored as positive. Unlabeled neurons were identified by their morphology (round soma and processes).

TUNEL assay

A TUNEL assay was used to screen for apoptotic nuclei under several growth conditions tested. The assay was performed as per the instructions supplied with the kit (Boehringer Mannheim).

Lineage tracing

In order to determine the fate of Neural crest-derived cells that did or did not exhibit calcium transient activity, we loaded 1-3 day old primary neural crest cultures, grown in standard culture medium, with OGB-1, and monitored the intracellular calcium levels of individual cells for 15 minutes (see Imaging Intracellular Calcium). For each recorded field (1 per culture), an individual cell (1 per field) was scored as active (exhibited one or more calcium spikes) or inactive and then intracellularly labeled using glass microelectrodes containing a solution of 6% lyseinated rhodamine dextran (10,000 MW, Molecular Probes) in 0.2 M KCl. The dye solution was injected ionophoretically. To insure that we injected the cell of choice, we overlaid the calcium image with the labeled image and showed that they overlapped. In addition, each injected culture was viewed within 4 hours of the injection to determine survival and verify that a single cell was injected. Following the injection, the culture was incubated overnight in a sympathetic neuron-inducing differentiation medium. This chemically defined medium is modified from Stemple and Anderson (Stemple and Anderson, 1992), in which we prepared the recipe without the addition of glycerol or nerve growth factor. The next day, the culture was switched back to the standard medium and cultured for two more days (3 days total), at which time the culture was fixed and immunolabeled for the neuronal marker, Hu. Lineage-tracked cells were identified and their phenotype determined.

Statistical Analysis

Student t-tests were used to compare means between two populations. For comparisons across more than two populations, we used the analysis of variance (ANOVA) to determine if there was a significant difference among the means compared (Sokal and Rohlf, 1969). If significance was established, an a posteriori test, the Student Newmann-Keuls, was applied to do a multiple comparison of the mean populations tested to determine which were the same and which were different (Sokal and Rohlf, 1969). Results of statistical analysis were calculated using statistical software (PRISM) on a Macintosh G3 computer (Apple).

A two-level mixed-model analysis of variance (MANOVA) was used to determine significant difference between neuronal populations based on the HVA calcium current profile. The two levels were as follows: The first level was between five subjects representing cell types and growth conditions (DRG, SCG, neural crest:mLIF(+somites), neural crest:hLIF(+somites), and neural crest:rCNTF(+somites)). The second level was within the total calcium current partitioned as the percent of the total current inhibited by the different channel blockers for the L-, N-, and P-type channels. Since the first level was between subjects and second level was within subjects, this analysis was defined as a mixed model. The numbers were entered into a statistical software program (SPSS) which computed the F-statistic and degrees of freedom for the within-subject effect and the between-subject effects. For the between-subjects effects to be interpretable, we determined the significance of interaction between the levels. Significance indicated that the distribution of total calcium current among the different HVA calcium currents corresponded to a particular cell-type. We then looked at the between-subjects effects and determined its significance. Once significance was established, an a posteriori test, the Student-Newman-Keuls, was applied to do a multiple comparison among the mean

channel types to determine which neuronal populations were the same and which were different.

CHAPTER I

Neurons differentiating from murine neural crest in culture exhibit sensory or sympathetic-like calcium currents

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ABSTRACT

The trunk neural crest gives rise to peripheral sensory and sympathetic neurons. In culture, neural crest cells can be induced to differentiate into either neuronal phenotype. Few studies have examined the differentiation of physiological properties in cultures of neural crest cells. Using whole-cell recordings, our study examined the effects of growth factors on high voltage activated calcium current profiles exhibited by neurons differentiating in culture. We compared these profiles with those exhibited by sensory and sympathetic neurons. Neural crest cells in culture gave rise to neurons with calcium current profiles identical to either sensory or sympathetic neurons, depending on the growth conditions. On average, the calcium current profile for sensory neurons was 23% (L), 51% (N), and 12% (P), while sympathetic neurons had a similar L-type current (20%), higher N-type (76%), and lower P-type (4%). Neural crest cells cultured with human leukemia inhibitory factor plus somite cells produced neurons with a sympathetic-like calcium current profile (L:17%, N:75%, and P:4%). However, murine leukemia inhibitory factor (L:25%, N:52%, and P:13%) and ciliary neurotrophic factor (L:18%, N:49%, and P:9%) plus somite cells produced neurons with sensory-like calcium current profiles. These growth conditions did not modify the calcium current profiles of neurons cultured from embryonic and neonatal ganglia. Similarly, murine leukemia inhibitory factor produced a greater percentage of neurons (57%) with sensitivity to capsaicin (sensory phenotype) than human leukemia inhibitory factor (3%). Physiological traits can be a useful tool for the determination of neuronal phenotype in culture where other traits may be less stable.

INTRODUCTION

Neurons differentiate in neural crest cultures under a variety of conditions (Anderson, 1993; Baroffio *et al.*, 1990; Le Douarin, 1982; Le Douarin and Dupin, 1993; Sieber-Blum and Cohen, 1980). Studies have described culture environments that promote the differentiation of many neuronal derivatives of the NC, including those of sensory, sympathetic and enteric lineages (Anderson, 1997; Gershon *et al.*, 1993; Murphy *et al.*, 1991). Typically, the classification of neuronal phenotype is based on the expression of cytochemical properties, such as neurotransmitters, cytoskeletal proteins, or transcription factors. However, cultured neurons modify the expression of many of these cytochemical properties which limits their usefulness in distinguishing neuronal phenotype (Kessler and Black, 1982; Landis, 1990; Leblanc, 1990). There have been only a handful of studies that have examined the differentiation of physiological properties in neural crest cultures (Bader *et al.*, 1983; Simonneau *et al.*, 1987); these studies could not specify the type of neuron under study since they examined properties shared by many types.

A number of physiological properties are unique to particular classes of neuron. We have, for example, previously used capsaicin to detect the differentiation of sensory neurons in murine neural crest cultures (Matsumoto, 1994b). A second well-characterized trait of peripheral neurons is the high voltage activated (HVA) calcium currents. HVA calcium currents are acquired during the initial stages of neuronal differentiation (Bader *et al.*, 1983; Godfraind and Govoni, 1995; Gottmann *et al.*, 1988; O'Dowd *et al.*, 1988). Different classes of Neural crest-derived neurons acquire HVA calcium currents autonomously, in a specific temporal sequence (Gottmann *et al.*, 1988). Once acquired, there appears to be little modification in the HVA calcium conductances other than a small increase in the overall current density (Bader *et al.*, 1983; Gottmann *et*

al., 1988; Spitzer, 1991). In addition, different populations of rat neurons have been shown to have distinct HVA calcium current profiles with respect to the relative contribution of three HVA calcium conductances (L-, N-, and P-type) to the total calcium current (Mintz *et al.*, 1992; Scroggs and Fox, 1992). Dorsal root ganglion (DRG) neurons, for example, exhibit a calcium current profile where 18% of the total calcium current is L-type, 43% N-type, and 23% P-type. Sympathetic neurons, on the other hand, exhibit a calcium current profile that is 7% L-type, 93% N-type with no detectable P-type current.

In the present study, we test the hypothesis that the calcium current profile of a neuron indicates its phenotype. Thus, neurons differentiating from trunk neural crest cells in culture ("NC-neurons") should acquire HVA calcium current profiles identical to sensory or sympathetic neurons. We examined the effects of three cytokines on neural crest neuronal differentiation: mouse recombinant LIF (mLIF), human recombinant LIF (hLIF), and rat recombinant ciliary neurotrophic factor (rCNTF). We compared the calcium current profiles of NC-neurons differentiated under these conditions with acutely dissociated neonatal sensory and sympathetic neurons and found that NC-neurons have calcium current profiles that are identical to neonate neurons. Sensory neurons shared calcium current profiles with NC-neurons cultured in mLIF or rCNTF, while sympathetic neurons have the same calcium current profile as NC-neurons cultured in hLIF. We compared these results with the expression of capsaicin-sensitivity (sensory phenotype; (Bevan and Szolcsányi, 1990; Winter, 1987)) in hLIF and mLIF NC-neurons, and found that the expression of capsaicin-sensitivity supported the calcium current data.

RESULTS

Murine sensory and sympathetic neurons exhibit distinct HVA calcium current profiles

Our first objective was to determine if sensory neurons exhibited HVA calcium current profiles that were different from sympathetic neurons. To do this, we examined the HVA calcium current profiles of acutely dissociated sensory and sympathetic neurons. Such measurements have been conducted in the rat and it has been shown that sensory and sympathetic neurons possess distinct HVA calcium current profiles (Mintz *et al.*, 1992). We found that sensory and sympathetic neurons in the mouse have a similar breakdown in their HVA calcium conductances (Fig. 1). We determined the HVA calcium current profile of acutely dissociated neonatal mouse sensory neurons from the dorsal root ganglion (DRG) and sympathetic neurons from the superior cervical ganglion (SCG), using whole-cell voltage clamp recordings. We used specific calcium channel blockers [10 μM nimod (L-type), 5 μM ω–CgTx (N-type), and 50 nM ω-Agatoxin-IVA (ω-Aga-IVA, P-type)] to measure the contributions of three calcium conductances. Figure 1 shows recordings from a sympathetic and a sensory neuron exemplifying the differences in the two neuronal phenotypes. In mouse sympathetic neurons, the majority of the HVA calcium current was N-type (Fig. 1A). The L-type current antagonist blocked most of the remaining current. Unlike the rat, we observed a small, but consistent, P-type current (Fig. 1A). Sensory neurons, in contrast, have a smaller N-type contribution and a larger P-type contribution than was observed in sympathetic neurons (Fig. 1B). On average, 76 ± 2.6% (SE) of the total calcium current in sympathetic neurons was N-type, 20 ± 2.5% L-type and 4 ± 1.1% was P-type (Fig. 1C). In sensory neurons, only 51 ± 3.2% of the total calcium current was N-type, with 23 ± 2.5% L-type and 12 ± 3.2% P-type. The

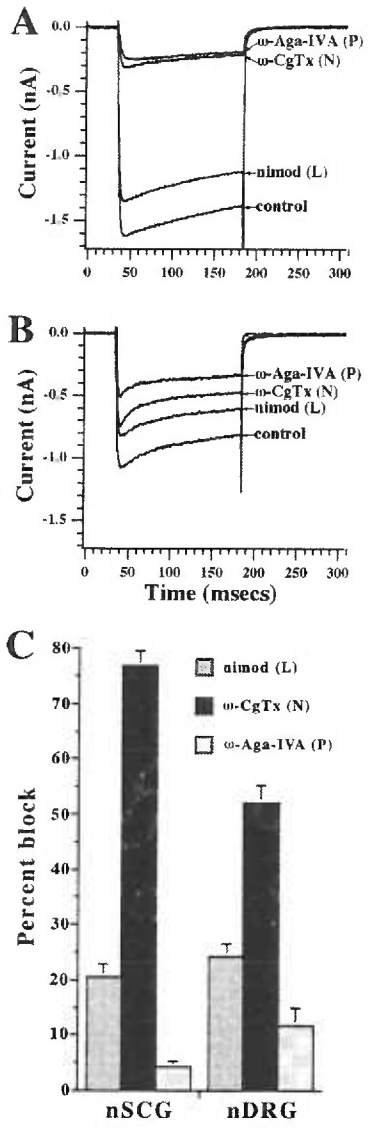

Figure 1. Acutely dissociated sympathetic and sensory neurons exhibit distinct distributions of L-, N-, and P-type calcium currents as measured by whole-cell voltage clamp recordings. Sample recordings from a sympathetic (A) and a sensory (B) neuron demonstrate the differences in sensitivity in these populations to three calcium channel blockers. HVA calcium currents were evoked by stepping from a holding potential of -90 mV to 0 mV for 150 ms. HVA calcium currents were isolated by a continuous superfusion of TTX (1.5 μM) and TEA (50 mM). K^+ currents were also suppressed by Cs-methanesulfonate in the intracellular solution (see Methods). Each of three HVA calcium currents was measured using specific pharmacological channel blockers (**L**, 10 μM nimod; **N**, 5 μM ω-CgTx; **P**, 50 nM ω-Aga-IVA). Summary graph (C) shows the mean (±SE) percent distribution of L-, N-, P-type calcium currents for each population tested. Note that sympathetic neurons (n=22) had a significantly larger ω-CgTx-sensitive current ($p<.001$) and a significantly smaller ω-Aga-IVA-sensitive current ($p<.05$) than sensory neurons (n=28).

three calcium channel antagonists blocked all of the HVA calcium currents in sympathetic neurons, while 14% of the calcium current in sensory neurons remained unidentified.

To determine if the differences seen between sensory and sympathetic neurons were statistically significant, we used a two-level mixed-model analysis of variance (MANOVA; first level between cell types and second level within calcium channel subtypes [L, N, and P]). For each cell type, we determined the calcium current profile for 14-16 individual neurons. As a population, sensory and sympathetic neurons exhibited distinct calcium current profiles ($p<.0001$). Using a two-tailed student t-test, we found significant differences in the mean N-type and P-type current percentages ($p<.001$ and .05, respectively), while the L-type current percentages were not significantly different.

NC-neurons exhibit calcium current profiles similar to either sympathetic or sensory neurons

Next, we examined the calcium current profiles of NC-neurons, grown under different growth conditions, to determine if their profiles were similar to either sensory or sympathetic neurons. To do this, we cultured neural crest cells in culture medium containing either hLIF or mLIF, since in preliminary experiments these two growth conditions supported the differentiation of neurons with N-type calcium current distributions most similar to sympathetic and sensory phenotypes, respectively (data not shown). We also cultured neural crest cells with rCNTF, because this cytokine acts through the same signal transduction pathway as LIF (Ip *et al.*, 1992) but may act on a different set of neural crest cells (i.e. those possessing the CNTF receptor α subunit) (Ip *et al.*, 1993). In addition, we co-cultured the neural crest cells with dissociated somite cells because its presence increased the number of neurons differentiated under these

growth conditions (data not shown). The somite cells had an added effect of increasing the N-type calcium current percentage for NC-neurons differentiated in the presence of hLIF or rCNTF (Table I). After 1-3 weeks in culture, we used whole-cell recordings to determine the calcium current profiles for NC-neurons and compared them to calcium current profiles of acutely dissociated sensory and sympathetic neurons.

Examples of the calcium currents and the effect of addition of each of the channel blockers are shown in figure 2. NC-neurons that differentiated in the presence of hLIF plus somite cells (hLIF(+S)) had HVA calcium current distributions similar to sympathetic neurons with most of the calcium current being N-type, a smaller contribution being L-type and little or no P-type (compare figs. 1A and 2A). However, NC-neurons differentiated in the presence of either mLIF plus somite cells (mLIF(+S)) or rCNTF plus somite cells (rCNTF(+S)) had calcium current distributions similar to sensory neurons with about half of the calcium current N-type, a larger P-type component than seen in the sympathetic and hLIF(+S) NC-neurons and a similar L-type component (compare figs. 1B and 2, B and C). On average, NC-neurons differentiated in the presence of hLIF(+S) had a calcium current profile similar to the sympathetic neurons with 17 ± 2.5% of the total calcium current sensitive to nimod, 75 ± 2.9% to ω-CgTx, and 4 ± 0.8% to ω-Aga-IVA (compare figs. 1C and 2D). However, NC-neurons differentiated in the presence of mLIF(+S) had higher nimod-sensitive current (25 ± 3.6%), lower ω-CgTx current (52 ± 3.6%), and a higher ω–Aga-IVA sensitive current (13 ± 2.1%). This calcium current profile was similar to sensory neurons (compare figs. 1C and 2D). NC-neurons differentiated in the presence of rCNTF(+S) had a profile similar to mLIF(+S) and sensory neurons (18 ± 2.8%, 49 ± 3.4%, and 9 ± 0.8%, respectively).

Table I. NC-neuron Ca^{2+} currents differentiated *in vitro*.

Growth Conditions	(n)	Ca^{2+} current types L	N
hLIF			
+S	(44)	12.1±1.5	75.3±1.9
-S	(14)	7.1±2.0	64.5±5.0
mLIF			
+S	(74)	13.4±1.4	54.0±1.9
-S	(3)	10.3±5.1	45.2±7.7
rCNTF			
+S	(49)	11.9±1.6	61.4±1.9
-S	(15)	7.0±1.1	48.7±4.5

Note: NC-neurons differentiated in the presence of these growth factors ± somites (S). (#) = number of neurons.

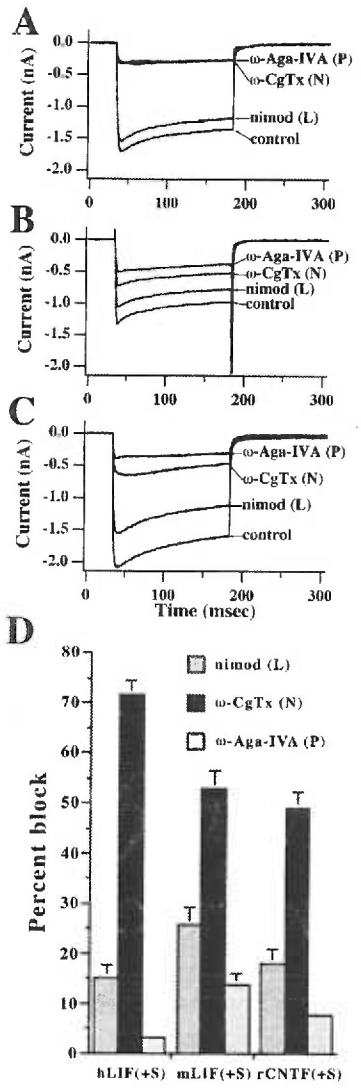

Figure 2. NC-neurons differentiated in the presence of hLIF(+S), mLIF(+S), or rCNTF(+S) exhibit distinct distributions of L-, N-, and P-type calcium currents. Sample recordings from an NC-neurons differentiated in the presence of hLIF(+S) (A), mLIF(+S) (B), and rCNTF(+S) (C) demonstrate the differences in sensitivity in these populations to three calcium channel blockers. HVA calcium currents were elicited by stepping the membrane potential from a holding potential of -90 mV to 0 mV for 150 ms in the presence of 1.5 μM TTX and 50 mM TEA. Each of three HVA calcium currents was measured using specific pharmacological channel blockers (**L**, 10 μM nimod; **N**, 5 μM ω-CgTx; **P**, 50 nM ω-Aga-IVA). Summary graph (D) shows the mean (±SE) percent distribution of L-, N-, P-type calcium currents for each population tested. The hLIF(+S) (n=19) mean calcium current profile is different from the mLIF(+S) (n=20) and rCNTF(+S) (n=17) calcium current profiles with a significantly larger ω-CgTx-sensitive current ($p<.05$) and a smaller ω-Aga-IVA-sensitive current.

The calcium current profiles noted in the sympathetic and sensory neurons were different from each other and similar to the profiles of the NC-neurons differentiated in the presence of either hLIF(+S) or mLIF(+S) and rCNTF(+S), respectively. To determine if the differences between these groups were statistically significant, we used a two-level mixed-model analysis of variance (MANOVA; first level between cell types and second level within calcium channel types). Five cell types were tested: sensory, sympathetic, neural crest (mLIF(+S)), neural crest (hLIF(+S)), and neural crest (rCNTF(+S)). For each cell type, we determined the calcium current profile for 14-20 individual neurons. Based on this analysis, there was a significant interaction between the channel types and the neuron type ($p<.0001$) and a significant difference between the populations tested ($p<.001$). Therefore, different classes of neurons exhibit unique calcium current profiles. To determine if the NC-neurons were sensory or sympathetic-like in their calcium current physiology, we did an a posteriori analysis (Student-Newman-Keuls test), which does a multiple comparison of the mean calcium current percentages for each cell type (Sokal and Rohlf, 1969). For the N-type current percentage, nSCG and hLIF(+S) were equal and greater than nDRG, mLIF and rCNTF, which were equal ($p<.05$). L-type and P-type current percentages were not significantly different. This more conservative analysis did not identify the difference in the P-type current percentage noted in the neonatal analysis. Exploring further, we overlaid the mean ± the 99%-confidence intervals for the sympathetic and sensory neuron N- and P- type data with a scatter plot of the NC-neuron data (Fig. 3). We found that most of the hLIF(+S) NC-neuron calcium current data cluster in the range of sympathetic neurons and the mLIF(+S) and rCNTF(+S) NC-neuron calcium current data predominantly lie within the sensory range. Some NC-neurons from each growth condition exhibited calcium current profiles of the opposing phenotypic range. Based on the significance of the MANOVA analysis and the comparison of mean current data, nSCG and hLIF(+S) calcium current profiles were the

same and different from the calcium current profiles of nDRG, mLIF(+S) and rCNTF(+S) neurons, which were the same.

Our finding that growth factors regulate the type of neuron found in neural crest cultures can result from a number of different processes. One possibility is that the growth conditions result in the selective survival of one class of neuron. To test this, we cultured neural crest cells in hLIF and mLIF (± S) and screened the cultures with a TUNEL assay after 24 hours. We screened for apoptotic nuclei at this time since, once differentiated, very few neurons subsequently die off under these growth conditions. Therefore, if an apoptotic process was removing a neuronal population, it would have to act during or soon after the neurons differentiate. We found a small percentage of labeled nuclei in both conditions (8 ± 3% hLIF; 6 ± 2% mLIF). To determine if the apoptotic nuclei represented the loss of a specific subpopulation of progenitor cells, we examined the cultures for immunoreactivity (-IR) to Mash-1, a transcription factor expressed by autonomic precursors (Sommer *et al.*, 1995). We detected Mash1-IR in mLIF and hLIF cultures at 1-3 days *in vitro* (not shown), suggesting that a selective loss of this class of progenitor cell was not occurring. Finally, it is possible that a single class of neuron is differentiating in neural crest cultures and the calcium current profile is modified by the growth factors tested. We address this possibility in the following section.

Calcium current profiles of sensory and sympathetic neurons do not change when cultured with hLIF or mLIF

The finding that the calcium current profile of NC-neurons could be environmentally regulated to resemble that of neonatal sensory or sympathetic neurons suggests that it may be an indicator of phenotype. For this to be true, it must be shown that a single population of neurons, sensory or sympathetic, does not modify their calcium currents in

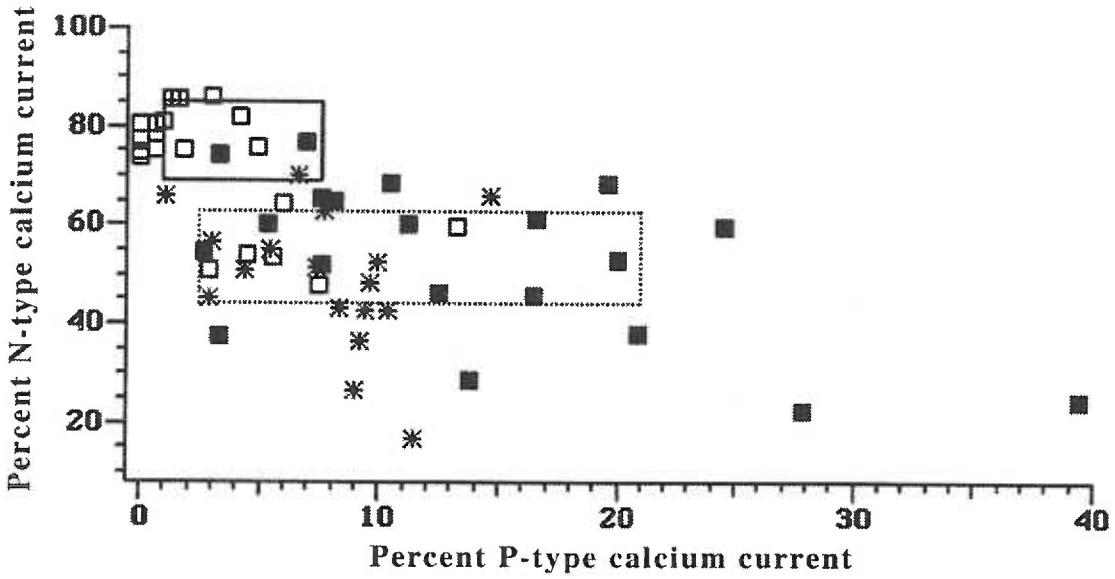

Figure 3. Environmental factors regulate calcium current profiles of NC-neurons to resemble sensory or sympathetic phenotypes. The 99%-confidence intervals for sympathetic (solid box) and sensory (dashed box) neurons for N- and P-type calcium current percentages are plotted here. A scatterplot of N- and P-type calcium current data for NC-neurons culture in hLIF(+S) (open squares), mLIF(+S) (closed squares), and rCNTF(+S) (asterisks) overlay the sympathetic and sensory confidence intervals. The N- and P-type calcium current data for hLIF(+S) NC-neurons cluster around the sympathetic range, while mLIF(+S) and rCNTF(+S) NC-neurons overlie the sensory range. Some individuals from each growth condition overlie the opposing range.

response to the same environmental stimuli that produces sensory- or sympathetic-like NC-neurons. To test this, we cultured neonatal neurons from DRG and SCG under conditions that produced sympathetic-like (hLIF) and sensory-like (mLIF) neurons from cultured neural crest cells. After 7-10 days in culture, we characterized the calcium current profiles and found that both neuron types were unchanged by the culture environment, with both long-term and acutely dissociated cells having identical calcium current profiles (Fig. 4; $p<.001$; MANOVA). To determine if plasticity of calcium currents was present at earlier stages of development, we cultured embryonic DRG neurons at a stage when the neurons are postmitotic but within 24 hours of their birth (embryonic day 12). The calcium current profile of these neurons cultured in hLIF(+S) or mLIF(+S) were also identical to acutely dissociated neonatal sensory neurons (Fig. 4).

mLIF(+S) and hLIF(+S) differentially affect the expression of a POU-domain transcription factor, Brn 3.0, and capsaicin-sensitivity in NC-neurons

Our physiological analysis of the neural crest cultures indicates that the different growth factors sustain neuronal populations that are predominately sensory-like (mLIF) or sympathetic-like (hLIF) in phenotype. Our recordings allowed us to analyze individual neurons in detail, however, we could only sample a small number of cells in a given culture. Therefore, we have used two cytochemical assays to screen the cultures for additional neuronal traits. The first assay screened for the presence of capsaicin sensitivity, a trait confined to a subpopulation of sensory neurons. Our second assay screened for immunoreactivity of a transcription factor specific for sensory neurons, Brn 3.0 (Fedtsova and Turner, 1995).

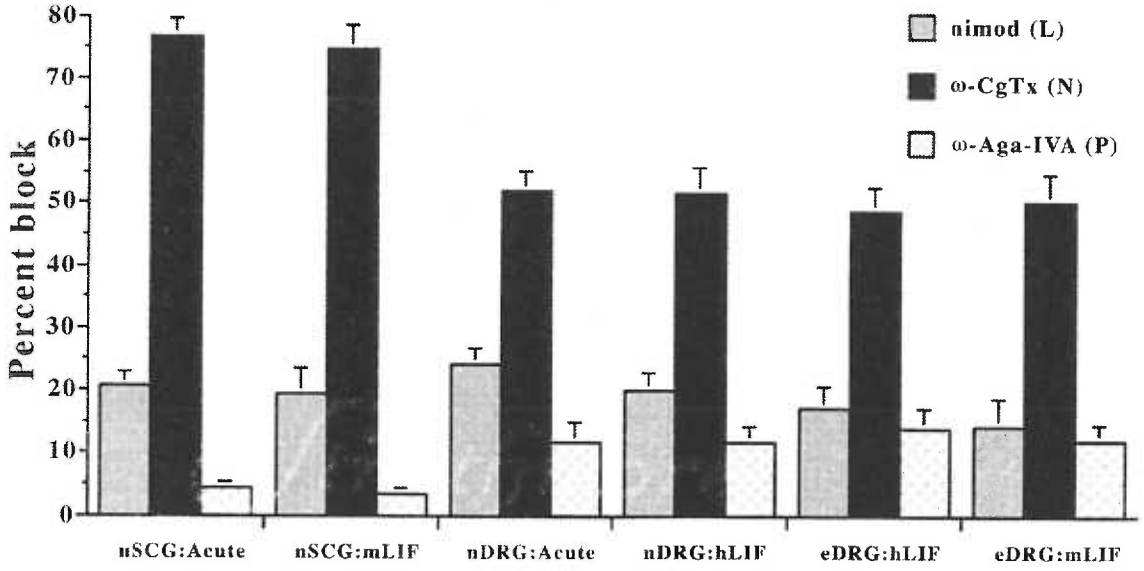

Figure 4. Sensory and sympathetic neurons do not change their HVA calcium current profiles in response to growth factors that influence NC-neuron phenotype. Calcium current profiles were determined for SCG and DRG neurons either acutely isolated or cultured in hLIF or mLIF for 1-2 weeks. Culturing neonatal DRG (nDRG) in the presence of hLIF or mLIF did not modify their mean (±SE) calcium current profile. Culturing embryonic day 12 DRG (eDRG) in the presence of hLIF(+S) or mLIF(+S) did not modify their mean (±SE) calcium current profile, which were identical to nDRGs (MANOVA; $p<.001$). Similarly, mLIF and hLIF had no effect on the mean (±SE) calcium current profile of neonatal SCG (nSCG) neurons.

A characteristic of a subpopulation of sensory neurons is the expression of capsaicin-sensitivity (Fitzgerald, 1983; Lynn *et al.*, 1984; Winter, 1987). Application of capsaicin results in the opening of a nonselective cation channel in a subpopulation of mammalian sensory neurons (Oh *et al.*, 1996). Our laboratory has previously shown that a small percentage of neurons differentiating from cultured neural crest cells in EE-medium, exhibit sensitivity to capsaicin (Matsumoto, 1994b). To determine if hLIF(+S) or mLIF(+S) differentially affect capsaicin responsiveness in neural crest cultures, we used a histochemical assay to determine if capsaicin-sensitive neurons were present in either growth condition (Fig. 5 A and B). neural crest cells cultured in hLIF(+S) had a small percentage (3-10%) of neurons expressing capsaicin-sensitivity (Fig. 5C). However, neural crest cells cultured in mLIF(+S), our sensory environment, had a greater percentage (57%) of the neurons expressing capsaicin-sensitivity, indicative of a sensory phenotype (Fig. 5C).

Recent studies have described the expression of transcription factors by specific types of neurons in the PNS (Bang and Goulding, 1996; Groves and Anderson, 1996). One of these, Brn 3.0, is expressed by virtually all sensory neurons of the DRG and is not observed any population of autonomic neurons (Fedtsova and Turner, 1995; McEvilly *et al.*, 1996). We first detected Brn 3.0 expression shortly after the birth of the neuron, and its expression is maintained indefinitely. We have used antisera raised against Brn 3.0 and found its expression in virtually all sensory neurons cultured from dissociated DRGs, regardless of the culture conditions (data not shown). When neural crest cultures were grown in hLIF or mLIF, we observed a distribution of Brn 3.0-immunoreactivity (IR) that correlated with our calcium current analysis (Fig. 6). Fewer than 5% of the neurons (±1.3) cultured in hLIF were Brn 3.0-IR (Fig. 6 A, B, D, E), while virtually all neurons (>98%) in mLIF were Brn 3.0-IR (Fig. 6 C, F). Thus, the distribution of Brn 3.0 and

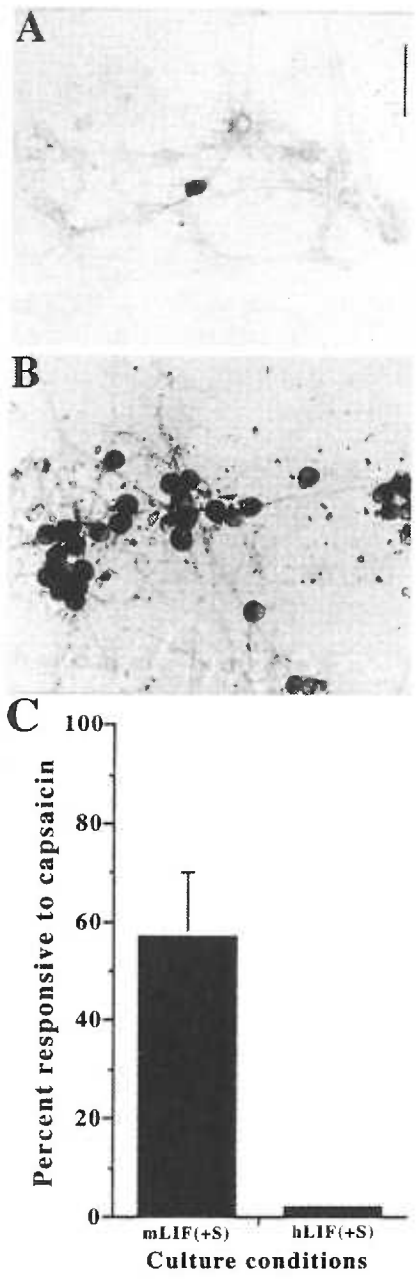

Figure 5. Neural crest cultured with mLIF(+S) produced a greater percentage of capsaicin-sensitive NC-neurons than hLIF(+S). Histochemical assay for capsaicin-sensitivity of NC-neurons cultured in hLIF(+S) (A) or mLIF(+S) (B); calibration = 80 μm. (C) Mean (±SE) percentage of NC-Neurons displaying capsaicin-sensitivity for NC cultured in either mLIF(+S) or hLIF(+S). mLIF(+S) had a greater percentage of capsaicin-sensitive NC-neurons than hLIF(+S), consistent with a sensory-like phenotype.

capsaicin-sensitivity are consistent with our calcium current analysis, indicating the differentiation of sensory-like (mLIF) and sympathetic-like (hLIF) neurons.

DISCUSSION

NC-neurons exhibit calcium current profiles of sensory and sympathetic neurons

Many classes of neuron in the central and peripheral nervous systems (CNS and PNS) exhibit distinctive calcium current profiles (Mintz et al, 1992). In the PNS, sensory neurons of the DRG and sympathetic neurons of the SCG have both qualitative and quantitative differences in their calcium currents. Quantitative differences are found with respect to the N-type calcium current contribution (sympathetic >> sensory), while a qualitative difference is found with respect to P-type calcium currents, which constitute a percentage of calcium current in sensory neurons but is absent in sympathetic neurons. We have shown in this study that similar differences are found in mouse sensory and sympathetic neurons, with the exception that mouse sympathetic neurons exhibit a small (4%) contribution from a P-type conductance (Fig. 1C). The difference in calcium currents even when taking into account that DRGs and SCGs are comprised of neurons subserving a variety of functions. Scroggs and Fox (1992), for example, have shown that although sensory neuron subpopulations exhibit varying L-type current percentages (52.9%, 6.6%, and 19.4%), N-type current percentages did not vary significantly across sensory phenotypes.

Our results indicate that NC-neurons exhibit sensory- or sympathetic-like calcium current profiles *in vitro*; these observed profiles are dependent on the culture conditions.

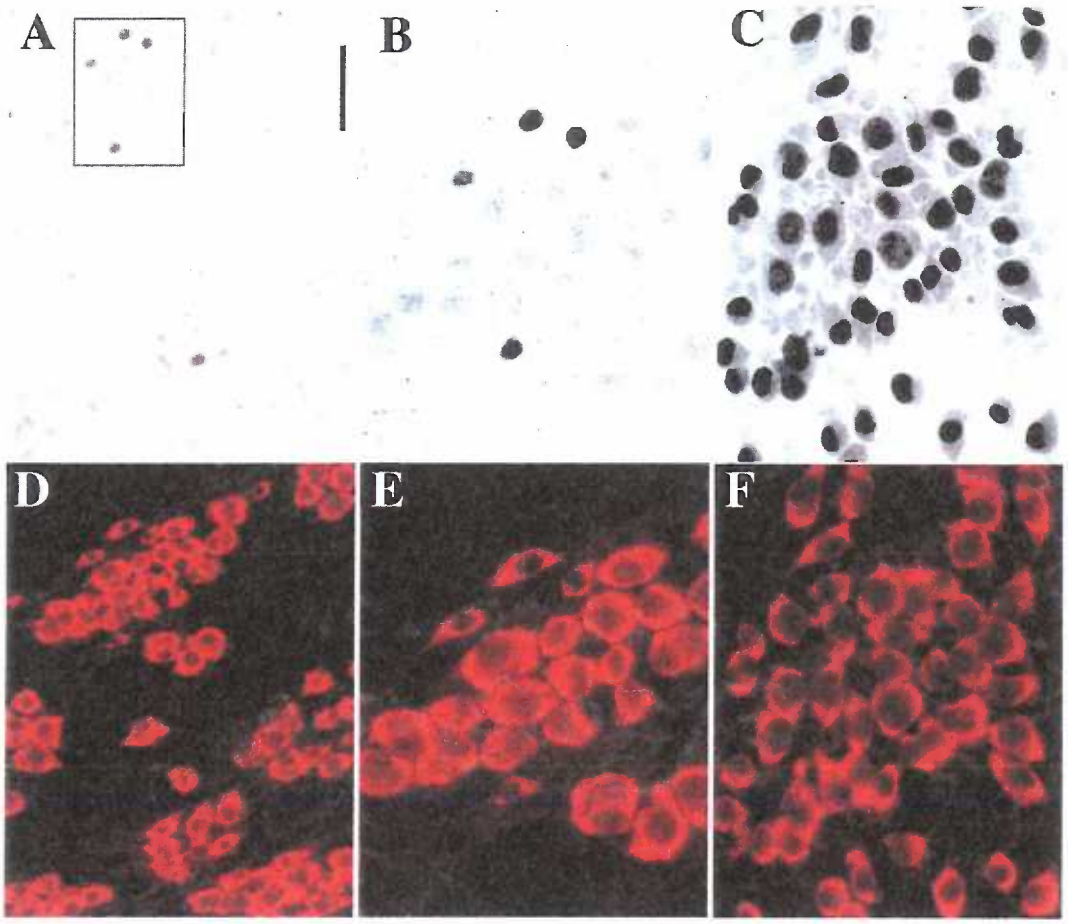

Figure 6. Neural crest cultured with mLIF produced a greater percentage of Brn 3.0-IR neurons than hLIF. Photomicrographs of NC cultures labeled with antisera raised against the sensory neuron specific transcription factor Brn 3.0 (A-C) and the pan-neuronal marker Hu (D-F). Brn 3.0-IR in the nuclei of select neurons was detected with HRP. Hu-IR, which is present in the cytoplasm of all neurons, was detected using rhodamine-conjugated secondary antisera. Cultures raised in hLIF (A, B, D, E) had a small percentage of Brn 3.0-positive neurons. The area outlined in (A) is shown at higher magnification in (B, E). In contrast, virtually all neurons display Brn 3.0-IR when NC cells were cultured in mLIF (C, F). The calibration bar equals 100 μm in (A, D) and 50 μM in (B, C, E, F).

We found that culturing neural crest cells in the presence of hLIF(+S) produced NC-neurons that exhibited calcium current profiles identical to sympathetic neurons , while culturing neural crest cells in the presence of mLIF(+S) or rCNTF(+S) produced NC-neurons with sensory-like calcium current profiles (Fig. 3). It is not clear from our experiments whether these culture conditions are acting instructively or permissively to produce these different classes of neuron. Attempts to address this question by combining growth conditions or switching from one to the other produced inconclusive results (data not shown). Examination of the cultures with a TUNEL assay did not reveal evidence for selective cell death; however, the loss of a small population of progenitor cells could have been easily missed.

Our study suggests that characteristic repertoires of HVA calcium currents can develop autonomously in sensory and sympathetic neurons and that the growth conditions we are using do not modify the relative contributions of these conductances to a neurons total HVA calcium current. We have shown that the HVA calcium current profiles of neonate and embryonic peripheral neurons were not plastic, demonstrating that our sensory-like (mLIF) or sympathetic-like (hLIF) growth conditions do not modify HVA calcium current profiles of postmitotic neurons (Fig. 4). Another study has shown in PC12 cells that modifying tyrosine kinase receptor expression or exposing the cells to different neurotrophins varies the extent of morphological differentiation and sodium current expression; however, the HVA calcium currents did not vary (Sherwood *et al.*, 1997). Apparently, once a PC12 cell is committed to differentiate into a neuron, its characteristic calcium current profile is irreversibly expressed. This has also been shown in *Xenopus* neurons and muscle, where once a factor initiated the differentiation process, the cell would develop characteristic voltage-dependent channels autonomously (Henderson and Spitzer, 1986). Not all calcium currents are insensitive to external factors. It has been shown, for example, that NGF can upregulate L-type channel expression in sympathetic

neurons (Tanaka and Koike, 1995) and, during target innervation, large diameter sensory neurons will downregulate P-type channel expression and upregulate Q-type channels.

Expression of a capsaicin response and Brn 3.0 immunoreactivity correlates with the distinctive calcium current profiles of NC-neurons

The appearances of a capsaicin response and Brn 3.0-IR in mLIF(+S) NC-neurons are also consistent with sensory neuronal differentiation (Fig. 5). A subpopulation of sensory neurons expresses capsaicin receptors (Oh *et al.*, 1996; Wood *et al.*, 1988); Nerve growth factor (Bevan and Winter, 1995; Winter *et al.*, 1988) and brain-derived neurotrophic factor (Winter, 1998) can upregulate the expression of these receptors in sensory neurons. The addition of either of these neurotrophins, however, will not induce the expression of capsaicin receptors in sympathetic neurons (Winter *et al.*, 1988). In our study, 57% of the mLIF(+S) NC-neurons displayed a response to capsaicin, while hLIF(+S) had only 3% of the NC-neurons display a response. We do not know if our growth conditions upregulate the expression of capsaicin receptors or select for the survival of neurons expressing the capsaicin receptor. Further studies are needed to address this question. Our findings that Brn 3.0-IR correlates with HVA calcium current profiles suggest a possible link between its expression and that of specific calcium channels. This is a link that can now be explored with the development of Brn 3.X knockout mice (Erkman *et al.*, 1996; McEvilly *et al.*, 1996; Xiang *et al.*, 1998; Xiang, 1998).

Growth factors that activate the same receptor subunits show different effects on the differentiation of cultured neural crest cells

Neural crest cells cultured in hLIF(+S) produced sympathetic-like neurons while mLIF(+S) produced sensory-like neurons and CNTF (+S) produced a mixed population

(Fig. 4, A and B). It is interesting that we observed these different effects since all three factors activate the same JAK/STAT signal transduction pathway via coordinated binding with the gp130 receptor subunit (Ip *et al.*, 1992; Kishimoto *et al.*, 1995). There are other reports of differences in the biological activities of species-specific LIFs and CNTF. Rat DRG neurons assayed in a survival assay, for example, were found to be nearly 100 times more sensitive to hLIF than mLIF with CNTF effective at intermediate doses (Simon *et al.*, 1995). The differential effects of hLIF and mLIF are very interesting since these proteins are highly homologous at the amino acid level (80%) with many of the differences being conservative substitutions (Gough *et al.*, 1988). There are, however, significant differences in the receptor-binding domain of these proteins. This difference, a key change of only six amino acids (Layton *et al.*, 1994b)), is responsible for hLIF's greater affinity (100-500X) and lower kinetic dissociation rate than mLIF's for the mLIF receptor, and the inability of mLIF to bind to the hLIF receptor (Layton *et al.*, 1994a; Owczarek *et al.*, 1993). This difference in ligand binding may alter signal transduction by changing the rate of receptor internalization, or by binding of other factors to the receptor complex (for reviews, (Hilton *et al.*, 1992; Marshall, 1995)). Consistent with the possibility that the affinity of the LIF ligand to the receptor is important is our observation that we could not mimic the effect of hLIF by altering the concentration of mLIF from 1-100 ng/ml (data not shown).

We observed an additional effect on NC-neuronal differentiation when co-culturing the neural crest cells with somite cells. Studies have shown that somite cells can influence the differentiation of neural crest cells (Elkabes *et al.*, 1994; Krull *et al.*, 1997; Matsumoto *et al.*, 1993). In our initial analysis of the calcium currents (table I), we noted that to achieve the highest average N-type calcium current contribution, both hLIF and somite cells were required (Table 1). When the somite cells were absent, however, the NC-neurons with the highest percentage of N-type current were lost and the distribution

shifted towards mLIF and the sensory neurons (Table 1). Therefore, the difference in the action of the LIFs and CNTF maybe indirect, acting on somite cells which then affect neural crest cell differentiation. In the future, it will be necessary to determine how hLIF(+S) confers a sympathetic neuronal phenotype and if an endogenous molecule is present that can emulate this action.

In conclusion, we have shown that NC-neurons exhibit HVA calcium current profiles identical to sensory or sympathetic neurons and that, once established, these profiles are not modified by the growth conditions that produced them. This physiological trait may prove to be useful as a marker for differentiated neurons. NC-neurons exhibit distinctive calcium current profiles before they express neurotransmitters, and, unlike some neurochemicals, the neurons do not change their profiles in culture.

ACKNOWLEDGEMENTS

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CHAPTER II

Spontaneous calcium transients are required for neuronal differentiation of murine neural crest

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ABSTRACT

We have shown that cultured mouse neural crest (NC) cells exhibit transient increases in intracellular calcium. Up to 50% of the cultured neural crest-derived cells exhibited calcium transients during the period of neuronal differentiation. As neurogenic activity declined, so did the percentage of active neural crest-derived cells and their calcium spiking frequency. The decrease in calcium transient activity correlated with a decreased sensitivity to thimerosal, which sensitizes inositol 1,4,5-triphosphate receptors. Thimerosal increased the frequency of oscillations in active neural crest-derived cells and induced them in a subpopulation of quiescent cells. As neurogenesis ended, Neural crest-derived cells became non-responsive to thimerosal. Using the expression of time-dependent neuronal traits, we determined that neurons exhibited spontaneous calcium transients as early as a neuronal phenotype could be detected and continued through the acquisition of caffeine-sensitivity, soon after which calcium transient activity stopped. A subpopulation of not neuronal neural crest-derived cells exhibited calcium transient activity within the same time frame as neurogenesis in culture. Exposing neural crest-derived cells to 20 mM Mg^{2+} blocked calcium transient activity and reduced neuronal number without affecting the survival of differentiated neurons. Using lineage-tracing analysis, we found that 50% of active Neural crest-derived cells gave rise to clones containing neurons, whereas inactive cells never gave rise to neurons. We hypothesize that calcium transient activity establishes a neuronal competence for undifferentiated neural crest cells.

INTRODUCTION

The neural crest consists of a heterogeneous population of multipotent progenitors from which all of the neurons of the peripheral autonomic nervous system and dorsal root ganglia are derived (Le Douarin, 1982). During embryogenesis, these multipotent neural crest cells become restricted to sublineages that generate different parts of the peripheral nervous (Henion and Weston, 1997; LaBonne and Bronner-Fraser, 1998; Stemple and Anderson, 1993; Weston, 1991). Studies have shown that lineage restriction of neural crest cells occurs prior to or soon after onset of migration (Baroffio *et al.*, 1990; Bronner-Fraser and Fraser, 1989; Bronner-Fraser and Fraser, 1988; Frank and Sanes, 1991; Fraser and Bronner-Fraser, 1991; Raible and Eisen, 1994; Sieber-Blum and Cohen, 1980; Vogel and Weston, 1988). This restriction in developmental potential creates intrinsic differences between early neural crest cells that plays an important role in the process of diversification. As yet, we do not understand the mechanisms that regulate lineage-restriction of neural crest cells.

One possible mechanism involves intracellular calcium signaling. Dynamic changes in intracellular calcium have been linked to many developmental processes including proliferation (Berridge, 1995; Petr *et al.*, 1998), differentiation (Ferrari *et al.*, 1996; Jones *et al.*, 1995; Sauer *et al.*, 1998; Spitzer, 1995), and maturation (Sato-Bigbee *et al.*, 1999; Spitzer and Ou, 1997). Calcium regulates many of these processes by modulating signal transduction pathways that activate transcription factors, such as CREB and SRF (Hardingham *et al.*, 1997; Miranti *et al.*, 1995; Sheng *et al.*, 1990). In PC12 cells, calcium regulates CREB activity by modulating the efficacy of the Ras-MAPK pathway at various levels: tyrosine kinase receptor (Rosen and Greenberg, 1996), Ras (Rosen *et al.*, 1994), and Raf (Farnsworth *et al.*, 1995). Experimental manipulations that inhibit the interaction between calcium and the Ras-MAPK pathway perturb various aspects of

neuronal differentiation of PC12 cells, including survival, differentiation and plasticity (for review, (Finkbeiner and Greenberg, 1996). Variations in the calcium signaling parameters, i.e., frequency, duration, amplitude and source of calcium, determine the signal transduction pathway activated and the extent to which it is activated (Bading *et al.*, 1997; Dolmetsch *et al.*, 1997; Dolmetsch *et al.*, 1998; Fields *et al.*, 1997; Sheng *et al.*, 1993). Calcium signaling is distinct between cell types, since the factors that regulate the calcium signaling parameters are differentially expressed (Bennett *et al.*, 1996; De Smedt *et al.*, 1997; DeLisle *et al.*, 1996; Fujino *et al.*, 1995; Giannini *et al.*, 1995; Lievremont *et al.*, 1996; Miyakawa *et al.*, 1999; Sharp *et al.*, 1999; Sugiyama *et al.*, 1994).

Calcium transients are observed during the differentiation of many types of cells; inhibiting these calcium transients affects many aspects of their development. In Xenopus, calcium transients exhibited by myocytes regulate cytoskeletal organization during myofibrillogenesis (Ferrari *et al.*, 1996). Calcium signaling in Xenopus neuroblasts is required for neurogenesis (Jones *et al.*, 1995; Jones and Ribera, 1994) and neuronal maturation: GABA expression (Spitzer *et al.*, 1993), maturation of a K^+ conductance (Desarmenien and Spitzer, 1991), and axon extension (Spitzer, 1995). In the mouse, primitive endodermal cells exhibit calcium transients to regulate exo/endocytotic vesicle shuttling during their differentiation into parietal and visceral endoderm (Sauer *et al.*, 1998). In the subventricular zone, mouse CNS progenitors exhibit calcium transients and the frequency of calcium transient activity increases as they migrate to the neuronal layers (Owens and Kriegstein, 1998).

There have been no published reports describing the role of calcium transients in the development of the mammalian PNS. In this study, we show that cultured mouse neural crest-derived cells exhibit spontaneous calcium transients. Furthermore, these cells exhibit calcium transients only during the period of neuronal differentiation. We have

determined, using immunocytochemistry and calcium pharmacology, that developing neurons exhibit calcium transient activity as well as a subpopulation of not neuronal neural crest-derived cells. Using elevated extracellular Mg^{2+}, we provide evidence that blocking calcium transient activity inhibits neurogenesis without effecting neuronal survival or cell proliferation. Finally, using lineage-tracing dyes, we show that neural crest-derived cells that exhibit calcium transients have the potential to differentiate into neurons, while inactive cells do not.

RESULTS

Cultured neural crest cells exhibit spontaneous calcium transients

Using calcium-imaging, we monitored intracellular calcium in primary neural crest cultures to determine if neural crest-derived cells exhibited transient increases in intracellular calcium. To do this, we loaded neural crest-derived cells with the calcium indicator dye, Oregon-Green 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid - 1 (OGB), and monitored their intracellular calcium levels for one hour, capturing an image every 3 seconds (see Methods). An example of a recorded field is shown in figure 1A. To demonstrate that the calcium fluctuations can be ascribed to individual cells, some cultures were fixed and labeled with propidium iodide to show that there was little overlap of individual cells (compare Figs. 1B and 1C). The generation of calcium transients appeared to be a property of individual cells. We did not observe synchronous activity among groups of cells, which would indicate a wave of activity traversing coupled cells. Furthermore, we did not detect the presence of dye-coupling (Lo, 1996; Huang *et al*, 1998), when we injected Lucifer yellow into active cells (not shown).

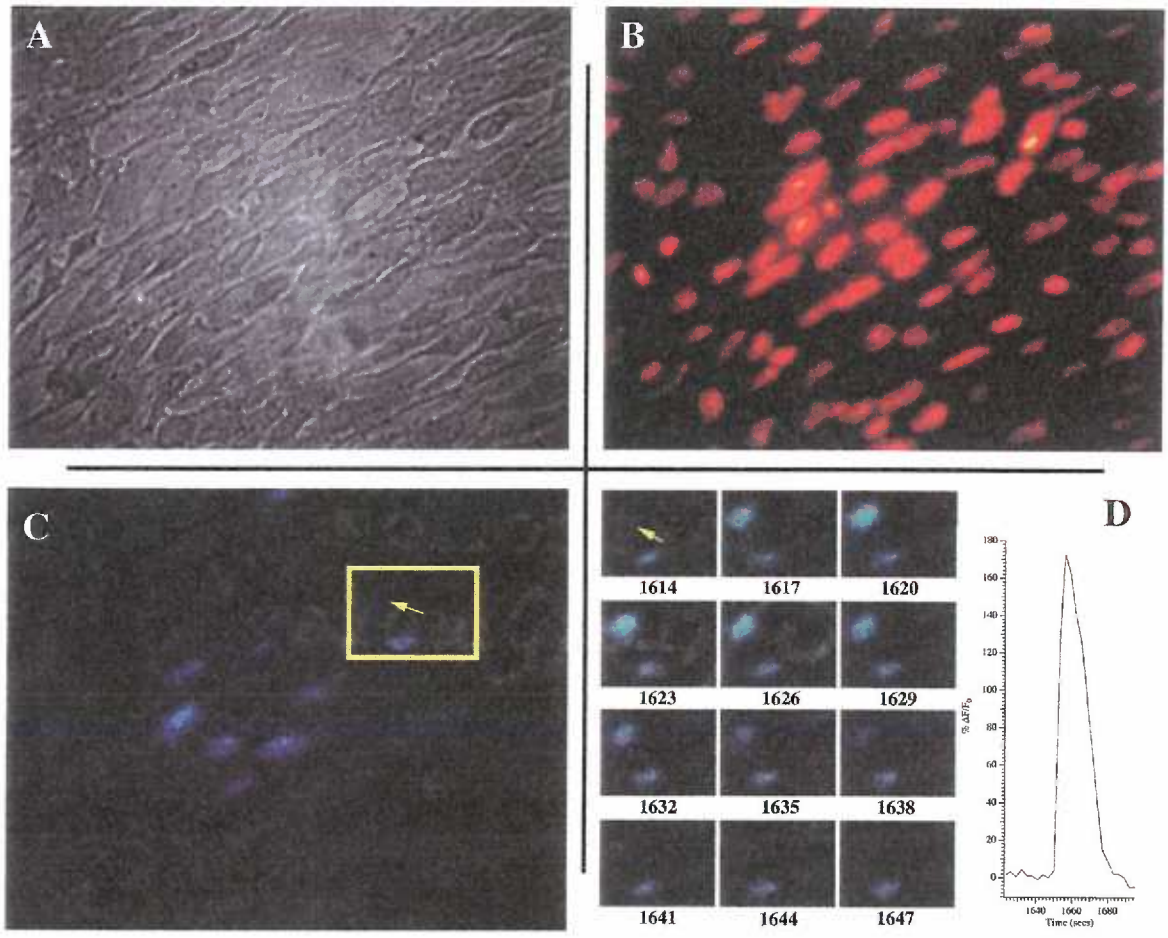

Figure 1. NC-derived cells exhibited spontaneous calcium transients in culture. (A). 40x phase image of a calcium-imaged field. (B). Propidium iodide labeling of the same field as A. Calcium transients can be ascribed to individual cells since there was little overlap in nuclear-labeling. (C). 40x fluorescent image of intracellular calcium using the calcium indicator dye, OGB. (D). Single cell exhibiting a calcium transient. Sequence of calcium images, starting at time 1614, showing a cell (yellow arrow) from the field in C (yellow rectangle) exhibiting a single calcium transient event. The pixel values were converted to % $\Delta F/F_0$ (see methods) and plotted versus time.

Many cells exhibited spontaneous increases in intracellular calcium. To analyze the data, the digitized pixel values were converted to percent change in fluorescent intensity over the baseline fluorescence (% $\Delta F/F_0$) and plotted versus time (Fig. 1D). We observed two types of calcium waveforms (Gu *et al.*, 1994): spikes and waves (Fig. 2A). Calcium spikes reached their peak within 10 seconds of onset and then declined over a period of 25 to 60 seconds. The average duration of a calcium spike was 46.3 ± 2.6 (mean ± SEM) seconds. Most calcium spiking cells (77%) exhibited 1-4 calcium spikes/hour (Fig. 3). However, many cells exhibited a higher frequency of calcium spiking (Fig. 2B), up to 96 spikes/hr (Fig. 3). The other calcium waveform exhibited by Neural crest-derived cells was the calcium wave, which had a relatively slow onset compared to the spike, >60 seconds, and a slow return to baseline, >60 seconds (Fig. 2A). The duration of these events was quite variable with a range of 120 to 360 seconds, much longer than the calcium spike. Some cells exhibited bursts of calcium spikes that may or may not have been accompanied by calcium waves (Fig. 2C).

Cultured Neural crest-derived cells exhibit spontaneous calcium transients during the period of neuronal differentiation.

Neural crest-derived cells exhibited spontaneous calcium transients during the period that neurons are born in this culture system (Matsumoto, 1994a). We have shown previously that neural crest cells differentiate into either sensory or sympathetic neurons, depending on the culture environment (Carey and Matsumoto, 1999a). Their ability to differentiate into neurons is restricted to the first six days in culture with the greatest sensitivity to differentiation factors occurring the first four days (Matsumoto, unpublished results). After seven days, few neurons are produced.

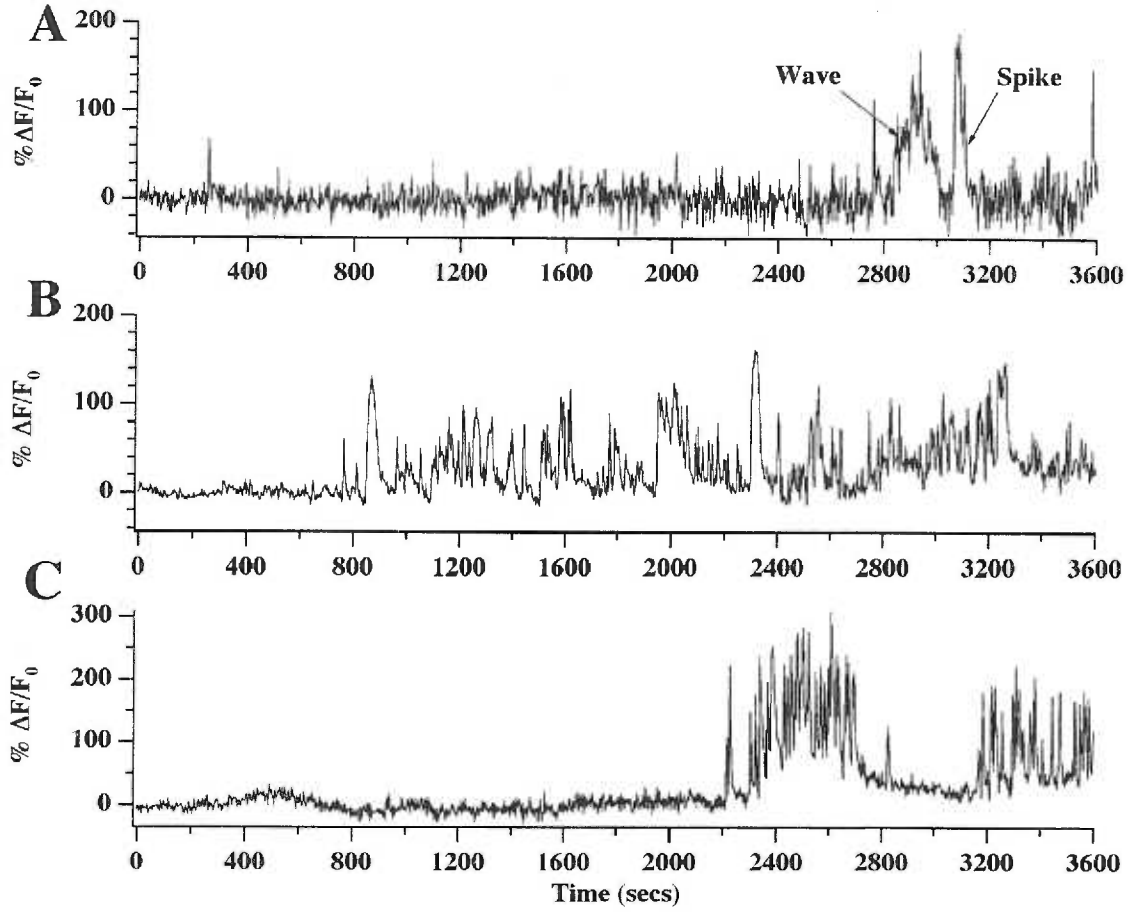

Figure 2. NC-derived cells exhibited calcium spikes and/or calcium waves with varying frequencies and patterns. (A). A single cell exhibiting a calcium spike and a calcium wave. Calcium waves were defined as calcium influxes that reached their peak values with a time course greater than 60 seconds and returned to baseline with a similar time course, total duration >180 seconds. Calcium spikes were defined as calcium influxes that reached their peak values within 5-15 and returned to baseline in less than 60 seconds, total duration <75 seconds. B. A single cell exhibiting a high frequency of calcium spiking activity. C. A single cell exhibiting two bursts of calcium spike activity. Bursts of calcium spikes may (first burst) or may not (second burst) be accompanied by an underlying calcium wave.

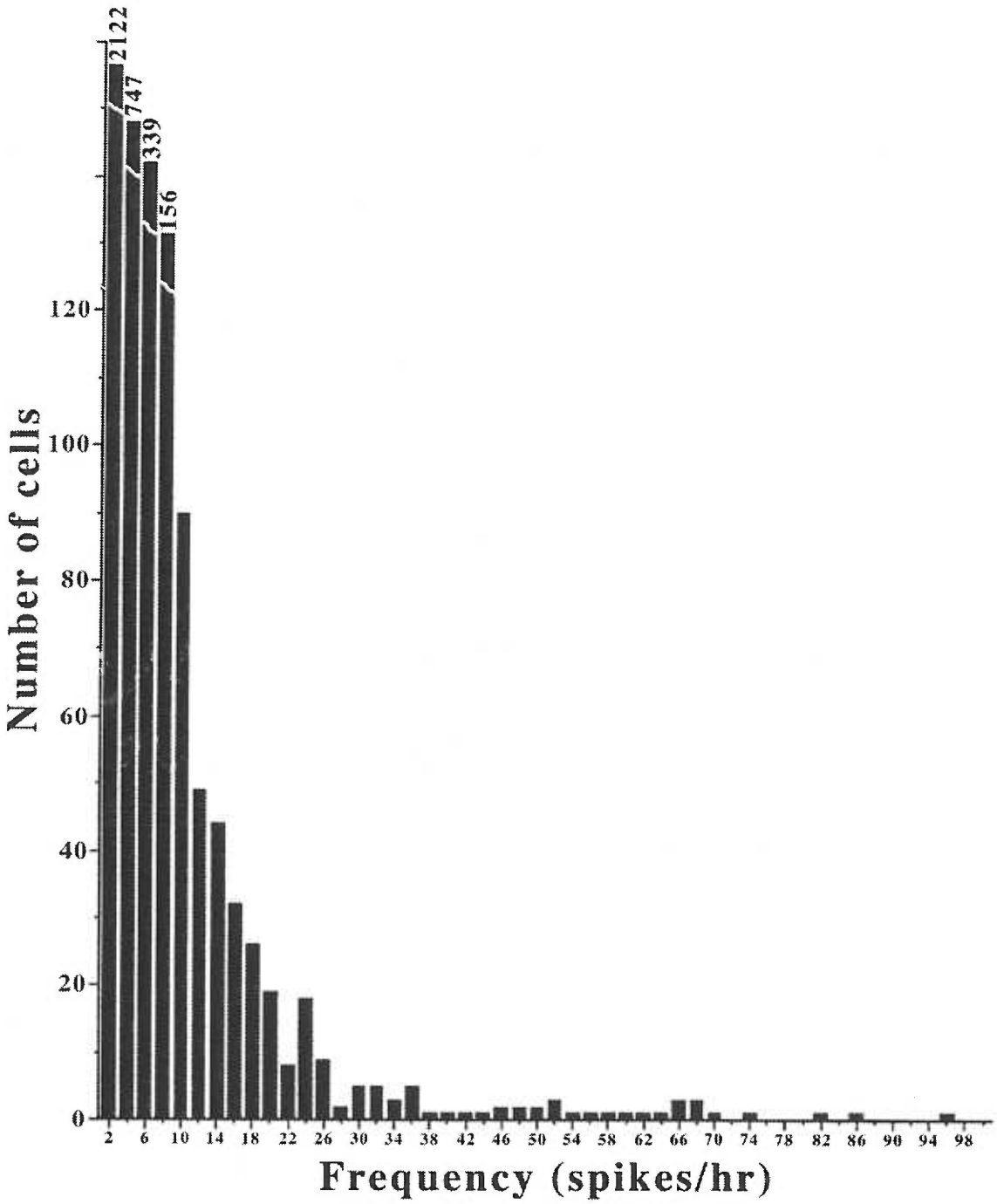

Figure 3. Distribution of calcium transient frequencies for all NC-derived cells that exhibited calcium transients for all days in culture. Most active NC-derived cells exhibited <3 calcium spikes/hr with some cells exhibiting up to 96 spikes/hr.

Neural crest-derived cells exhibited spontaneous calcium transient activity with the same time course as this neurogenic period. During the first 2 days in culture, 47.6 ± 3.5% of the cells in a recorded field exhibited calcium transient activity (Fig. 4A). The percentage of active cells decreased slightly during days 3 and 4 (38.4 ± 4.0%). The average percentage of active cells dropped significantly after 4 days in culture, falling to values below 20% ($p<.001$). Similarly, the frequency of calcium spikes in active cells was highest the first six days in culture (Fig. 4B). During the first 2 days in culture, Neural crest-derived cells averaged 3.7 ± 0.1 spikes/hr. The frequency of spikes in our sampled cells increased significantly to 4.8 ± 0.2 spikes/hr the next two days ($p<.001$), and decreased to 3.5 ± 0.5 spikes/hr on days 5 through 6 (Fig. 4B). Calcium-spiking frequency declined significantly after 6 days to an average frequency of 2.5 spikes/hr ($p<.001$). By day 10 in culture, most active cells exhibited only 1 calcium spike/hr (not shown).

Thimerosal induces calcium oscillations in neural crest-derived cells during the period of neuronal differentiation.

Less than 50% of the neural crest-derived cells in a recorded field exhibited calcium transients during days 1-2 in culture (Fig. 4A). To determine whether inactive neural crest-derived cells were capable of calcium transient activity, we used thimerosal to induce calcium oscillations. Thimerosal is a thiol reagent that sensitizes the inositol 1,4,5,-triphosphate (IP_3) receptor (IP_3R) inducing cytoplasmic calcium oscillations in those cells capable of IP_3-mediated calcium-induced calcium release (Bootman *et al.*, 1992; McDougall *et al.*, 1993). We have determined that the IP_3R channel is responsible for the primary influx of calcium during a calcium transient event (Carey and Matsumoto, submitted). During days 1-2 in culture, almost all neural crest-derived cells, active and quiescent, responded to thimerosal (Fig. 5). This indicated that while less than 50% of

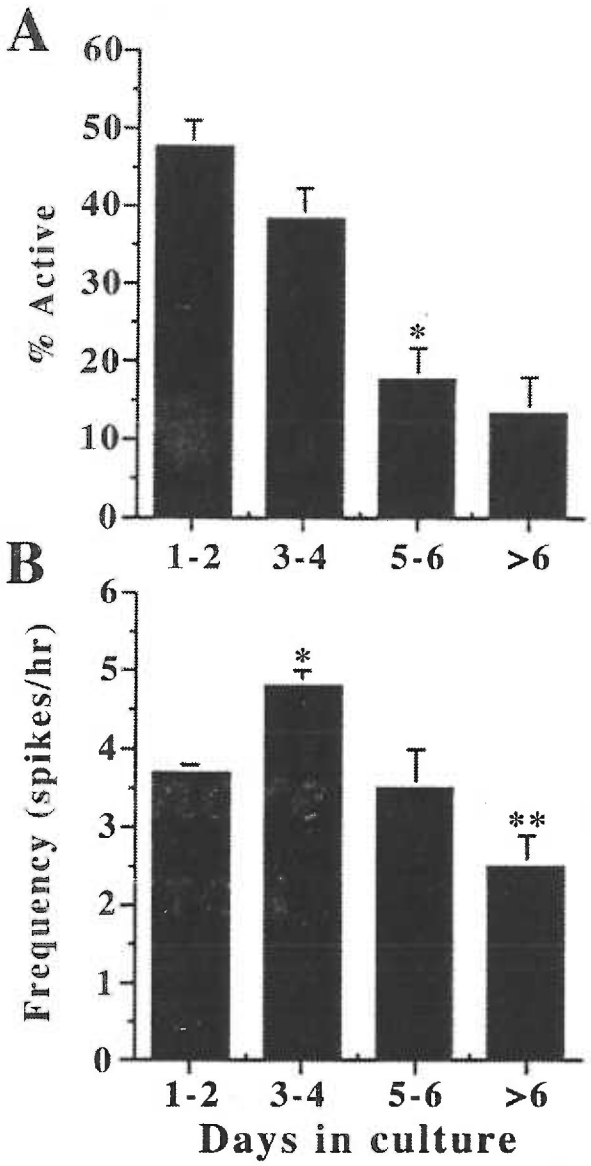

Figure 4. Calcium transient activity decreases with time in culture. Intracellular calcium levels were monitored for 1 hour (every 3 seconds), and we determined for every cell in the recorded field if it exhibited a calcium transient and the frequency at which it exhibited these calcium transients. This was repeated in cultures that were up to 12 days old. A. Mean percent (±SEM) of NC-derived cells that exhibited calcium transients (% Active) versus days in culture. Most calcium transient activity occurred in the first four days of culture (40-50%). After day 4, the percentage of active cells significantly decreased ($p<.001$,*). B. Mean calcium spike frequency (±SEM) for NC-derived cells that exhibited calcium transients versus days in culture. The mean calcium spike frequency peaked days 3-4 (4.7 spikes/hr, $p<.001$,*) and declined after day 6 (2.5 spikes/hr, $p<.001$,**).

recorded Neural crest-derived cells exhibited spontaneous calcium transients at this stage most Neural crest-derived cells had the potential to do so.

The ability to induce calcium oscillations with thimerosal was restricted to a subpopulation of neural crest-derived cells over time in culture (Fig. 3). Thus, while most neural crest-derived cells (active and inactive) responded to thimerosal initially, the percentage of inactive cells that responded to thimerosal dropped to 50% by day 3 (Fig. 5B). This percentage continued to decline with only 6% of the inactive cells sensitive to thimerosal by day 7. Active cells continued to respond to thimerosal through day 4, but dropped off significantly by day 7 (28.6 ± 10.3%). After day 7, All Neural crest-derived cells failed to respond to thimerosal (not shown). It is not clear, why neural crest-derived cells lose their sensitivity to thimerosal. However, the decline in sensitivity corresponded to a loss of neurogenic potential within the population as a whole.

Neurons differentiated in culture exhibited spontaneous Ca^{2+} transients in a stage dependent manner.

As neural crest-derived cells undergo neuronal differentiation, they sequentially acquire many neuronal traits. For example, neurons begin to express the RNA binding protein, Hu, during the earliest stages of neurogenesis (Marusich *et al.*, 1994; Marusich and Weston, 1992). Within 12-48 hrs of their terminal mitosis, neurons begin to express voltage dependent calcium currents (VDCC) (Bader *et al.*, 1983; Gottmann *et al.*, 1988). In our cultures, the first indication of neuronal differentiation is the expression of Hu-immunoreactivity (Hu-IR) followed by acquisition of VDCCs and then sensitivity to caffeine (Fig. 6G). By day 10, all cultured neurons exhibit these three characteristics (not shown).

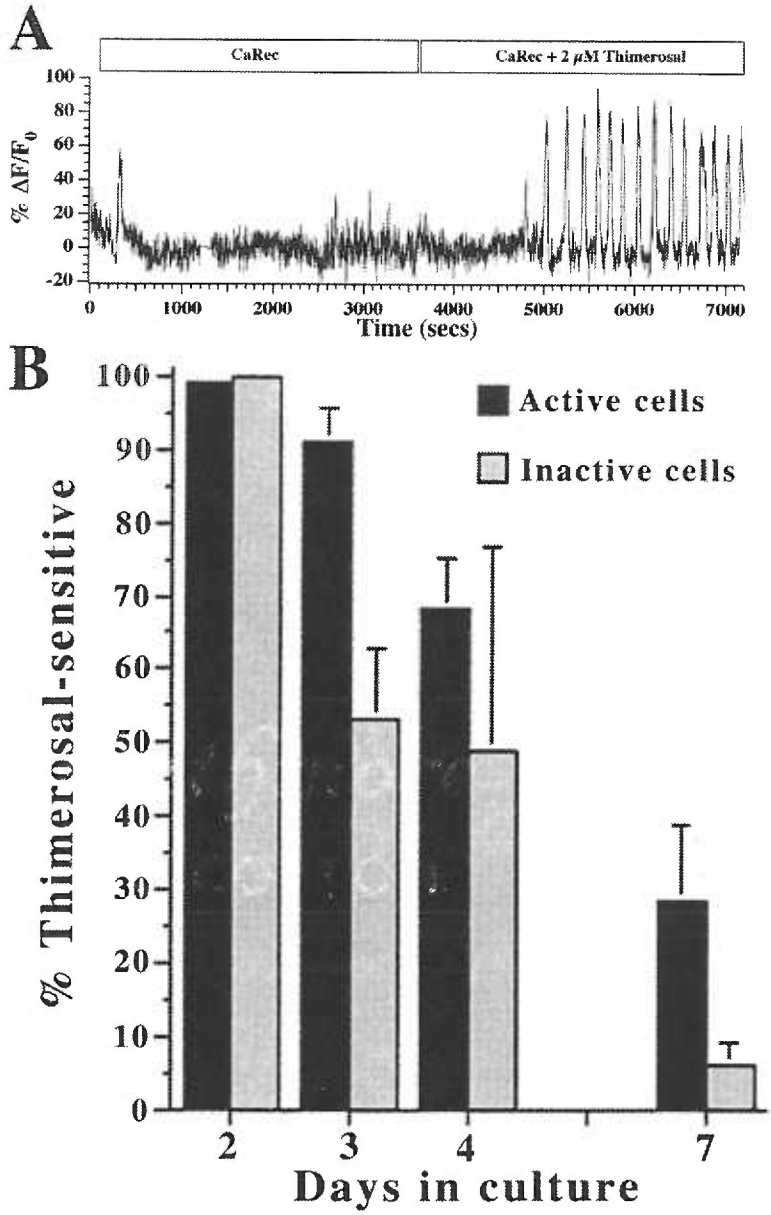

Figure 5. Thimerosal induction of calcium oscillations in cultured NC-derived cells declined over time. A. Intracellular calcium levels were monitored for 1 hour in the presence of normal CaRec. In the 2nd hour, cultures were perfused with CaRec + 2 μM thimerosal. Addition of thimerosal to the perfusion medium induced calcium oscillations in many NC-derived cells. B. The mean percentage of NC-derived cells that responded to thimerosal declined over time in culture. At day 2 in culture, all NC-derived cells in a recorded field responded to thimerosal. By day 3, cells that exhibited calcium transient activity during the first hour (Active cells) continued to respond to thimerosal, while 50% of the cells that did not exhibit calcium transients the first hour (inactive) failed to respond to thimerosal. From day 4 to day 7, both populations declined in sensitivity to thimerosal. After Day 7, no NC-derived cells responded to thimerosal (data not shown).

We can correlate calcium transient activity with the progression of neuronal maturation using these traits. We did this by monitoring intracellular calcium levels for 1 hour and then perfusing, sequentially, 20 mM caffeine and 90 mM KCl, to determine the presence of ryanodine receptor (RyR)-dependent calcium release and VDCC, respectively. Subsequently, we fixed and immunolabeled these cultures with a neuronal marker, Hu (Marusich *et al.*, 1994; Marusich and Weston, 1992). Using a grid coordinate system, we correlated calcium transient activity with the expression of these markers, an example of which is shown in figure 6. The cell identified by the white arrow exhibited calcium transient activity (not shown), responded to caffeine (compare Figs. 6 A and B), responded to high K^+ (compare Figs. 6 A and C), and exhibited Hu-immunoreactivity (Hu-IR; compare Figs. 6 D-F).

Neurons exhibited calcium transient activity throughout the early stages of development and stopped soon after the acquisition of a caffeine response. During the first 4 days in culture, greater than 50% of the recorded neurons exhibited calcium transient activity (Table 1: All). This neuronal population was heterogeneous with respect to their stage of development: Hu-IR only (H only), Hu-IR and a high K^+ responsive (HK), and Hu-IR, a high K^+ responsive and a caffeine responsive (HKC). During days 1-2 in culture, a greater percentage of HK and HKC neurons exhibited calcium transient activity than the earliest neurons (H-only) (Table 1). After day 2, the percentage of neurons that exhibited calcium transients were similar for all stages of development tested. Calcium transient activity in the neuronal population dropped to 33.8 ± 12% after day 4 (Table 1: All). This was due to shift in the composition of the neuronal population to a more mature developmental stage. The percentage of active H-only neurons remained high (75 ± 25%) while HK (36.5 ± 13%) and HKC neurons (32.7 ± 14%) declined. After day 6, few neurons (<10%) exhibited calcium transients (Table 1) with all activity ceased by day 10 (not shown).

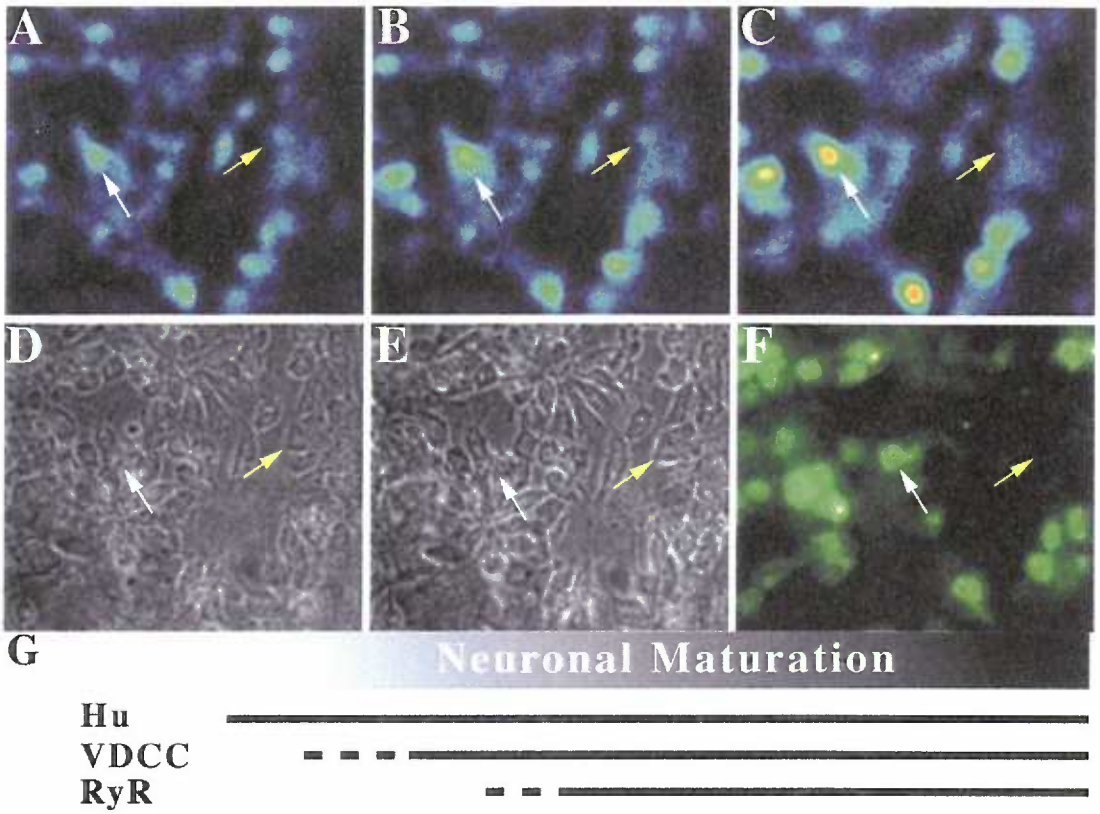

Fig. 6. Correlation of Hu-IR, calcium pharmacology, and calcium transient activity in cultured NC-derived cells. Intracellular calcium levels were monitored for 1 hour. Baseline calcium levels are shown in (A). The cultures were then sequentially exposed to CaRec + 20 mM caffeine (B) and 90 mM KCl modified CaRec solution (C) to determine if these cells had caffeine-sensitive calcium stores and/or high voltage-activated calcium channels, respectively. After calcium-imaging, the cultures were fixed and immunolabeled for the neuronal marker, Hu. Phase images before (D) and after fixation (E) were done to compare the effects of fixation. We then took a fluorescent image of the Hu-labeling (F). We compared the calcium transient activity of each cell with the calcium pharmacology and Hu-labeling, by orienting the culture using the grid coordinates (shadow of which may be seen in D and E). The white arrows identify a cell that exhibited calcium transient activity, responded to both caffeine and high KCl (compare A with B and C, respectively), and was a neuron (Hu+, F). The yellow arrows identify a cell that did not respond to either caffeine or high KCl and was not a neuron (Hu-). (G) Time course of calcium transient activity and acquisition of select neuronal characteristics during the course of maturation.

The frequency of calcium transient activity increased soon after neurogenesis and declined as the neurons matured. During days 1-2, neurons exhibited 2.6 ± 0.3 spikes/hr (Table 1: All). Calcium spiking frequency was similar for all neuronal developmental stages, ranging from 2.0 – 3.1 spikes/hr. After day 2, calcium spiking frequency increased to 4.0 ± 0.6 spikes/hr. This increase was due to an elevation in the calcium spiking frequency for H-only (3.9 ± 0.7 spikes/hr) and HK neurons (6.0 ± 1.8 spikes/hr). HKC neurons did not exhibit an increased spiking frequency, since this population was primarily made up of early differentiating neurons (Matsumoto, 1994a). During days 5-6, calcium spiking frequency increased further to 6.0 ± 1.5 spikes/hr, because of the high calcium spiking frequencies of HK (5.7 ± 2.1 spikes/hr) and HKC neurons (7.0 ± 2.3 spikes/hr). After day 6, the calcium spiking frequency for all neuronal stages decreased to 2.3 ± 0.5 spikes/hr. At this point, most neurons had matured (HKC; 1.6 ± 0.3 spikes/hr). H-only and HK neurons continued to exhibit elevated calcium spiking frequencies (5.0 ± 2.1 and 3.5 ± 2.5 spikes/hr, respectively). In summary, neurons exhibited calcium transient activity early in development that increased in frequency, as they acquired high K^+ and caffeine responses, and then stopped as they continued to mature.

Calcium transient activity in neural crest-derived cells correlated with neurogenic potential in culture.

We have shown that cultured neural crest cells can give rise to either sensory or sympathetic neurons depending on the growth conditions (Carey and Matsumoto, 1999a). For both neuronal populations, neurons are generated over the first 4 days in culture. The production of neurons then gradually declines until by day 7 neurogenesis ends. Although both sensory and sympathetic progenitors are present in these cultures, the growth conditions we used only supported sensory neuronal differentiation. The

Table 1. Calcium transient activity in neurons

% Active

Days in	Neurons			
Culture	All	H-only	HK	HKC
1 to 2:	56.4 ± 5.4	31.7 ± 8.1	73.9 ± 8.1	66.4 ± 7.3
	(16)	(16)	(15)	(15)
3 to 4:	52.1 ± 11	40.8 ± 16	55.0 ± 12	47.1 ± 13
	(9)	(7)	(8)	(8)
5 to 6:	33.8 ± 12	75 ± 25	36.5 ± 13	32.7 ± 14
	(6)	(2)	(6)	(6)
≥6:	9.8 ± 5.4	25 ± 25	22.9 ± 20	6.7 ± 3.0
	(6)	(4)	(5)	(6)
Frequency				
1 to 2:	2.6 ± 0.3	2.0 ± 0.2	2.2 ± 0.2	3.1 ± 0.5
	(303)	(62)	(88)	(153)
3 to 4:	4.0 ± 0.6	3.9 ± 0.7	6.0 ± 1.8	2.7 ± 0.6
	(70)	(15)	(21)	(34)
5 to 6:	6.0 ± 1.5	2.0 ± 0.6	5.7 ± 2.1	7.0 ± 2.3
	(74)	(5)	(38)	(31)
≥6:	2.3 ± 0.5	5.0 ± 2.1	3.5 ± 2.5	1.6 ± 0.3
	(19)	(3)	(2)	(14)

Note: Neurons (All) were analyzed based on calcium pharmacology: HK = responded to 90 mM KCl, HKC = responded to both caffeine and KCl, and H-only = did not respond to either caffeine or KCl. Data reported as mean ± SEM. Numbers in parentheses are total number of cultures (% Active) or total number of cells (Frequency).

sympathetic progenitors do not differentiate unless exposed to a different culture medium (Matsumoto, unpublished results). However, sympathetic progenitors maintain their competence to differentiate for 6 days in this culture environment (Matsumoto, 1998; Matsumoto, unpublished results).

Calcium transient activity in non-Hu-IR (H-) neural crest-derived cells, i.e., neural crest-derived cells that are not neuronal, coincided with the culture's sensitivity to a "sympathetic differentiation" media. Using the method described previously (Fig. 6), we monitored calcium transient activity in H(-)neural crest-derived cells over time in culture. Peak calcium transient activity for H(-)neural crest-derived cells occurred during days 1-4 with 35% of these cells exhibiting calcium transients (Table 2: All). A higher percentage of H(-)neural crest-derived cells that expressed excitable cell traits [responded to caffeine (C-only), to high K^+ (K-only), or both (KC)] exhibited calcium transient activity than cells that lacked either trait (U) (Table 2). After day 4, the percentage of active H(-)neural crest-derived cells declined to $14.9 \pm 4.4\%$ (Table 2: All). U H(-)neural crest-derived cells continued to exhibit a lower percentage of active cells ($9.6 \pm 3.0\%$) than cells that expressed excitable traits (28 – 33%; Table 2). By day 7, the percentage of all active H(-)neural crest-derived cells dropped below 10%. Calcium spiking frequency Table 2. Calcium transient activity in H(-)neural crest-derived cellswas highest days 1-4 (3-4 spikes/hr) with all H(-)neural crest-derived cells exhibiting similar frequencies (Table 2: all). After day 4, calcium spiking frequency declined slightly to 2.4 spikes/hr and continued to decline to less than 2 spikes/hr after day 6. This time course of calcium transient activity coincided with sympathetic differentiation potential with peak activity through day 4 and gradually declining until day 7, when both activities essentially end. Speculations on the identity of H(-)neural crest-derived cells that exhibited spontaneous calcium transient activity and those that exhibited excitable traits are reserved for the discussion.

Table 2. Calcium transient activity in H(-)NC-derived cells

%Active

Days in	H(-)Neural crest-derived cells				
Culture	All	U	C-only	K-only	KC
1 to 2:	33.9 ± 5.0	31.8 ± 5.0	84.0 ± 7.6	66.9 ± 12	78.8 ± 11
	(20)	(19)	(9)	(12)	(8)
3 to 4:	35.8 ± 5.6	27.7 ± 5.2	47.8 ± 3.8	49.5 ± 9.1	57.7 ± 6.1
	(14)	(14)	(10)	(11)	(10)
5 to 6:	14.9 ± 4.4	9.6 ± 3.0	30.7 ± 14	28.5 ± 12	32.9 ± 16
	(8)	(8)	(5)	(7)	(5)
>6:	6.4 ± 4.3	7.6 ± 5.8	14.3 ± 14	11.2 ± 7.2	0
	(6)	(5)	(4)	(5)	(4)
Frequency					
1 to 2:	3.1 ± 0.1	3.1 ± 0.1	3.4 ± 0.3	2.9 ± 0.3	3.0 ± 0.4
	(588)	(412)	(78)	(62)	(36)
3 to 4:	3.2 ± 0.2	2.7 ± 0.3	2.9 ± 0.3	3.7 ± 0.4	4.3 ± 0.5
	(469)	(180)	(82)	(128)	(79)
5 to 6:	2.4 ± 0.2	2.5 ± 0.3	2.5 ± 0.7	2.3 ± 0.3	2.4 ± 0.3
	(112)	(42)	(10)	(39)	(21)
>6:	1.5 ± 0.2	2.2 ± 0.6	2.0 ± 1.0	1.5 ± 0.2	_
	(26)	(14)	(2)	(10)	_

Note: H(-)neural crest-derived cells (all) were analyzed based on calcium pharmacology: C-only = responded to 20 mM caffeine, K-only = responded to 90 mM KCl, KC = responded to both caffeine and KCl, and U = did not respond to either caffeine or KCl. Data reported as mean ± SEM. Numbers in parentheses are total number of cultures (%Active) or total number of cells (Frequency).

Elevated Mg^{2+} blocks calcium transient activity in neural crest-derived cells and inhibits neurogenesis in culture

To determine if calcium transient activity is required for neuronal differentiation, we cultured neural crest cells in the presence of 20 mM Mg^{2+} to block the production of calcium transients. Studies have shown that elevated intracellular Mg^{2+} inhibits intracellular calcium release through the IP3R by a noncompetitive interaction (Kasahara *et al.*, 1993; Volpe and Vezu, 1993). Perfusing CaRec plus 20 mM $MgCl_2$ on Neural crest-derived cells inhibited calcium transient activity (compare Figs. 7A and 7B). This concentration of Mg^{2+} blocked responsiveness to thimerosal, as well (compare Figs. 7B and 7C). Overall, the addition of 20 mM $MgCl_2$ to the perfusion medium significantly reduced the mean percentage of active cells from 55.1 ± 12.1% to 5.4 ± 1.8% (Fig. 7D, $p<.001$).

Culturing neural crest cells with 20 mM Mg^{2+} reduced the number of neurons generated (Fig. 8). Under the current culture conditions, neurons differentiate in the first 6 days of culture from postmitotic neural crest cells (Matsumoto, 1994a). The neurons can be identified at day 4 in culture by labeling with antisera against neuron-specific enolase (NSE) (Figs. 8A-D). On average, neural crest cells cultured in control medium produced 2700 neurons/culture (Fig. 8E). Addition of 20 mM Mg^{2+} to the culture medium significantly reduced the number of neurons to less than 1000 neurons/culture (Fig. 9E, $p<.01$). The effect of elevated Mg^{2+} on neuronal survival was assessed by culturing embryonic day 12 dorsal root ganglion (DRG) cells ± 20 mM Mg^{2+}. All of the neurons in these cultures are postmitotic at plating when grown in the culture conditions used in this study (Matsumoto, 1998). The survival of the embryonic DRG neurons in Mg^{2+} was not significantly different than the control cultures over 5 days in culture (87 ± 4% vs. 89 ± 6%). We also pulsed cultures with BrdU (10μM) for 12 hours to determine if elevated

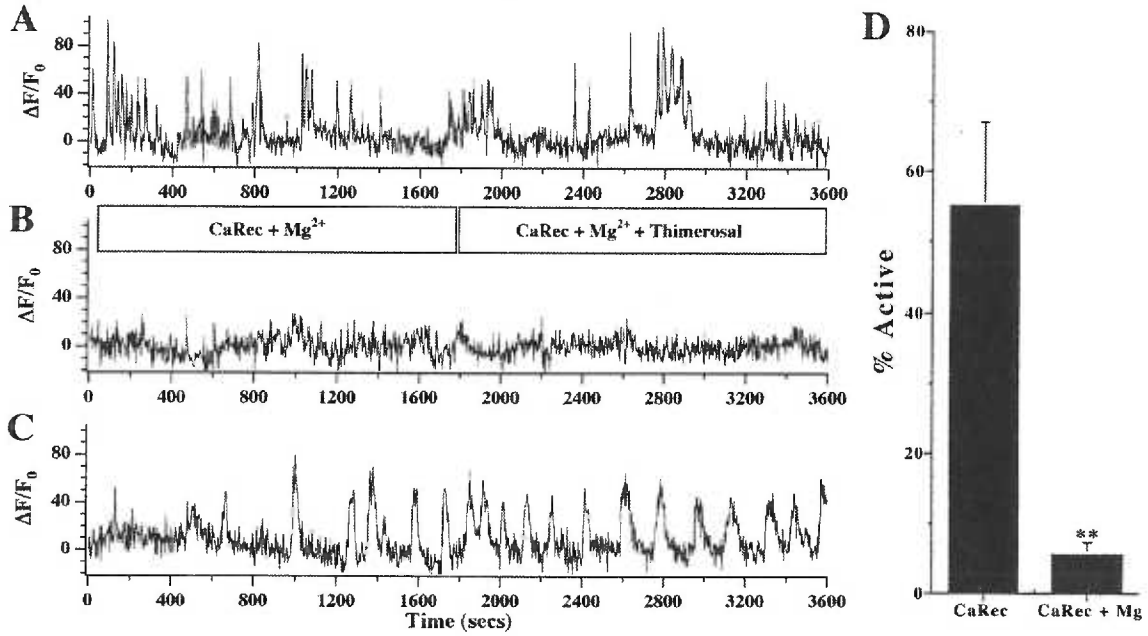

Figure 7. 20 mM Mg^{2+} blocked spontaneous calcium transient activity and thimerosal-induced calcium oscillations in cultured NC-derived cells. Intracellular calcium levels were monitored for 1 hour in the presence of normal CaRec (A). During the 2nd hour, cultures were perfused with CaRec + 20 mM $MgCl_2$ for 30 minutes and then switched to CaRec + Mg^{2+} + 2 μM thimerosal for 30 minutes (B). For the 3rd hour, cultures were perfused with CaRec + thimerosal, in the absence of Mg^{2+} (C). NC-derived cells that had exhibited calcium transients failed to exhibit these transients and respond to thimerosal in the presence of Mg^{2+}. Removal of Mg^{2+} relieves the inhibition on thimerosal-induced calcium oscillations. (D) Addition of 20 mM Mg^{2+} significantly reduced the mean percentage (±SEM) of active NC-derived cells ($p<.01$, **).

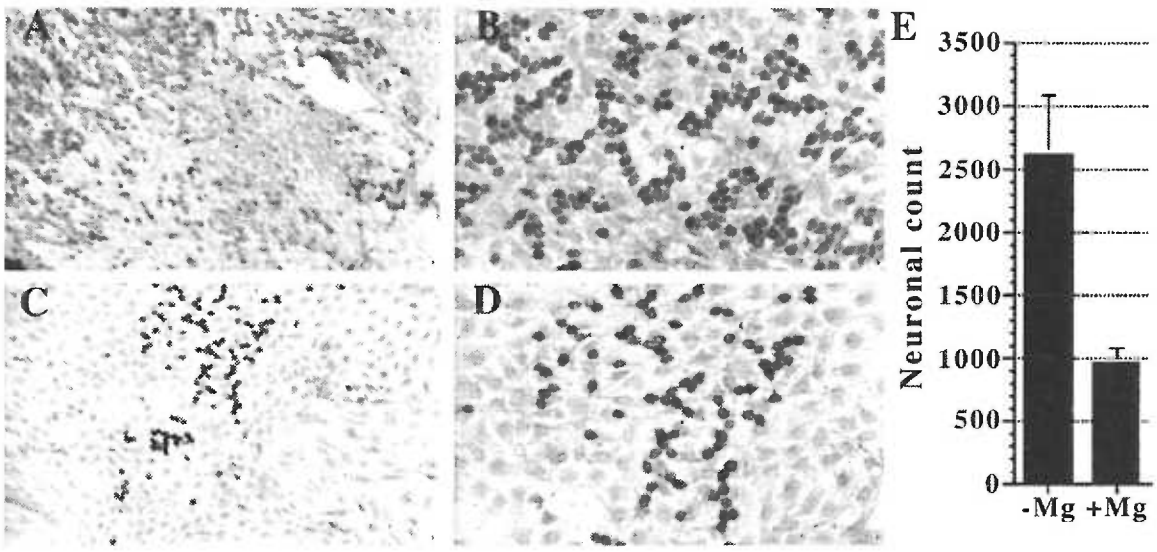

Figure 8. 20 mM Mg^{2+} blocked neurogenesis in NC cultures. NC cells were cultured with normal culture medium ± 20 mM $MgCl_2$ for 4 days, starting at plating of the neural tubes. The cultures were then fixed and immunolabeled with a neuronal marker, NSE. The NSE-labeling was visualized using a biotinylated secondary. (A). 20x phase image of a control NC culture (-Mg^{2+}). (B) 40x phase image of the same field as in (A). (C) 20x phase image of NC culture with Mg^{2+}. (D) 40x phase image of the same field as (C). Note the presence of dark-stained neurons under both conditions, but a noticeable absence of the lighter stained neurons (compare A and C). E. Mean (±SEM) number of neurons differentiated in the presence and absence of Mg^{2+}. Culturing NC cells in the presence of 20 mM Mg^{2+} significantly reduced the number of neurons differentiated ($p<.01$, *).

Mg^{2+} inhibited cell proliferation. There were no obvious differences in the proportion of cells labeled ± 20 mM Mg^{2+} (not shown). However, it is unlikely that we would detect a selective inhibition of a small number of cells, i.e., a select inhibition of neuronal progenitors.

Active neural crest-derived cells can give rise to neurons

To determine directly if cells that exhibit calcium transients are neuronal progenitors, we used lineage-tracing analysis to follow the fate of active and inactive neural crest-derived cells. Active and inactive cells were injected ionophoretically with a 10,000 MW lyseinated-rhodamine dextran. Anode break was used to determine if the impaled cell was a neuron. Once injected, the culture was exposed to a sympathetic differentiation medium (See Methods) overnight, switched to our standard medium for two days, and then fixed and immunolabeled for Hu-IR. Exposure to this medium optimizes the differentiation of sympathetic as well as sensory neuronal progenitors. Comparing Hu-IR with the lineage marker, we found that active neural crest-derived cells gave rise to clones containing neurons, while inactive cells did not. In figure 9, a single active cell (Fig. 9 A-C) gave rise to 3 cells (Fig. 9 D and E), two of which were Hu-IR (Fig. 9 E-G). Overall, 50% of lineage-traced active cells gave rise to clones containing neurons (Fig. 10). Inactive cells, however, were never observed to give rise to clones containing neurons (Fig. 10). The mean clone size for active (2.4 ± 0.4) and inactive (3.6 ± 0.6) labeled Neural crest-derived cells was not significantly different ($p>.05$), indicating similar mitotic activity between the populations labeled. The clone sizes ranged from 1 to 9 cells for both groups with neuron containing clones having a clone size greater than 1.

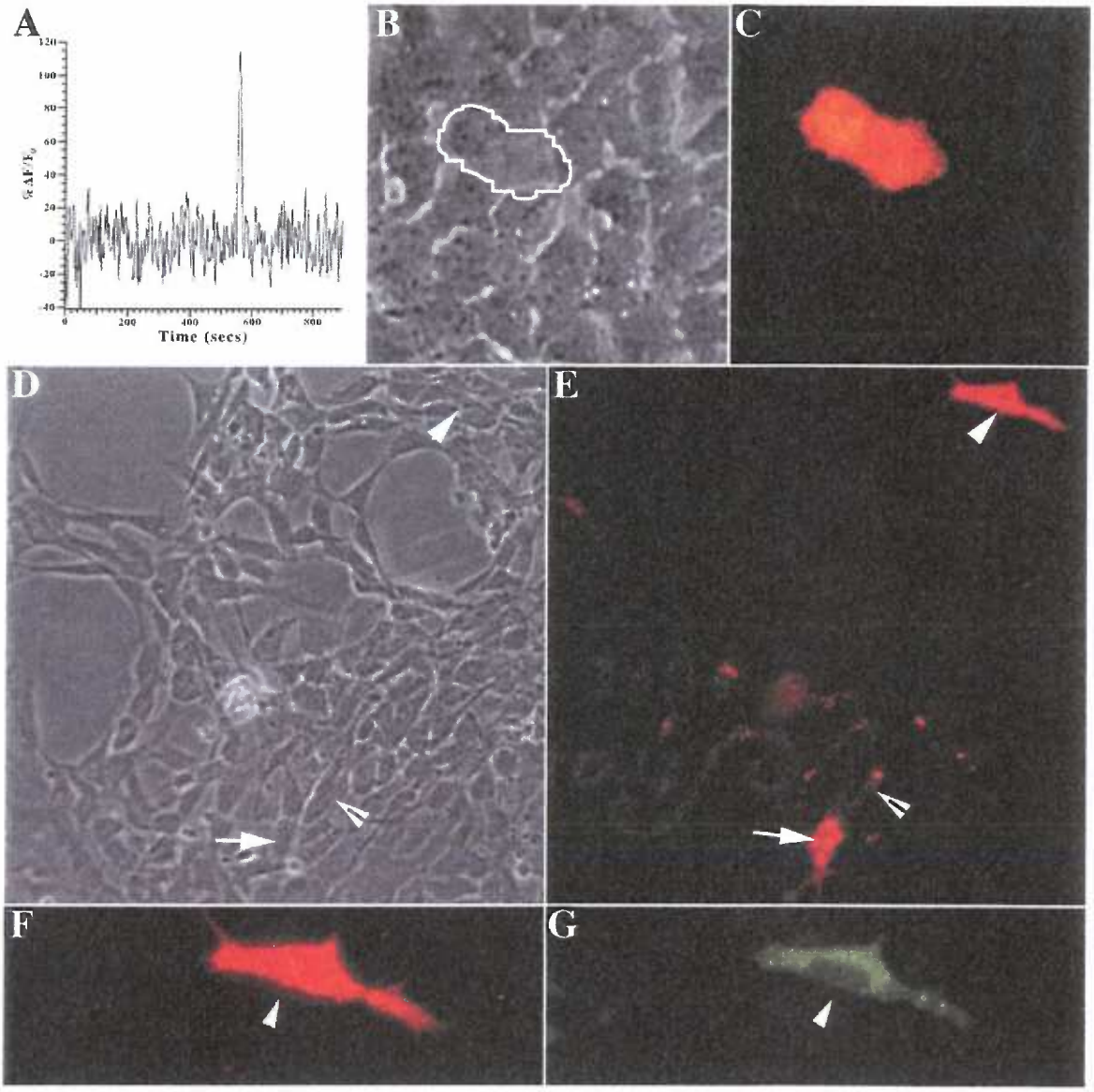

FIG. 9. NC-derived cells that exhibit calcium transients can give rise to neurons. Calcium levels in cultured NC-derived cells were monitored for 15 minutes. An active cell (A) was selected (B, outlined) and injected with a fixable lineage tracking dye (C), lyseinated rhodamine dextran (10000 MW). Culture was then exposed to sympathetic-inducing differentiation media overnight, switched back to the standard culture medium, and then fixed 3 days later. Fixed cultures were immunolabeled for the neuronal marker, Hu. 20x phase image (D) of NC-derived cells that retained the lineage label (arrowhead, inverted arrowhead and arrow) (E). 40x fluorescent image (F) of labeled cell (arrowhead) expressing Hu-IR (G). Arrow denoted cell in D + E also exhibited Hu-IR, while the inverted arrowhead denoted cell was not Hu-IR.

DISCUSSION

Cultured Neural crest-derived cells exhibited spontaneous calcium transient activity similar to what has been described in other models of neuronal differentiation. As with these other models, neural crest-derived cells exhibited calcium transients for a limited time during the course of development, beginning with a low frequency (3 spikes/hr), increasing as neurons mature (6 spikes/hr), and then decreasing at the end of differentiation (Table 1). Similar patterns have been observed in the developing CNS. In the Xenopus cord, for example, immature neurons initially exhibit on average 1 calcium spike/hr, increasing to an average of 3 spikes/hr 5-10 hours after neurogenesis, and then declining to 1 spike/hr (Gu and Spitzer, 1995; Spitzer *et al.*, 1995). In the mammalian CNS, Owens and Kriegstein (1998) have shown that cells in the neocortical ventricular zone (neuronal precursors) exhibited spontaneous calcium transients, and as these cells progressed developmentally both the incidences of calcium transient activity and their frequency increased significantly.

In this study, we have shown that developing peripheral neurons exhibit calcium transient activity. Using the expression of time-dependent neuronal traits, we determined that neurons exhibited spontaneous calcium transients from the onset of expression of the earliest known neuronal characteristic (H-only cells) and continued through the acquisition of VDCCs (HK cells) and caffeine-sensitivity (HKC cells). As the neurons matured, they stopped exhibiting spontaneous calcium transients. The reason for the reduced calcium transient activity in neurons imaged during the first 2 days in culture (Table 1) was probably due to the degree of maturation of these neurons. We have previously shown that under these culture conditions two sets of sensory neurons differentiated: ED (early differentiating) and LD (late differentiating) (Matsumoto, 1994a). The ED neurons arise within 24 hours of plating, while the LD neurons

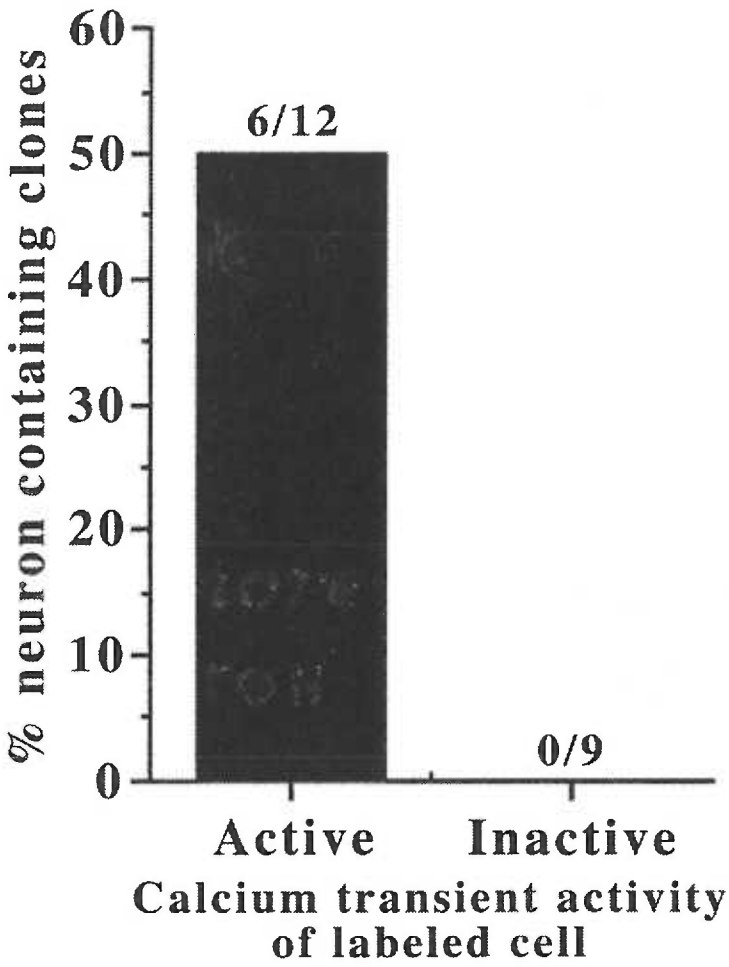

Figure 10. Active and inactive NC-derived cells differ in their neuronal potential. This is a summary of the data described in figure 9. Each clone was determined to contain a neuron, if any of the dye-labeled progeny coexpressed Hu-IR. For each population (active or inactive), the % neuron containing clones was determined as the number of Hu-IR containing clones divided by the total number of clones analyzed times 100. A total of 12 active clones and 9 inactive clones were analyzed. The difference between the populations was significant ($p<.01$).

differentiate 2 days to 6 days after plating. It is likely that the low level of calcium transient activity observed during days 1-2 reflect the more mature ED neurons, while more active neurons imaged from days 3-6 were the more recently differentiated LD neurons (Table 1). This modulation in calcium spiking frequency may be regulating neuronal maturation as seen in Xenopus spinal neurons (Gu and Spitzer, 1995).

In our study, it is important to note that only a subpopulation of neural crest-derived cells exhibited calcium transients. Our hypothesis is that the (H-)neural crest-derived cells that exhibit calcium transients are neuronal progenitors. In support of this, we found that (H-) Neural crest-derived cells exhibited calcium transients during the same time period as neuronal progenitors can be induced to differentiate into (Matsumoto, 1998). In addition, neurogenesis and calcium transient activity (both %active and frequency) were highest the first 4 days in culture and sharply declined from days 5-6 with both activities ending after day 6 (Table 2). Similarly, the mechanism for generating calcium transients was lost with the same time course. This was indicated by the loss of sensitivity to thimerosal after day 6 (Fig. 5B). Thimerosal activates the same calcium oscillatory pathway used to generate spontaneous calcium transients (Carey and Matsumoto, submitted). At this time, we cannot rule out that these active cells may represent other differentiating cell types, i.e., Schwann cells, melanocytes, or smooth muscle. We have, however, monitored calcium levels in sciatic nerve explants, which contain only glial progenitors (Walter, 1994), and found no calcium transient activity (Matsumoto, unpublished results).

Neural crest-derived cells that expressed excitable traits had increased calcium transient activity (% active and frequency) over cells that did not. The expression of such traits may act to increase calcium transient activity by providing a means of calcium entry (VDCCs and RyR) that can increase the probability of a calcium transient event through a calcium-induced calcium release mechanism. A similar mechanism for regulating

calcium transient activity has been described in Xenopus spinal neurons (Gu and Spitzer, 1993). The function of this increased activity could modulate many cellular parameters from growth factor receptor activation (Rosen and Greenberg, 1996) to gene transcription (Buonanno and Fields, 1999). Both Hu+ and Hu- neural crest-derived cells were found to express excitable traits and increased calcium transient activity. The identity of the Hu- cells is unknown. However, it is possible that these cells are sympathetic neuronal progenitors that have begun to express some neuronal traits prior to overt differentiation (Rohrer and Thonen, 1987).

Blocking calcium transient activity, using 20 mM Mg^{2+} (Fig. 7) inhibited neurogenesis (Fig. 8) with no effect on neuronal survival or overall cell proliferation. Elevated extracellular Mg^{2+} blocks calcium transient activity (Fig. 7). This blocking action seems to be selective for the mechanism for generating calcium transients in Neural crest-derived cells (Carey and Matsumoto, in preparation). It has been shown, for example, that Mg^{2+} noncompetitively inhibits the release of intracellular calcium regulated by the IP_3R (Kasahara *et al.*, 1993; Volpe and Vezu, 1993). Although Mg^{2+} can effect other calcium channels (Neuhaus and Cachelin, 1990; Valdivia *et al.*, 1995), the concentration of Mg^{2+} we used had no effect on ryanodine receptors or VDCC (not shown), while it inhibited thimerosal-sensitization of IP_3R (Fig. 7). It is possible, however, that elevated Mg^{2+} may be affecting neurogenesis by inhibiting the proliferation of a select population of neural crest-derived cells.

All of our experiments have consistently found that calcium transients are generated under conditions that support neurogenesis, and are not observed when neuronal differentiation is absent. Thus, a culture of Neural crest-derived cells examined in the first week exhibits calcium transients, as neurons are differentiating but not later, after neurogenesis is complete. This pattern holds true for other culture conditions as well.

When neural crest-derived cells are cultured at low density, for example, they do not exhibit calcium transient activity and we do not observe the generation of neurons (Carey and Matsumoto, unpublished results). Other studies have also shown that calcium transient activity is required for neuronal differentiation. For example, inhibition of calcium signaling in Xenopus neuroblasts blocked neurogenesis (Jones *et al.*, 1995; Jones and Ribera, 1994). During neuronal maturation, inhibiting calcium spikes in Xenopus spinal neurons during their peak transient activity period inhibits expression of GABA-IR (Spitzer *et al.*, 1993), modification of K^+-conductance (Desarmenien and Spitzer, 1991), and regulation of axonal outgrowth (Holliday *et al.*, 1991).

It is unclear how neurogenesis is inhibited when calcium transients are blocked (Fig. 9). Blocking calcium transient activity may prevent the neural crest-derived cells from responding to neuronal differentiation signals. A possible mechanism for this has been described for neuronal differentiation of PC12 cells. Studies have shown that calcium modulates the activity of the Ras-MAPK signaling cascade at various levels, increasing the responsiveness of this pathway to activating signals (Finkbeiner and Greenberg, 1996; Ghosh and Greenberg, 1995; Rosen *et al.*, 1994). One level described is at the growth factor-activated tyrosine kinase receptor. Rosen et al (Rosen and Greenberg, 1996) has shown that calcium influx induces the dimerization of tyrosine kinase receptors, increasing the sensitivity of the receptor to its ligand. Another potential mechanism has been described for regulating neuronal differentiation of neural crest stem cells. In a recent study (Lo *et al.*, 1999), it was shown that Phox2a regulates specification of neurotransmitter identity and was required for neurogenesis. Phox2a actions were dependent on signal transduction pathways that activate the transcription factor CREB, i.e., cAMP and intracellular Ca^{2+}. As seen in both PC12 cells and neural crest stem cells, calcium can regulate of various signal transduction pathways to enhance growth factor signaling and transcription factor activation. In this way, calcium signaling may infer

competence for neuronal differentiation. In our study, we blocked intracellular calcium release to inhibit calcium dependent signaling during neuronal differentiation. The problem is that intracellular calcium release is required for a variety of cellular processes: cell proliferation, transcription, and motility. To better ascertain the role of calcium transient activity during development, pharmacological manipulations need to be directed at prospective downstream effectors of calcium, e.g., CAMKIV, CREB, PKC, etc (Hardingham *et al.*, 1998; Krebs, 1998; Santella and Carafoli, 1997; Tao *et al.*, 1998).

Finally, we provide direct evidence that calcium transient activity is part of the neuronal lineage. We found that a high percentage of active neural crest-derived cells gave rise to clones containing neurons, while inactive cells did not (Table III). However, only 50% of the labeled active cells produced clones containing neurons. There are two possible explanations for why some active cells did not produce neurons. The first is that calcium transients may be necessary, but not sufficient to induce neuronal differentiation. Other factors may be required in conjunction with calcium signaling to induce neuronal differentiation. Alternatively, it is possible that all of the active cells would have given rise to neurons in time. We only allowed the culture to go for 3 days after labeling before we analyzed the resultant progeny, while neuronal differentiation extends several days past this time point. This could result in labeled cells being fixed prior to an opportunity to differentiate. Although we cannot address the specific role calcium plays in regulating neuronal differentiation, the experiments in thus study suggests that its role is an important one.

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CHAPTER III

Calcium transient activity in cultured murine neural crest cells is regulated at the IP_3 receptor

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ABSTRACT

We have shown that cultured neural crest cells exhibit spontaneous calcium transients that are required for neurogenesis. In this study, we determined the mechanism that generates these calcium transients. Extracellular Ca^{2+} modulates calcium transient activity. Superfusing a general calcium channel blocker (La^{3+}) or removing extracellular Ca^{2+}, reduced the percentage of cells exhibiting calcium transients by 26.2% and 40.5%, respectively, and calcium spiking frequency (4.5 to 1.0 and 2.5 to 1.0 spikes/30 minutes, respectively). Intracellular calcium stores also contribute to the generation of calcium transients. Depleting endoplasmic reticulum (ER) calcium stores also reduced the percentage of active cells (15.7%) and calcium spiking frequency (2.8 to 1.5 spikes/30 minutes). Superfusing ryanodine (100 μM), which blocks calcium release regulated by the ryanodine receptor (RyR), had no effect on calcium transient activity. Blocking inositol 1,4,5-triphosphate receptor (IP_3R)-dependent calcium release, using 20 mM Mg^{2+}, abolished calcium transient activity. Mg^{2+} did not block caffeine-sensitive calcium release (RyR-dependent) or voltage-dependent calcium channels. Mg^{2+} also suppressed thimerosal-induced calcium oscillations (IP_3R-dependent). Small increases in the intracellular calcium concentration ($[Ca^{2+}]_i$), elevated the percentage of active cells and the calcium spiking frequency, while larger increases in $[Ca^{2+}]_i$ blocked transients. Reducing intracellular IP_3 levels reduced the percentage of active cells and the calcium spiking frequency. We conclude that the mechanism for generating spontaneous calcium transients in cultured neural crest cells fits the model for IP_3R-dependent calcium excitability of the ER.

INTRODUCTION

During the process of differentiation, many cell types exhibit spontaneous calcium transients; these transients have been shown to regulate many aspects of their normal development. In Xenopus, for example, calcium transients exhibited by myocytes regulate cytoskeletal organization during myofibrillogenesis (Ferrari *et al.*, 1996). Calcium signaling in Xenopus neuroblasts is required for neurogenesis (Jones *et al.*, 1995; Jones and Ribera, 1994) and neuronal maturation, including GABA expression (Spitzer *et al.*, 1993), maturation of a K^+ conductance (Desarmenien and Spitzer, 1991), and axon extension (Spitzer, 1995). In the mouse, primitive endodermal cells exhibit calcium transients that regulate exo/endocytotic vesicle shuttling during their differentiation into parietal and visceral endoderm (Sauer *et al.*, 1998). Mouse CNS progenitors in the subventricular zone exhibit calcium transients prior to and during migration to neuronal layers (Owens and Kriegstein, 1998).

Recently, we found that mouse neural crest cells exhibit spontaneous calcium transients in culture; this calcium transient activity was required for neuronal differentiation (Carey and Matsumoto, in press). Using lineage-tracing dyes, we found that active cells could give rise to neurons, while inactive cells did not. Finally, when calcium transient activity was blocked in culture, neurogenesis was prevented. Thus, spontaneous calcium transient activity is required for neuronal differentiation of mouse neural crest cells in culture. Its unclear, however, what mechanism generated these transients and how they were regulated.

Previous studies indicate that both extracellular calcium and intracellular calcium stores are required for generating calcium transients. In non-excitable cells, studies have shown that intracellular calcium stores provide the bulk of calcium influx during a transient

(Holliday *et al.*, 1991; Stachecki and Armant, 1996a), while extracellular calcium replenishes depleted stores (Jaconi *et al.*, 1997; Randriamampita and Tsien, 1993). Extracellular calcium influx may also trigger calcium transient events via calcium-induced calcium release (CICR) (Spitzer, 1994c). This CICR of intracellular calcium stores can be mediated by either the inositol 1,4,5-triphosphate (IP_3) or ryanodine receptors (IP_3R and RyR, respectively) (Dupont and Goldbeter, 1993; Keizer and Levine, 1996). During development, many studies have identified the IP_3R as the mediator of calcium influx for spontaneous calcium transient activity (Carroll and Swann, 1992; Sauer *et al.*, 1998).

Our hypothesis is that spontaneous calcium transient activity in cultured neural crest-derived cells is regulated at the IP_3R. In this study, we show that regulated release of intracellular calcium by the IP_3R underlies the primary influx of calcium during a calcium transient event. Furthermore, we provide evidence that extracellular calcium modulates calcium transient activity by acting as a source of calcium to trigger transient activity. Similarly, we show that intracellular levels of IP_3 modulate calcium transient activity.

RESULTS

Spontaneous calcium transient activity is dependent on extracellular and intracellular calcium stores

Using calcium imaging, we have shown that mouse neural crest-derived cells exhibit spontaneous calcium transient activity in culture (Carey and Matsumoto, in press). To identify the primary source of calcium influx during a calcium transient event, we monitored calcium transient levels every 3 secs for 30 minute intervals in the presence and absence of several intracellular and extracellular calcium channel blockers.

We inhibited extracellular calcium entry by blocking extracellular calcium channels or by removing extracellular calcium. To block extracellular calcium channels, we added the La^{3+} to our normal superfusion medium (CaRec). La^{3+} blocks all types of calcium channels. In the presence of La^{3+}, we saw a reduction in calcium transient activity (Fig. 1A, Table 1). We monitored calcium levels for 30 minutes in normal CaRec and then for 30 minutes in either normal CaRec or CaRec + 30 μM La^{3+}. La^{3+} significantly reduced the percentage of active cells, i.e., cells displaying at least one calcium transient, by 26.2 ± 7.5% compared to cultures maintained in normal CaRec ($p<.05$; Table 1). The difference in percent reduction was significant ($p<.05$). The calcium spiking frequency was also significantly reduced from 4.5 ± 0.3 spikes to 1.0 ± 0.1 spikes per 30 minute interval ($p<.0001$; Table 1). Removal of extracellular La^{3+} returned calcium transient activity to normal levels (N=5). La^{3+} is capable of blocking intracellular calcium channels and quenching calcium-indicating dyes (Gomez *et al.*, 1995; Meszaros and Volpe, 1991; Palade *et al.*, 1989). To control for this, we exposed cells sequentially to 20 mM caffeine and 90 mM KCl. In normal CaRec, both agents produced a clear calcium signal (Fig. 1B). In the presence of 30 μM La^{3+}, Neural crest-derived cells continued to exhibit a caffeine response, while the KCl response was blocked (Fig. 1C). This result indicates that La^{3+} is acting extracellularly to block voltage-dependent calcium channels (VDCC) but did not reach significant intracellular concentrations to inhibit the caffeine-response or quench the dye.

When we removed extracellular calcium, both the percentage of active cells and calcium spiking frequency was significantly reduced. Superfusion with a modified CaRec (0 mM Ca^{2+}/5 mM Mg^{2+}) significantly reduced the percentage of active cells by 40.5 ± 4.5% compared to controls (2.7 ± 0.1%, $p<.05$; Table 1). 5 mM Mg^{2+} was required for the cells

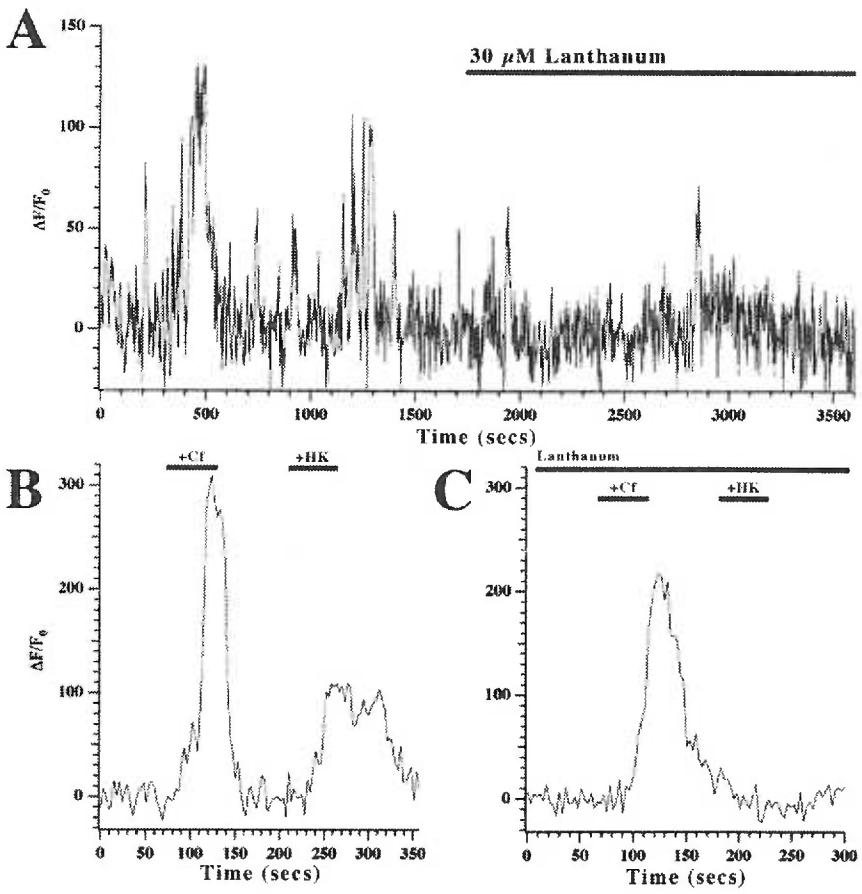

Figure 1. La^{3+} decreases calcium transient activity in cultured NC-derived cells. NC-derived cells were loaded with 2 μM OGB-1 and intracellular calcium levels determined at 3-second intervals. (A) Calcium transient activity in an individual cell monitored for 30 minutes in normal CaRec followed by 30 minutes in CaRec + 30 μM La^{3+}. (B) Calcium influxes in response to 20 mM caffeine (Cf) and 90 mM K^+ (HK) in normal CaRec. (C) Calcium influxes in response to Cf and HK in CaRec + 30 μM La^{3+}. La^{3+} blocks extracellular calcium entry, but does not enter the cell to inhibit intracellular calcium release.

Table 1. Source of calcium and calcium transient activity

	Reduction in % Active		# of events/interval	
Extracellular Ca^{2+}:	Control	Experimental	Before	After
0 Ca^{2+}/5 Mg^{2+}	2.7 ± 0.1%	40.5 ± 4.5%	2.5 ± 0.3	1.0 ± 0.3
	(3)	-	(102)	-
30 μM La^{3+}	6.3 ± 2.5%	26.2 ± 7.5%	4.5 ± 0.3	1.0 ± 0.1
	(4)	-	(366)	-
Intracellular Ca^{2+}:				
10 μM DHtBQ/ 1 nM Thapsigargin	6.2 ± 1.9%	15.7 ± 2.7%	2.8 ± 0.2	1.5 ± 0.1
	(3)	-	(304)	-

Note: For reduction in % Active, cultures were monitored for 30 minutes in normal CaRec and then 30 minutes in normal CaRec (Control) or CaRec + Blocker (Experimental). For # of events/interval, calcium spiking frequency was determine for individual cells before and after treatment. The average (±SEM) calcium spiking frequency was determined for all cells that were active under either condition. The numbers in parentheses are number of cultures (% Active) and # of cells (# of events/interval).

to remain attached to the dish. Calcium spiking frequency also significantly decreased from 2.5 ± 0.3 spikes/30 minutes to 1.0 ± 0.3 spikes/30 minutes ($p<.001$; Table 1). Superfusion with 5 mM Ca^{2+}/5 mM Mg^{2+} did not effect calcium transient activity (not shown). Based on these two results, extracellular calcium entry appears to play a role in regulating calcium transient activity of neural crest-derived cells. However, the continued presence of calcium transient activity in the absence of extracellular calcium influx indicates that it is not the primary calcium source.

We next determined if intracellular calcium stores contribute to the generation of calcium transients. The endoplasmic reticulum (ER) is the primary source of calcium for regulated intracellular calcium release (Meldolesi and Villa, 1993). To determine if ER-dependent calcium stores are required for calcium transient activity, we depleted the ER of calcium using the drug, 2,5-Di(tert-butyl)-1,4-benzohydroquinone (DtBHQ) or thapsigargin. Both drugs block the Ca^{2+} ATPase pump, inhibiting calcium entry into the ER. This blockade results in a slow release of calcium and depletion of the ER stores (Inesi and Sagara, 1992; Moore *et al.*, 1987). Calcium transient activity was monitored in normal CaRec for 30 minutes (Fig. 2A) and then for 30 minutes in CaRec ± the inhibitor (Fig. 2B). We observed a consistent reduction in the amplitude of the calcium transient events, and a small, but significant reduction in the percentage of active Neural crest-derived cells (6.2 ± 1.9% vs. 15.7 ± 2.7% for controls, $p<.05$; Table 1). Calcium spiking frequency was also reduced (2.8 ± 0.2 spikes/30 minutes to 1.5 ± 0.1 spikes/minutes, $p<.0001$; Table 1). As with the role for extracellular calcium, intracellular calcium stores may play a role in calcium transient production.

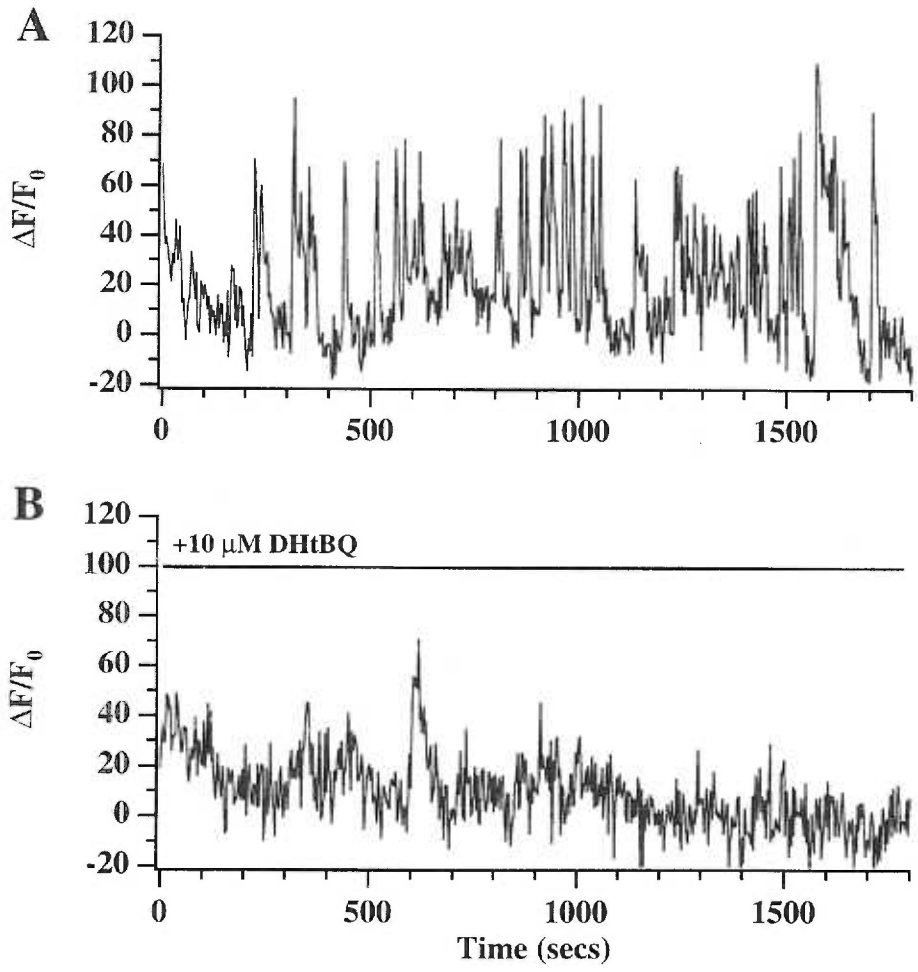

Figure 2. Depletion of ER calcium stores with DtBHQ reduces calcium transient activity. NC-derived cells were loaded with 2 μM OGB-1 and intracellular calcium levels determined at 3-second intervals. Calcium transient activity in an individual cell monitored for 30 minutes in normal CaRec (A) and then switched to CaRec + 10 μM DtBHQ for 30 minutes (B).

IP_3R regulates calcium release during calcium transient events

To further elucidate the role of ER calcium stores in calcium transient activity, we inhibited specific intracellular calcium release sites. The two major classes of receptors that regulate the release of ER calcium stores are the IP_3R and the RyR (for review, (Meldolesi and Villa, 1993)). To determine if the RyR is involved in regulating calcium transient production, we monitored calcium transient activity in the presence of its antagonist, ryanodine (100 μM). Since ryanodine blocks open RyR channels at this concentration (Ferrari *et al.*, 1996), we incubated neural crest cultures for 2 hours prior to imaging, to facilitate blockade of the channel. Calcium transient activity was then monitored in the continued presence of the ryanodine. Figure 3A shows an example of an individual cell that exhibited calcium transient activity in the presence of the ryanodine. Although spontaneous calcium transient activity was unaffected, this concentration was sufficient to inhibit the caffeine-sensitive release of intracellular calcium (Fig. 3B). Cultures exposed to 100 μM ryanodine showed no significant change in the percentage of active cells or calcium spiking frequency (Fig. 5 A and B). This indicates that regulated release of intracellular calcium by the RyR is not required during a calcium transient event.

Next, we determined if regulated calcium release by the IP_3R was necessary for calcium transient activity. To do this, we blocked IP_3R-mediated calcium release by superfusing cultures with a modified CaRec containing 20 mM Mg^{2+} (See Methods). This concentration is sufficient to noncompetitively inhibit IP_3R mediated calcium release (Volpe and Vezu, 1993). The resting intracellular concentration for Mg^{2+} is 0.4-0.6 mM (Volpe and Vezu, 1993). The addition of 20 mM Mg^{2+}, extracellularly, results in an increase in the intracellular Mg^{2+} concentration to approximately 2 mM (Kasahara *et al.*, 1993). At this concentration, Mg^{2+} may also affect VDCCs and the RyR

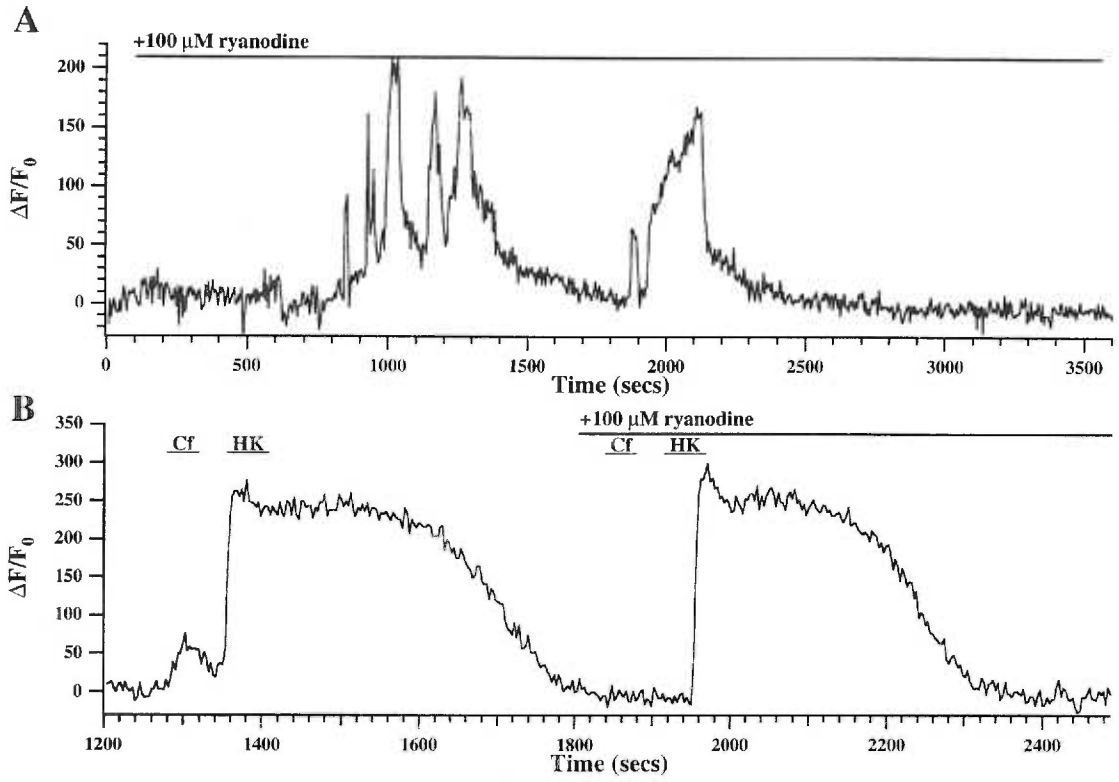

Figure 3. Ryanodine does not affect the spontaneous calcium transient activity of culture NC-derived cells. NC cultures were incubated for 2 hours in 100 µM ryanodine. The cultures were then loaded with 2 µM OGB-1 and the calcium transient activity determined. (A) Calcium transient recording of an individual cell in CaRec + 100 µM ryanodine. Calcium recording of an individual cell responding to 20 mM caffeine (Cf) or 90 mM KCl (HK) ± 100 µM ryanodine. Ryanodine (100 µM) inhibits caffeine-sensitive calcium release, but does not inhibit spontaneous calcium transient activity (B).

(Pessah *et al.*, 1987; Volpe *et al.*, 1990); however, control experiments in which we applied 90 mM KCl or 20 mM caffeine in the presence of 20 mM Mg^{2+} indicate that there is little effect on these conductances (not shown). When we exposed neural crest cultures to 20 mM Mg^{2+}, we found that virtually all calcium transient activity was blocked. Figure 4 shows an example of an individual Neural crest-derived cell that exhibited spontaneous calcium transient activity that was subsequently blocked when exposed to 20 mM Mg^{2+} and then reversed when returned to normal CaRec. Mg^{2+} significantly reduced the percentage of active cells from 48% to 5% ($p<.001$; Fig. 5A). Calcium spiking frequency also significantly decreased from 2.2 events/30 minutes to 1 event/30 minutes, for those cells that continued to exhibit calcium transients ($p<.001$; Fig. 5B). This inhibition was reversed upon removal of the Mg^{2+} (Fig. 4).

To support the conclusion that the effect of Mg^{2+} on calcium transient activity is directed at the IP_3R, we analyzed its effect on thimerosal-induced calcium oscillations. Thimerosal induces calcium oscillations in many cells types by sensitizing the IP_3R, increasing the probability of intracellular calcium release (Bootman *et al.*, 1992). When applied to neural crest cultures, thimerosal induced calcium oscillations in both active and inactive neural crest-derived cells (Fig. 6A and B) (Carey and Matsumoto, 1999b). In 5 experiments, we blocked calcium transient activity with 20 mM Mg^{2+} and then added 2 μM thimerosal in the continued presence of 20 mM Mg^{2+} (Fig. 6C and D). In the presence of Mg^{2+}, thimerosal had no effect. When Mg^{2+} was washed out, calcium oscillations were produced (Fig. 6E). Thimerosal may act on the RyR as well (Abramson *et al.*, 1995), however, we did not observe inhibition of thimerosal-induced calcium oscillations in the presence of 100 μM ryanodine (not shown).

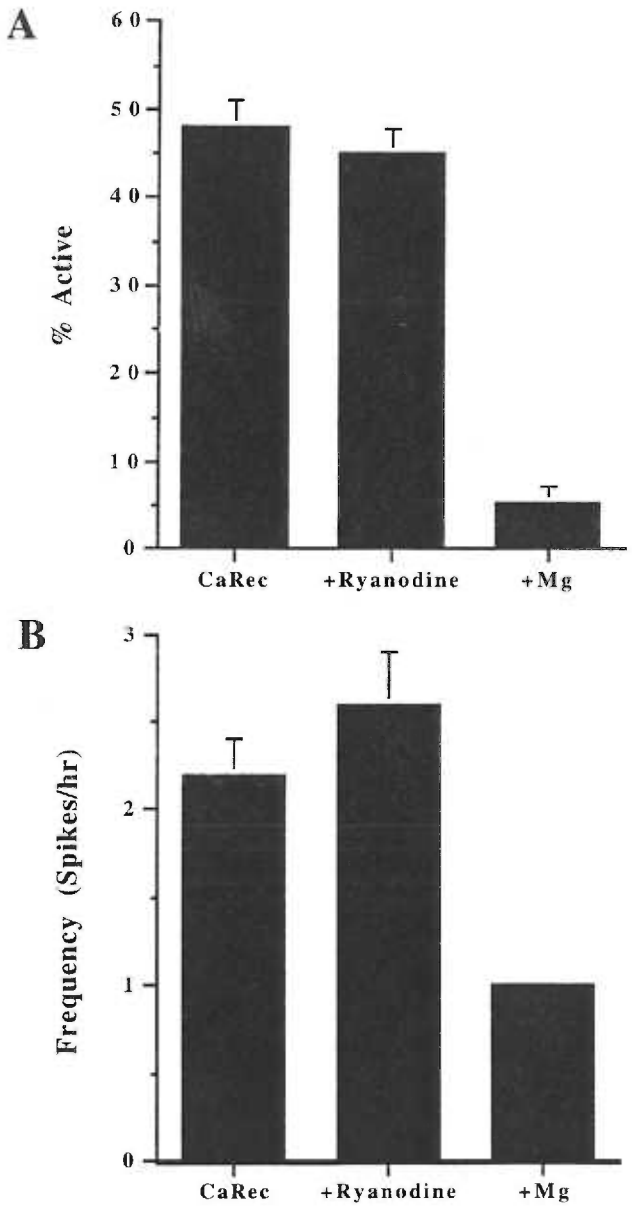

Figure 4. Mg^{2+} (20 mM) inhibits calcium transient activity. Cultured NC-derived cells were loaded with 2 μM OGB-1 and intracellular calcium levels monitored at 5 second intervals. Calcium transient recording of an individual cell monitored for 900 secs in normal CaRec, switched to a modified CaRec + 20 mM Mg^{2+} for 1800 secs, and then to normal CaRec for 1800 secs. Note: the inhibition of calcium transients by Mg^{2+} (20 mM) was reversible.

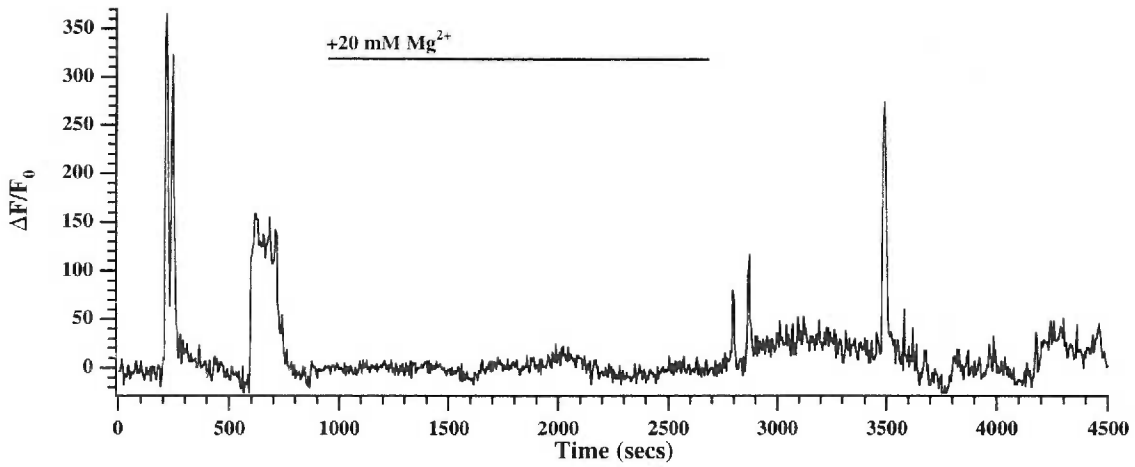

Figure 5. The IP_3R, not the RyR, regulates calcium transient activity. Summary of calcium transient activity for NC-derived cells recorded in normal saline (CaRec), CaRec + 100 μM ryanodine (ryanodine), or a modified CaRec + 20 mM $MgCl_2$ (Equimolar concentrations of Na^+ and Mg^{2+} were exchanged to maintain osmolarity). Mean percentage (+ SEM) of NC-derived cells that exhibited spontaneous calcium transient activity. Inhibition of IP_3R regulated release of calcium using 20 mM Mg^{2+} reduced calcium transient activity, while 100 μM ryanodine did not.

Intracellular calcium and IP_3 levels modulate calcium transient activity

The IP_3R and the Ca^{2+} ATPase transporter confer Ca^{2+} excitability to the ER (Berridge, 1997a; Keizer *et al.*, 1995; Li *et al.*, 1995). The excitability of the ER resembles plasma membrane excitability of neurons because it is driven by an ionic gradient across its membrane that is maintained by an ATP-dependent active transport with an excitation variable (intracellular calcium concentration) that controls a nonlinear dynamic release of Ca^{2+} (Li *et al.*, 1995). The IP_3R acts like a voltage-dependent ion channel, triggering an all-or-none release of intracellular calcium during a spontaneous event (Missiaen *et al.*, 1991). Whereas voltage is the primary determinant for opening probability of a voltage-dependent ion channel, the primary coactivators of the IP_3R are calcium and IP_3 (Bootman and Berridge, 1995). To determine if the calcium transient activity exhibited by cultured Neural crest-derived cells was dependent on ER excitability, we varied the intracellular levels of IP_3R coactivators and observed the effects on calcium transient activity.

We had noted previously that blocking extracellular calcium influx with La^{3+} decreased calcium transient activity (Fig. 1A). This result may indicate that calcium influx is acting as a trigger to induce CICR at the IP_3R. To test this, we monitored calcium transient activity in normal CaRec followed by superfusion with a modified CaRec in which Na^+ was replaced with choline. The removal of Na^+ reverses the plasma membrane Na^+/Ca^{2+} exchanger , resulting in a small increase of intracellular calcium levels (Pan and Kao, 1997). In these experiments, we observed a $5.3 \pm 1.4\%$ reduction in the percentage of active cells in normal CaRec, while there was a $14.8 \pm 11.2\%$ increase in active cells in 0 Na^+. Substituting tetraethylammonium chloride (TEA) for Na^+ produced a similar increase in active cells ($16.1 \pm 8.0\%$). These results are summarized in Table 2.

Interestingly, Neural crest-derived cells did not alter their calcium transient activity when we modulated the plasma membrane excitability. For example, increasing plasma membrane excitability with 50 mM TEA, which is sufficient to block voltage-dependent K^+ channels, had no effect on calcium transient activity (Table 2). Reducing the plasma membrane excitability with 1.5 μM tetrodotoxin also had no effect on calcium transient activity (Table 2). Therefore, increasing intracellular calcium levels can increase calcium transient activity, while modulating membrane excitability has no effect.

Although membrane excitability may not affect calcium transient activity, it has been shown in other systems that VDCC may provide a source of calcium to trigger calcium transient events (Gu and Spitzer, 1993; Scamps *et al.*, 1998; Wang and Gruenstein, 1997). To test if VDCCs modulate calcium transients, we applied a number of inhibitors onto neural crest cultures. Although La^{3+}, which blocks all calcium conductances, decreased calcium transient activity (Table 1), the selective inhibition of specific calcium channel subtypes did not result in a change in calcium transient activity (Table 2). Thus, while extracellular calcium is important for the production of calcium transients, its entry via voltage-dependent channels is not required.

Calcium transients are inhibited by manipulations that cause large increases in intracellular calcium concentrations. For example, raising intracellular calcium levels with either elevated K^+ (50 mM) or with the calcium ionophore, A23187 (1 μM) reduced calcium transient activity (not shown). This is also consistent with an IP_3R mechanism, in that calcium has been shown to act as an coactivator of the IP_3R at concentrations below 300 nM and an inhibitor at concentrations above 300 nM (Bezprozvanny *et al.*, 1991).

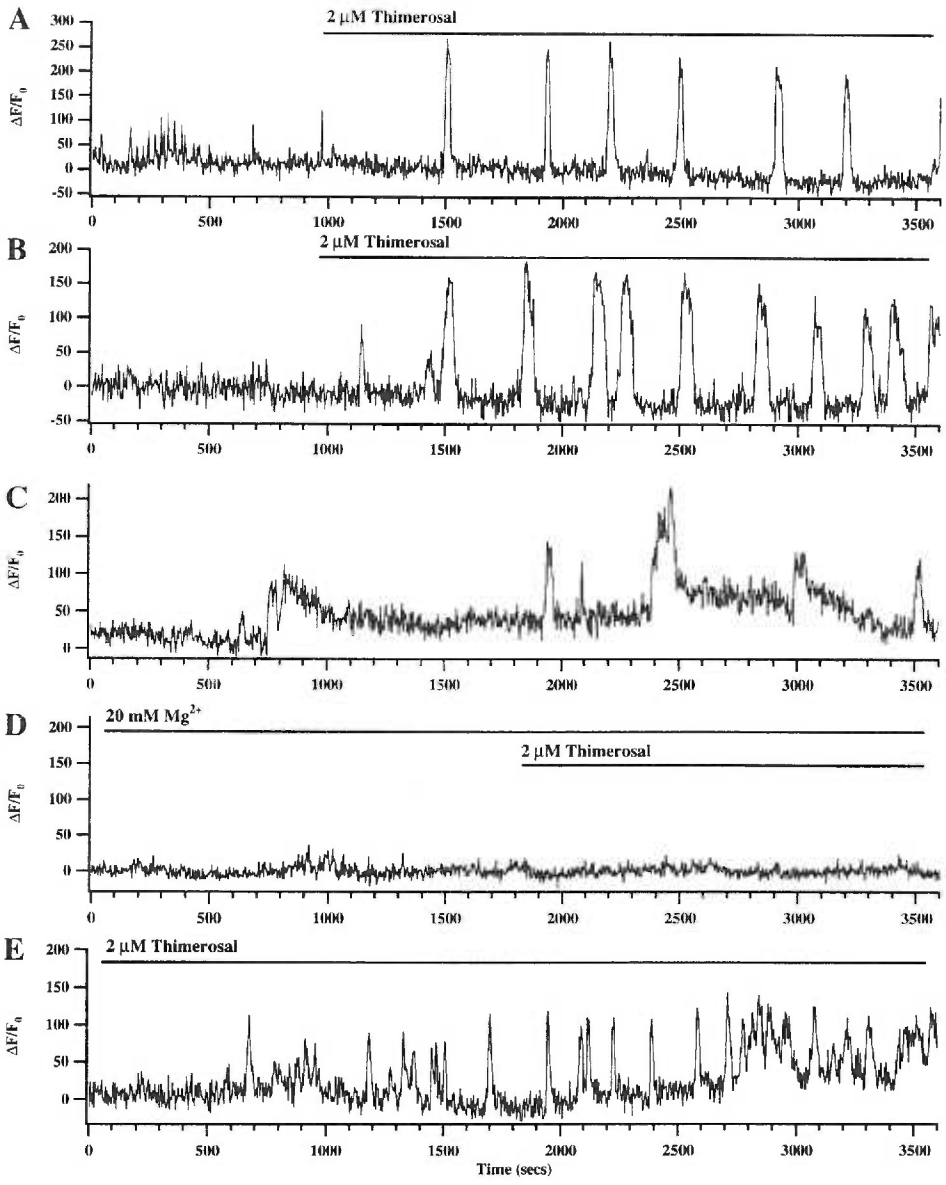

Figure 6. Mg^{2+} inhibits thimerosal-induced calcium oscillations in cultured NC-derived cells. NC-derived cells were loaded with 2 μM OGB-1 and intracellular calcium levels monitored at 3 second intervals. (A) Calcium transient recording of an active NC-derived cell monitored for 900 secs in normal CaRec and then switched to CaRec + 2 μM thimerosal for 2700 secs. (B) Calcium transient recording of an inactive NC-derived cell monitored for 900 secs in normal CaRec and then switched to CaRec + 2 μM thimerosal for 2700 secs. (C-E) Individual cell monitored for 1 hour in normal CaRec (C), switched to modified CaRec + 20 mM Mg^{2+} for 30 minutes (D, 1-1800 secs), switched to modified CaRec + 20 mM Mg^{2+} + 2 μM thimerosal for 30 minutes (D, 1801-3600 secs), and then switched to CaRec + 2 μM thimerosal (E). 20 mM Mg^{2+} reversibly inhibits thimerosal-induced calcium oscillations.

Table 2. Summary of pharmacological effects on calcium transient activity

Site of action	Action	Pharm. agent	Effects on Ca^{2+} transients
Ext. calcium:			
general	Deplete	0 mM Ca^{2+}/5 mM Mg^{2+}	Sig. decrease
general	Block	30 μM La^{3+}	Sig. decrease
VDIC			
L-type Ca^{2+} channels	Block	10 μM Nimod	No Δ
N-type Ca^{2+} channels	Block	10 μM Flu	No Δ
T-type Ca^{2+} channels	Block	5 μM Mib, 12 μM NDGA, 2 mM Ni^{2+}, 200 mM Amil	No Δ
Na^+ channels	Block	1.5 μM TTX	No Δ
K^+ channels	Block	50 mM TEA	No Δ
		150 mM TEA	Slight increase
Na^+/Ca^{2+} exchanger	Reverse	150 Choline, 150 TEA	Slight Increase
Int. calcium:			
ER stores	Deplete	10 μM DHtBQ, 1 nM Thap	Sig. decrease
RyR	Block	100 μM Ryanodine	No Δ
IP_3R	Block	20 mM Mg^{2+}	Blocks
IP_3R	Sensitize	2 μM thimerosal	Induce oscillations
PLCγ	Inhibit	2 μM U73122	Slight decrease
PLCγ	Excite	50 mM Imipramine HCl	No Δ

Note: Abbreviations – Amiloride (Amil), Dihydro-tert-butylquinone (DHtBQ), Flunarazine (Flu), Mibefradil (Mib), Nimodopine (Nimod), Nordihydroguaiaretic acid (NDGA), Tetraethylammonium chloride (TEA), Tetrodotoxin (TTX), Thapsigargin (Thap), and voltage-dependent ion channels (VDIC).

As with calcium, modulating intracellular IP_3 levels varied calcium transient activity in neural crest-derived cells. We used the phospholipase C γ inhibitor, U73122, to reduce intracellular IP_3 levels (Jin *et al.*, 1994). Figure 7A is a calcium recording of an individual cell that was exposed to 1 μM U73122, which reduced the calcium spiking frequency. On average, the decrease in the percentage of active cells was not significant over control conditions (compare 12.4 ± 2.8% and 6.5 ± 3.3%; Fig. 7B). However, calcium spiking frequency was significantly reduced from 3.4 ± 0.6 spikes/30 minutes to 1.9 ± 0.3 spikes/30 minutes ($p<.01$; Fig. 7C). Attempts to increase intracellular IP_3 levels using imipramine HCl had no effect on calcium transient activity (Table 2).

DISCUSSION

We have previously shown that neural crest-derived cells, like many other differentiating cell types, exhibit spontaneous calcium transients that are required for specific aspects of their development (Ferrari *et al.*, 1996; Gu *et al.*, 1994; Reinhard *et al.*, 1995; Sauer *et al.*, 1998; Stachecki and Armant, 1996a). Interestingly, many of these cell types generate calcium transients prior to the expression of excitable traits, i.e., VDCCs. Inexcitable cells generate calcium transients using the Ca^{2+} excitability of the ER (Berridge, 1997a; Li *et al.*, 1995). In these cells, Ca^{2+} excitability is defined as the ability of a small increase in $[Ca^{2+}]_i$ to trigger a large release of intracellular calcium, i.e., a calcium transient (Li *et al.*, 1995). This excitability resembles electrical excitability of the plasma membrane of neurons. Like the plasma membrane, ER excitation is driven by a transmembrane concentration gradient of nonpermeable ions that are released upon excitation. In both cases, the gradient is reestablished by an ATP-dependent active transport. Unlike voltage-dependent plasma membrane channels, the excitation variable that controls the changes in ER membrane permeability is $[Ca^{2+}]_i$ (Li *et al.*, 1995).

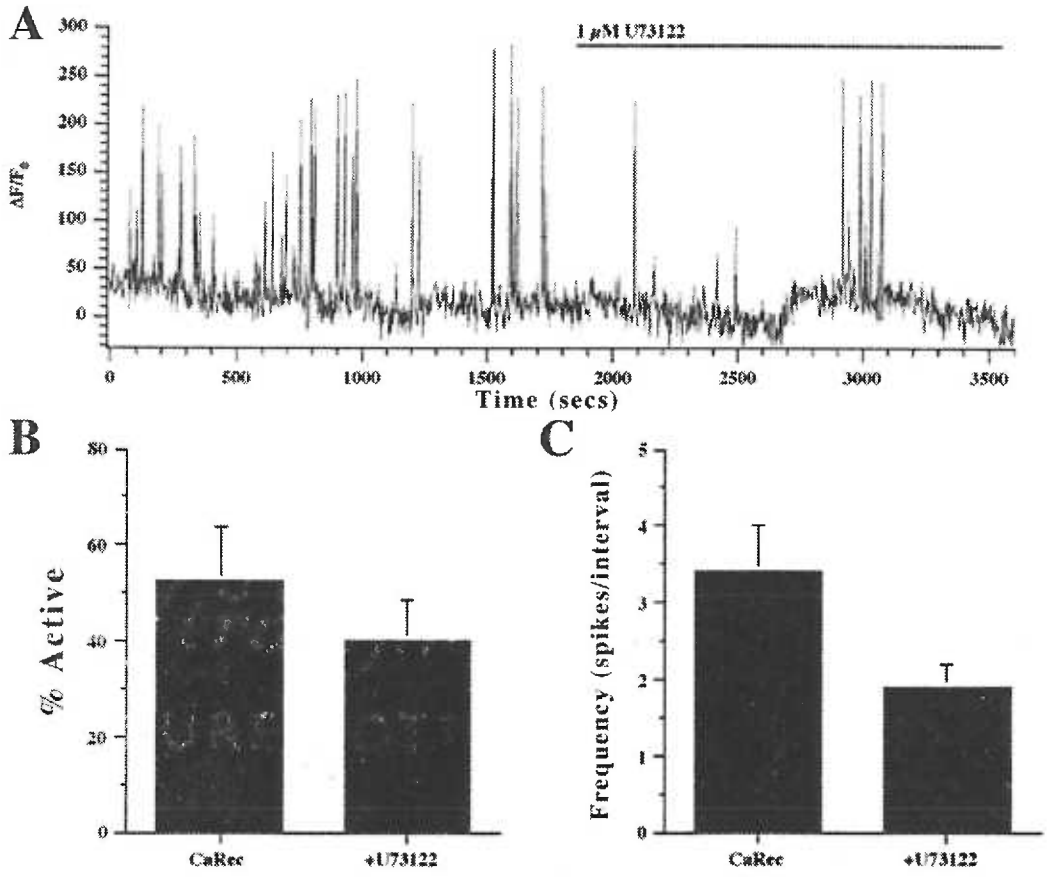

Figure 7. Inhibition of PLCγ reduces spontaneous calcium transient activity in cultured NC-derived cells. NC-derived cells were loaded with 2 μM OGB-1 and intracellular calcium levels monitored at 3 second intervals. (A) Calcium transient recording of an individual cell monitored for 30 minutes in normal CaRec and then monitored for 30 minutes in normal CaRec + 1 μM U73122. (B) Mean (+SEM) change in percentage of active NC-derived cells for cultures monitored in normal CaRec and CaRec + U73122. (C) Mean (+SEM) calcium spiking frequency for cultures monitored in normal CaRec and CaRec + U73122. NC-derived cells exhibited a significant reduction in calcium spiking frequency when exposed to 1 μM U73122 ($p<.01$).

Typically, the IP_3R mediates the release of Ca^{2+} from ER stores in inexcitable cells (for review, (Jacob, 1990)). A model for regulation of calcium transient behavior at the IP_3R is shown in figure 8 (Furuichi and Mikoshiba, 1995). The primary coactivators of the IP_3R are calcium and IP_3 (Picard *et al.*, 1998). When activated, the receptor will initiate an "all or none" release of intracellular calcium stores (Iino *et al.*, 1993; Missiaen *et al.*, 1991; Parker and Ivorra, 1990). The duration of the transients are regulated by the elevated intracellular calcium concentration and the time-dependent inactivation of the receptor (Hajnoczky and Thomas, 1997). This process allows for repeated calcium release (Berridge and Dupont, 1994; Li *et al.*, 1995; Parker and Yao, 1991). The frequency of these events is dictated by the sensitization of the receptor, regulated by its phosphorylation states, intracellular IP_3 concentration, intracellular calcium concentration, and the receptor isoform expressed (Fig. 8). This variability in receptor sensitization accounts for complex calcium signals that are regulated by the IP_3R (Thomas *et al.*, 1996).

Spontaneous calcium transient activity exhibited by neural crest-derived cells fits the model for IP_3R-regulated calcium excitability. First, the calcium transient activity is dependent on IP_3R-regulated release of calcium. Application of 20 mM Mg^{2+} inhibited spontaneous calcium transients and thimerosal induced calcium oscillations (IP_3R-dependent) without affecting VDCCs and caffeine-sensitive calcium release (RyR dependent) (Figs. 4-6). Although depleting ER calcium stores using Ca^{2+} ATPase pump inhibitors did not completely suppress IP_3R-dependent calcium transient activity, it is possible that depleted ER calcium stores activated store-operated calcium conductances located on the plasma membrane, which could be interpreted as calcium transients (Parekh and Penner, 1997; Rabasseda *et al.*, 1990; Razani-Boroujerdi *et al.*, 1994). Secondly, modulating the intracellular concentrations of the IP_3R coactivators varied

calcium transient activity. Small increases in intracellular calcium levels, for example, increased calcium transient activity, while robust increases decreased calcium transient activity (Table 2). Reducing intracellular calcium levels by blocking calcium influx (La^{3+}, Fig. 1) or removing extracellular calcium (Table 1) decreased calcium transient activity, as well. Reduced IP_3 levels decreased the frequency of calcium transients, while increasing IP_3 levels had no effect (Fig. 7 and Table 2). This last observation suggests that there is a limit to IP_3's ability to enhance excitability in neural crest-derived cells. However, manipulations that demonstrate no effect are questionable, since we did not control for the efficacy of the manipulation, e.g., we did not measure IP_3 levels after imipramine addition.

Cellular factors that regulate IP_3R activity (Fig. 8) determine the calcium transient frequency, which in turn govern the activation of downstream events. Activation of Ca^{2+}-dependent signal transduction pathways is dependent on the frequency of the calcium oscillations. For example, in developing Xenopus spinal neurons, calcium transient activity is regulated by the voltage-dependent influx of calcium to trigger intracellular calcium release. This cellular arrangement produces calcium transients at a select frequency (approx. 3 events/hr) that, if varied, modifies several aspects of neuronal development, i.e., neurite extension and GABA-immunoreactivity. In cultured dorsal root ganglion neurons, activation of the transcription factors CREB and MAPK was strongly dependent on calcium transient frequency. Interestingly, we have preliminary

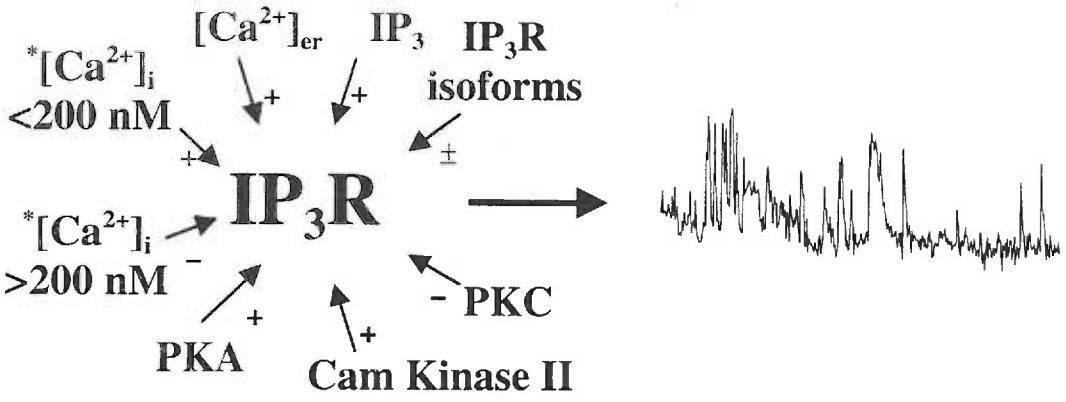

Figure 8. IP_3R model for calcium transient activity. Modulation of IP_3R activation level regulates cellular calcium transient activity. + interaction increases IP_3R activation level and supports calcium transient activity. – interaction decreases IP_3R activation level and inhibits calcium transient activity. []'s indicate concentration. *-The open probability of the IP_3R displays a bell-shaped curve for dependence on $[Ca^{2+}]_i$ with a maximum probability at approximately 200 nM.

results in which we found that young neurons and a subpopulation of neural crest-derived cells, both of which typically express calcium transients, expressed activated CREB immunoreactivity (unpublished results). Understanding how calcium transients are generated and what effectors they activate, will provide insight as to their role in neural crest development.

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DISCUSSION

Summary of experiments and results

Through the experiments presented in this dissertation, I have examined neuronal differentiation of cultured neural crest cells with respect to neuronal commitment and neurogenesis. Using whole-cell recording, I established that neurons differentiated in culture exhibited identical voltage-dependent calcium current (VDCC) profiles to neurons differentiated in vivo. Furthermore, I showed that growth factors regulated the differentiation of the neuronal phenotype but were unable to modify the VDCC profiles in established neurons. To show that other characteristic neuronal features coincided with VDCC profiles, I established that neurons exhibiting sensory VDCC profiles also expressed capsaicin sensitivity and Brn 3.0-IR.

Calcium transient activity was tested for its role in neuronal differentiation of cultured neural crest cells. I monitored intracellular calcium levels and found that neural crest-derived cells exhibited calcium transient activity during the period of neurogenesis in culture. Using pharmacology and immunocytochemical correlations, I showed that young neurons and a subpopulation of (H-) neural crest-derived cells exhibited calcium transients. When calcium transients were blocked in culture, neurogenesis was inhibited. Lineage-tracing studies of active and inactive cells showed that only neural crest-derived cells exhibiting calcium transients were capable of giving rise to neurons. Lastly, the IP_3R regulated the primary release of Ca^{2+} during a calcium transient event. Modifying intracellular Ca^{2+} and IP_3 levels modulated this calcium transient activity.

Conclusions

1. Neurons differentiated in vitro and in vivo exhibit identical VDCC profiles.

2. VDCC profiles are established soon after neurogenesis.

3. Neural crest-derived cells exhibit calcium transients.

4. Calcium transient activity correlates with neuronal differentiation.

5. Calcium transient activity is required for neuronal differentiation.

6. IP_3R regulates calcium transient activity in cultured neural crest cells.

The results from my thesis research support a model in which specification of neuronal phenotype occurs prior to differentiation and calcium transient activity is required for neuronal differentiation to proceed.

Classification of a neuronal phenotype

A difficult issue in the study of neuronal development is the classification of neurons. The problem is establishing identifiable markers that are unique to a specific population of neurons. Many studies utilize the expression of specific neurotransmitters to identify neurons in culture. However, it has been clearly shown that neurotransmitter expression is modifiable characteristic. For example, substance P (SP) has been used to identify sensory neurons in culture, since nociceptive neurons in the dorsal root ganglion express

this protein (Ernsberger and Rohrer, 1988; Murphy *et al.*, 1994). However, LIF and CNTF can upregulate SP in sympathetic neurons, a neuronal population that does not normally express SP in vivo. This makes it difficult to assign a phenotype to neurons cultured in LIF or CNTF (Kessler and Black, 1982; Lentz *et al.*, 1993). Many other neurotransmitters are sensitive to growth factors, as well (Ji *et al.*, 1996; Sterne *et al.*, 1998; Wirth *et al.*, 1998).

More recently, transcription factors have been associated with particular neuronal lineages. Mash1 and Phox2 are required for the differentiation of many noradrenergic neurons (Hirsch *et al.*, 1998). Cranial neurons and dorsal root ganglion neurons require the expression of the neurogenins (Fode *et al.*, 1998; Ma *et al.*, 1998; Sommer *et al.*, 1996). Brn 3.0 is expressed by sensory and motor neurons (McEvilly *et al.*, 1996). However, transcription factors are not uniquely expressed among neuronal lineages, which limits their usefulness in distinguishing neuronal phenotypes. Although many neuronal lineages share transcription factors, these factors regulate the differentiation of unique phenotypes because of the cellular context in which they are expressed (Brunet and Ghysen, 1999).

A phenotypic characteristic that may be used to distinguish neurons is ion channel repertoire. Most neurons exhibit a unique ion channel profile, associated with their function in the nervous system (Mintz *et al.*, 1992). These profiles are established very early in development. For example, once primary induction of amphibian spinal neurons has occurred, the maturation of the action potential is cell-autonomous (Henderson and Spitzer, 1986). When comparing the development of VDCC and Na^+ currents in dorsal root and ciliary ganglion cells, both were shown to have a distinctive pattern of development and expression (Gottmann *et al.*, 1988). Some conductances may be more closely linked to the differentiation process, however. When comparing the neuronal

differentiation of two strains of PC12 cells, both strains exhibited neurite extension and an identical increase in expression of calcium currents, while only one strain showed an increase in the expression of a Na^+ current (Sherwood *et al.*, 1997). The results from the study indicated that calcium current expression was linked to neuronal differentiation of PC12 cells, while Na^+ currents were not.

Ion channel profiles make a good marker for neuronal classification, because the analysis is based on a physiological feature that is distinctive with respect to the function of a neuron (Gonzalez Burgos *et al.*, 1995; Swandulla *et al.*, 1991; Waterman, 1996; Yu *et al.*, 1992). For example, sensory and sympathetic neurons utilize a distinctive array of neurotransmitters in their synaptic communication. Sympathetic neurons primarily release a classical small molecule neurotransmitter, while sensory neurons depend largely on neuropeptides. Small molecule neurotransmitters are packaged in vesicles associated with active zones of the synaptic terminal; they require only short, localized increases in intracellular calcium levels to stimulate vesicular release. Neuropeptide-containing vesicles are not docked at specific sites and it has been established that they require a more sustained increase in cytoplasmic calcium levels for their release (May *et al.*, 1995). Because of these synaptic transmission requirements, sympathetic neurons primarily express N-type VDCC (Hirning *et al.*, 1988), which are closely associated with active zones and inactivate with prolonged depolarization (Gonzalez Burgos *et al.*, 1995). Sensory neurons primarily express VDCC that exhibit sustained activation with depolarization (Evans *et al.*, 1996). This allows the calcium levels rise to levels necessary for neuropeptide release.

Calcium transients and differentiation

Calcium transients play many important roles throughout development. Calcium transients regulate blastocoele formation (Stachecki and Armant, 1996a; Stachecki and Armant, 1996b); oocyte activation (Whitaker and Swann, 1993); exo/endocytotic vesicle shuttling in primitive endodermal cells (Sauer *et al.*, 1998); and myofibrillogenesis in embryonic Xenopus myocytes (Ferrari *et al.*, 1996). In all of these examples, calcium transient activity is a cell autonomous process. This intrinsic signalling mechanism affects the cell's response to extrinsic factors during development.

In neurons, calcium transient activity has been shown to play a significant role in regulating membrane excitability, neurotransmitter release, synaptic plasticity, aging, and neuronal-glial interactions (for reviews, (Henzi and MacDermott, 1992; Kostyuk and Verkhratsky, 1994)). However, most of these studies have been directed at the function of calcium transients in mature neurons. For years, it has been known that calcium can affect the differentiation, survival and phenotype of developing neurons (Chalazonitis and Fischbach, 1980; Collins *et al.*, 1991; Walicke and Patterson, 1981). More recently, it has been shown that neuronal precursors and immature neurons exhibit calcium transient activity and that this activity is required for their maturation (Holliday *et al.*, 1991; Jones *et al.*, 1995; Ribera and Spitzer, 1991; Spoerri *et al.*, 1990).

The most studied and well understood system exhibiting calcium transient activity is the Xenopus nervous system. Several studies have shown that calcium signaling is required from the onset of neurogenesis. Jones and Ribera (1994) showed that decreasing Ca^{2+} influx by overexpressing a potassium channel in the developing Xenopus embryo resulted in a reduced number of morphologically differentiated neurons (Jones and

Ribera, 1994). In a later study, they showed that suppressing steady state intracellular calcium levels, using BAPTA, also reduced the number of neurons generated (Jones *et al.*, 1995).

Xenopus spinal neurons continue to be dependent on calcium signaling during neuronal maturation. Holliday and Spitzer (1990) observed spontaneous calcium transient activity in young neurons that declined as the neurons matured. This calcium transient activity was found to regulate the phenotype of neurons (Holliday *et al.*, 1991). If calcium transient activity is blocked during a critical 6-12 hours after onset of neurogenesis, GABA-IR is suppressed (Spitzer *et al.*, 1993), a K^+ conductance fails to mature (Desarmenien *et al.*, 1993), and neurites extend abnormally (Holliday *et al.*, 1991). These three neuronal characteristics were also sensitive to the frequency of the calcium transients. A minimum of 2 events per hour were required for GABA expression and maturation of the K^+ conductance, while neurons exhibited decreased neurite outgrowth at calcium frequencies greater than 3 events/hr (Gu and Spitzer, 1995).

IP_3R and calcium signalling

Understanding how calcium transients are regulated requires an understanding of how they are generated. In general, nonexcitable cells that generate spontaneous calcium transients rely on the IP_3R to regulate the release of intracellular calcium stores (Korngreen *et al.*, 1997; Thomas *et al.*, 1996). A variety of conditions modulate the activity of the IP_3R, which allows for complex patterns of Ca^{2+} signaling. In mouse pancreatic acinar cells, low concentrations of IP_3 evoke repetitive local calcium spikes, while relatively high concentrations produce repetitive calcium waves (Petersen *et al.*, 1991). Calmodulin-dependent protein kinase II and PKA phosphorylation increases IP_3R

activity, while PKC phosphorylation inhibits calcium release (Bird *et al.*, 1993; Ferris *et al.*, 1991a; Ferris *et al.*, 1991b; Komalavilas and Lincoln, 1996; Matter *et al.*, 1993; Nakade *et al.*, 1994; Quinton and Dean, 1992; Volpe and Alderson-Lang, 1990). Furthermore, cells expressing different IP_3R isoforms exhibit different patterns of calcium transient activity (Miyakawa *et al.*, 1999). IP_3R2 is the most sensitive to IP_3 and is required for long lasting calcium oscillations. IP_3R1 mediates less regular pattern of calcium oscillations. IP_3R3 is least sensitive to IP_3 and Ca^{2+} and tends to generate monophasic calcium transients.

IP_3R-dependent calcium signaling has the potential to play a significant role in the development of specific lineages. Cells expressing different isoforms of the IP_3R exhibit a distinctive pattern of calcium transient activity (Cardy *et al.*, 1997; Hagar *et al.*, 1998; Iino, 1999; Putney, 1997; Wojcikiewicz, 1995; Wojcikiewicz and Luo, 1998; Yoneshima *et al.*, 1997). Furthermore, these isoforms are expressed in tissue-specific and developmentally regulated manner (Danoff *et al.*, 1991; De Smedt *et al.*, 1997; Fujino *et al.*, 1995; Nakagawa *et al.*, 1991; Sharp *et al.*, 1999; Sugiyama *et al.*, 1994). IP_3R1 is predominantly expressed in the brain. IP_3R2 and IP_3R3 are ubiquitously expressed and are found in the nonneuronal cells of the CNS. Expression of these receptors is regulated by differentiation signals. In mouse CNS neurons, NeuroD-related factor activates the expression of IP_3R1 in developing neurons (Konishi *et al.*, 1999). In hematopoietic cells, IP_3R expression changes when these cells are induced to differentiate (Sugiyama *et al.*, 1994). Finally, the IP_3R signaling environment is linearly inherited. The bulk of the signaling machinery is located on the ER membrane (Rossier and Putney, 1991), which is distributed equally during cell division.

Model for calcium transient activity and neuronal differentiation

This study shows that neural crest cells exhibit calcium transients and this activity is required for neurogenesis. A model depicting cellular requirements for neuronal differentiation of neural crest cells is shown in Figure 1. In this model, calcium transients alone are insufficient to produce neurons (Fig. 1A). Environmental signals in the form of cytokines and growth factors are also required. Our experiments have shown that neural crest cells grown in the absence of growth factors produce calcium transients for a short period in culture, but neurons are not born or die soon after their birth. Furthermore, growth factors alone are insufficient to induce neurogenesis (Fig. 1B). Preliminary results have shown that neural crest cells cultured at low density do not exhibit calcium transients and do not differentiate into neurons, although growth factors are present. Therefore, calcium transient activity and the appropriate differentiation signals are required for neuronal differentiation (Fig. 1C).

My studies support the hypothesis that calcium transient activity makes cells competent to respond to instructive environmental signals. Calcium transients may do this by increasing the sensitivity of the molecules responsible for transducing the differentiation signal. In one study, calcium influx through L-type VDCC led to tyrosine phosphorylation of the epidermal growth factor receptor in the absence of growth factor and subsequent activation of the MAPK signaling pathway (Rosen and Greenberg, 1996). Furthermore, calcium levels have been shown to increase the activity of the Ras/MAPK pathway directly, converting a mitotic signal into a differentiating signal (Marshall, 1995). In summary, calcium transients are cell-autonomous events that allow neural crest cells to respond to neurogenic signals.

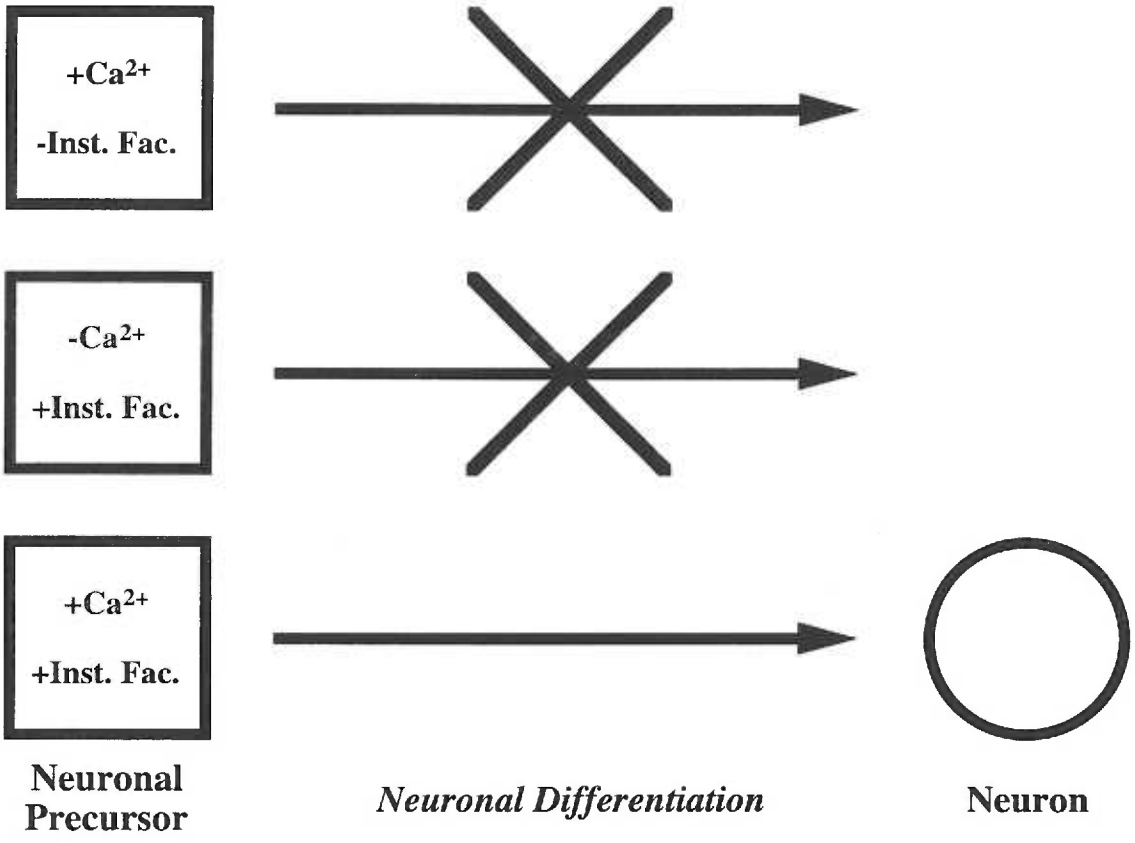

Figure 1. A model for calcium transient activity and neuronal differentiation. A '+' indicates the presence of a parameter. A '-' indicates the absence of a parameter. Calcium transient activity is indicated by the Ca^{2+}. Inst. Fac. represent the various instructional factors which are required for neuronal differentiation.

Future directions

The purpose of this study was to elucidate the role of calcium transient activity in neuronal differentiation of cultured neural crest cells. I have shown that undifferentiated neural crest cells exhibit spontaneous calcium transients. Furthermore, cells differentiating into neurons increase their calcium transient activity, while cells contributing to separate lineages stop producing this activity. As neurons mature, the calcium transient activity declines in these cells, such that all neural crest-derived cells are incapable of producing calcium transients once neurogenesis is complete. To further define the role of calcium transient activity in neurogenesis, subsequent research will need to be directed at determining the mechanisms that regulate this activity, neuronal characteristics that are dependent on this activity, and effectors systems modulated by this activity.

Since most undifferentiated neural crest cells can express calcium transients, these cells regulate this activity by either varying the frequency of expression or stopping the activity altogether. To determine what factors regulate calcium transient frequency, analyses should be focused on factors that regulate IP_3R agonist levels, i.e. intracellular calcium and/or IP_3 levels. One idea would be to determine how cellular density effects calcium transient activity and autonomic neurogenesis. Since high cellular density is associated with elevated calcium levels, we may be able to use conditioned medium or membrane extracts to isolate factors that raise intracellular calcium levels, inducing calcium transient activity and subsequent autonomic neurogenesis. To understand how neural crest-derived cells stop calcium transient activity, investigations should be directed at identifying mechanisms that essentially irreversibly modify the receptor (i.e., covalent modifications or changes in IP_3R isoform expression). Several studies have shown that

IP_3Rs are developmentally regulated (Parrington *et al.*, 1998; Toescu, 1995). Recently, a NeuroD-like factor was shown to upregulate the expression of a neuronal isoform (IP_3R1), during neuronal differentiation (Konishi *et al.*, 1999).

Since neural crest cells increase their calcium transient activity as neuronal differentiation proceeds, calcium transients may be involved in regulating the expression of several neuronal characteristics. Studies in Xenopus have shown that panneuronal (neurite extension) and phenotype-specific traits (GABA-IR and ion channel modulation) are sensitive to the frequency of calcium spikes (Gu and Spitzer, 1995; Gu and Spitzer, 1997). The neural crest model used in this thesis can be used to characterize calcium-dependent traits in mammalian neuronal differentiation. Furthermore, we can take advantage of the separate neuronal lineages (sensory and autonomic) to determine if lineage-specific neuronal traits require distinct patterns of calcium transient expression.

To determine how calcium transient activity regulates distinct aspects of neurogenesis, we need to identify the downstream effectors responsible for mediating calcium transient action on neurogenesis. Several signal transduction pathways involved in neuronal development are known to be modulated by intracellular calcium. Elevated intracellular calcium levels can enhance growth factor signalling pathways by increasing the intrinsic activity of tyrosine kinase receptors (Rosen and Greenberg, 1996) and the small g-protein, ras (Gawler, 1998). Furthermore, calcium transient activity has been shown to increase the activity of CREB, a transcription factor activated by the MAPK signalling pathway, by activating CAMKIV (Bito, 1998; Chawla *et al.*, 1998; Deisseroth *et al.*, 1998; Fields *et al.*, 1997). In cultured neural crest, we have preliminary evidence, which suggests that neural crest cells exhibiting calcium transient activity express the phosphorylated form of CREB. Future studies, using pharmacology and

immunocytochemistry, are necessary to determine the mechanism that mediates calcium transient regulation of neuronal differentiation.

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