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Neuronal class-specific patterns of calcium activity and target dependence of neurotransmitter expression in the developing spinal cord

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Neuronal Class-Specific Patterns of Calcium Activity and Target Dependence of Neurotransmitter Expression in the Developing Spinal Cord

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Biological Sciences by Qian Xiao

Committee in Charge:

Professor Nicholas Spitzer, Chair
Professor Darwin Berg
Professor Anirvan Ghosh
Professor Mark Tuszyinski
Professor Binhai Zheng

2009
The Dissertation of Qian Xiao is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2009
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I want to thank all my friends for their friendship and support. Particularly I want to thank Jiajuan Liu for being my best friend and for the almost daily online
conversation that allowed us to share our lives with each other. I want to thank all the friends I met in my 3 years volunteering with the Rural China Education Foundation, particularly Weiji Ma, a great friend, my first mentor in the NGO world and a neuroscientist himself.

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Chapter III is a preprint of “Xiao Q, Spitzer NC. Muscle-dependent regulation of neurotransmitter specification and embryonic neuronal calcium spike activity. (In revision)” of which I am first author.
VITA

2003  B.S. in life sciences,
University of Science and Technology of China

2004 – 2007  Teaching Assistant, Division of Biological Sciences, University of California, San Diego

2004 – 2009  Research Assistant, Division of Biological Sciences, University of California, San Diego

2007  Ph.D. in biological sciences, University of California, San Diego

PUBLICATIONS

Xiao Q and Spitzer NC Muscle-dependent regulation of neurotransmitter specification and embryonic neuronal calcium spike activity. In revision.


FIELD OF STUDY

Major Field: Biological Sciences

Studies in Developmental Neurobiology
Professor Nicholas C. Spitzer
ABSTRACT OF THE DISSERTATION

Neuronal Class-Specific Patterns of Calcium Activity and Target Dependence of Neurotransmitter Expression in the Developing Spinal Cord

by

Qian Xiao

Doctor of Philosophy in Biological Sciences
University of California, San Diego, 2009

Professor Nicholas Spitzer, Chair

The proper functioning of all nervous systems requires efficient communication among neurons. Neurotransmitters provide the fundamental basis for neural communication. Establishing the expression of the appropriate neurotransmitters in different populations of neurons is a critical challenge for neural development. This is a complicated process that involves multiple mechanisms, including intrinsic genetic coding, early neuronal activity and signal transduction cascades driven by neuronal targets.
Embryonic *Xenopus* spinal neurons generate spontaneous transient elevations of intracellular calcium. Different classes of neurons exhibit this calcium spike activity in distinct patterns, and altering this activity modulates transmitter expression. However neurotransmitter expression is sensitive to this neuronal spike activity only during an early period of development. Both calcium spiking activity and the sensitivity of activity-dependent transmitter specification decrease and disappear as embryos mature. I showed that spinal transection causes neurons to resume calcium spike activity, and that enhancing this activity leads to increased expression of the inhibitory transmitter GABA. These results indicate a potential reopening of the sensitive period that exists earlier in development.

Using a neuron-muscle co-culture system, I showed that muscle, as a neuronal target, plays important role in regulating transmitter expression. Muscle contact refines transmitter expression in cultured neurons by reducing the expression of non-cholinergic transmitters, GABA, glycine and glutamate, while leaving choline acetyltransferase (ChAT) expression unaltered. Trophic factors that are normally released at synapses and delivered retrogradely along axons appear to be sufficient to induce such changes. Further investigation revealed that muscle electrical activity is required for muscle-dependent suppression of non-cholinergic transmitter expression. In addition, muscle contact also suppresses neuronal spontaneous calcium activity and abolishes activity-dependent transmitter specification at early embryonic stages.
CHAPTER I

INTRODUCTION
**Calcium-dependent electrical activity in early development**

Using electrical recordings or optical imaging methods, spontaneous calcium-dependent electrical activity is widely observed at early stages of neuronal development in various vertebrate and invertebrate model systems (Spitzer, 2006). This activity is critical in determining many aspects of neuronal development, including neuronal proliferation, migration, and differentiation.

*Spontaneous calcium spikes in developing Xenopus laevis*

Spontaneous calcium-dependent spikes are characteristic of the early development of *Xenopus* spinal neurons, both *in vitro* and *in vivo* (Gu & Spitzer, 1993; Borodinsky et al. 2004). In contrast with sodium-dependent action potentials that last for only a few milliseconds in adult animals, these spikes rely on depolarization-induced calcium influx from voltage-gated calcium channels and can last for as long as half a minute. Low voltage-activated calcium currents initially depolarize the membrane potential, triggering the activation of voltage-gated sodium and high voltage-activated calcium channels. Influx of calcium ions leads to calcium-induced calcium-release from intracellular compartments (Holliday et al., 1991).

Spontaneous calcium spikes can be suppressed by removal of extracellular calcium ions, pharmacological blockade with antagonists of various voltage-dependent calcium channels, and overexpression of potassium channels that cause hyperpolarization (Gu & Spitzer, 1993; Borodinsky et al. 2004). Depolarization by high extracellular KCl activates voltage-dependent calcium channels and leads to increased level of intracellular calcium (Holliday and Spitzer, 1990). Both the
incidence and frequency of spontaneous calcium spikes can be enhanced by overexpression of sodium channels or by incubation with drugs such as veratridine that prevent sodium channel inactivation.

**Developmental regulation of spontaneous calcium activity**

In *Xenopus laevis* spinal neurons, spontaneous calcium spike activity is normally first detected at the neural tube stage (stage 20) (Spitzer & Lamborghini, 1976; Holiday & Spitzer, 1990; Gu & Spitzer, 1993; Borodinsky et al. 2004) prior to synapse formation (Hayes & Roberts, 1973). *In vitro*, spontaneous calcium activity is detected in spinal neurons plated from neural plate stage (stage 15), as early as 5 hr following plating.

The onset of spontaneous calcium activity is driven by a combination of intrinsic and extrinsic factors. The genetically programmed expression of the β3 subunit of the Na⁺, K⁺-ATPase is temporally regulated in early development of both *Xenopus laevis* and its closely related cousin *Xenopus tropicalis*. This regulation, although different between the two amphibian species, coincides with the peak of spontaneous calcium activity in their spinal neurons (Good et al., 1990; Messenger & Warner, 2000; Chang & Spitzer, 2009). Disrupting this developmental regulation depolarizes the membrane potential in *Xenopus tropicalis* and suppresses spontaneous calcium activity at later stages (Change & Spitzer, 2009). On the other hand, recent study of the promiscuously expressed neurotransmitters, GABA and glutamate, shows that environmental factors such as these transmitters expressed early in development
can act through metabotropic receptors and downstream kinase signaling pathways to drive spontaneous calcium spike activity (Root et al., 2008).

Spontaneous calcium spiking activities peak during a critical time window that spans approximately 10 hr in vivo and 5 hr in vitro before both incidence and frequency decline significantly (Borodinsky et al., 2004, Spitzer & Lamborghini, 1976). With further development, voltage-dependent delayed rectifier potassium currents are developmentally upregulated and the long-duration calcium-dependent impulses are converted into brief sodium-dependent action potentials (Lockery and Spitzer, 1992).

Distinct classes of neurons exhibit distinct patterns of calcium activity with different incidence and frequency, and these patterns change as development progresses (Borodinsky et al., 2004). In chapter II, I analyze different parameters, such as the amplitude, duration, and frequency of spontaneous calcium spikes, to characterize calcium activity for each neuronal class. I found that by analyzing frequency and amplitude, patterns of calcium activity imaged in four different classes of neurons can be distinguished from each other.

NEUROTRANSMITTER SPECIFICATION IN EARLY EMBRYONIC DEVELOPMENT

Efficient communication among neurons ensures normal functioning of the nervous system. Over the course of development, neurons establish the basis of neural communication as they acquire the proper expression pattern of both neurotransmitters and their matching receptors. This developmental process is an immensely critical and
complicated one that requires synergistic interplay among genetic coding, electrical activity and environmental factors.

**Genetic regulation of transmitter specification**

It is widely accepted that distinct expression patterns of intrinsic transcription factors can establish distinct neuronal identities (Lee & Pfaff, 2001; Ma, 2006) and there are numerous studies showing that neurotransmitter expression can be determined by transcription factors. Ectopic expression of transcription factors elicits ectopic expression of neurotransmitters. In chick embryos, misexpression of homeobox gene *MNR2* in interneurons drives the motor neuron phenotype by inducing the expression of choline acetyltransferase (ChAT), the enzyme that synthesizes motor neuron transmitter acetylcholine (Tanabe et al., 1998). *Ist*, the Drosophila homolog of *islet-1* and *islet-2* genes, encodes the transcription factor that mediates motor neuron differentiation in vertebrates. This transcription factor also determines axon pathfinding and targeting of a group of embryonic neurons, and regulates dopamine and serotonin synthesis (Thor & Thomas, 1997).

Moreover, in many cases, genetically coded factors do not act alone, but interact with each other to ensure expression of a specific transmitter phenotype. For example, in the mouse spinal cord, the combined expression of *Nkx2.2*, *Lmx1b*, and *Pet-1* is required to drive differentiation of the serotonergic phenotype (Cheng et al., 2003). The role of complex transcription factor networks in transmitter specification is probably best demonstrated by the well balanced development of GABAergic and glutamergic phenotypes in the mouse spinal cord. In the ventral spinal cord a lim-only
protein, LMO4, can induce GABAergic V2b-interneurons in collaboration with a bHLH factor, SCL; at the same time, LMO4 can block a homeodomain factor, Lhx3, from generating glutamatergic V2a-interneurons (Joshi et al., 2009). In addition, some transcription factors have more than one role in the development of the nervous system. In the dorsal spinal cord, a basal GABAergic differentiation state is initially defined by \( Lbx1 \), but glutamatergic cell fate is promoted by the antagonistic roles of \( Tlx1 \) and \( Tlx3 \) on \( Lbx1 \). In addition, a bHLH gene, \( Ptf1a \), can in turn suppress \( Tlx3 \) expression, and thus allow \( Lbx1 \) to promote GABAergic differentiation (Cheng et al. 2004, 2005; Glasgow et al. 2005; Hoshino et al. 2005; Ma, 2006).

Activity-dependent regulation of transmitter specification

Spontaneous calcium spike activity is critical in regulating inhibitory and excitatory transmitter phenotypes in embryonic \( Xenopus \) spinal neurons. In culture, the incidence of neurons expressing GABA or glutamic acid decarboxylase (GAD), the synthetic enzyme for GABA, increases following enhanced calcium activity (Gu & Spitzer, 1995; Watt et al., 2000). In vivo, spontaneous calcium activity regulates transmitter expression in a homeostatic fashion. Suppression of activity in young embryos increases the incidence of neurons expressing excitatory transmitters (glutamate and acetylcholine) and decreases the incidence of neurons expressing inhibitory transmitters (GABA and glycine). On the other hand, enhancing activity leads to the opposite effects (Borodinsky et al., 2004). Changes in transmitter expression in presynaptic neurons caused by altered activity are also matched by respective changes in receptor expression on the postsynaptic side, and functional
synapses as well as effective transmission are established between neurons and targets (Borodinsky & Spitzer, 2007).

This activity-dependent specification of transmitter occurs only during a relatively brief developmental time window both in vitro and in vivo. In vitro, transmitter expression can be specified only during a 5 hr critical period that occurs 5 hr after neural plate neurons are cultured (Borodinsky et al., 2004). In vivo, the sensitive period extends from about 19 hr to 35 hr after fertilization (Root et al., 2008). Transmitter expression is most responsive to changes in calcium activity between neural tube formation (stage 20) and an early larval stage (stage 28), and sensitivity decreases during early tailbud stages and disappears when the embryo approaches stage 35 (Root and Spitzer, 2004). The weakening and disappearance of the sensitive period for activity-dependent transmitter specification coincides with the termination of the period of spontaneous calcium spike activity.

In chapter III I show that spontaneous calcium activity can be re-triggered in later stage embryos following axotomy. Axotomy may also reopen the sensitive period for activity-dependent transmitter specification. It remains to be determined whether neuronal injury, removal of target, or both contribute to the return of spontaneous calcium spike activity and respecification of neurotransmitters.

**TARGET REGULATION OF NEURONAL DEVELOPMENT**

Establishment of the correct connections between neurons and their targets depends on multiple developmental programs that involve cell survival and apoptosis, axon and dendrite growth, synaptogenesis, and transmitter specification. These events
are all two way processes in which both the neurons and their targets communicate extensively with each other to ensure proper alignment. The expression of members of neurotrophin families and other target derived factors peaks early in neuronal development and declines at later times (Timmusk et al., 1993), suggesting their critical roles in promoting, guiding and refining neuronal development (Zweifel et al., 2005).

A series of ablation and transplantation experiments in amphibian and avian embryos have demonstrated the importance of targets on neuronal survival (Levi-Montalcini, 1987). Extirpation of the embryonic chick wing bud causes death of spinal motor neurons and transplantation of exogenous wing buds leads to increased numbers of neurons by enhancing neuronal survival. In vitro experiments with the compartmentalized Campenot chamber system show that trophic factors such as NGF can be retrogradely transported from distal axons to the neuronal soma and promote both survival and axon extension of cultured sympathetic neurons (Campenot, 1977). The size and complexity of dendritic arbors of sympathetic neurons are positively influenced by the size of target field and axotomy leads to retraction of dendrites (Voyvodic, 1989; Ruit et al, 1990). A subset of mammalian sympathetic ganglion neurons undergo a switch from a noradrenergic to a cholinergic phenotype after they innervate sweat glands, and transplantation experiments show that this developmental change is triggered and regulated by targets (Furshpan et al., 1976; Landis & Keefe, 1983; Nawa & Patterson, 1990; Francis & Landis, 1999).

Muscle tissue expresses many important trophic factors during development, including NT-3/4/5, BDNF, NGF, GDNF, CNTF, and ILF. Muscle-derived factors
play crucial roles in establishing motor neuron identities. Neuronal expression of ChAT and acetylcholinesterase (AChE) depend on muscle factors and axotomy or muscle removal often results in loss of their expression (Lams et al., 1988; Jiang et al., 2003). Application of trophic factors including NT-4/5, BDNF and GDNF can partially rescue such losses (Kou et al., 1995; Fernandes et al., 1998). Muscle-derived factors are also critical in establishment of presynaptic electrophysiological properties by refining quantal release of acetylcholine, shaping mature action potentials and regulating ion channel expression (Dryer et al., 2003; Liou & Fu, 1997; Nick & Ribera, 2000).

In chapter III, I demonstrate the role of muscle targets on transmitter specification and early neuronal calcium spike activity. I found that muscle derived factors prevent neurons from expressing non-cholinergic neurotransmitters and reduce spontaneous calcium activity. I also showed that muscle factors abolish activity-dependent transmitter specification. I further studied the mechanisms of muscle-dependent regulation of transmitter specification and revealed that retrogradely transported factors and muscle activity are involved in this process. In the end, I propose a model with which I integrate the developmental roles of intrinsic genetic coding, spontaneous calcium spike activity and target derived factors in establishing the appropriate expression of neurotransmitters.
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CHAPTER II

IDENTIFICATION OF DISTINCT CALCIUM SPIKE ACTIVITY PATTERNS IN DIFFERENT CLASSES OF NEURONS
**INTRODUCTION**

Different classes of embryonic *Xenopus* spinal neurons generate different frequencies of calcium spikes at different stages of development (Borodinsky et al., 2004). Calcium spike activity contributes to the specification of the neurotransmitter, but the way in which information is coded in the pattern of calcium spikes is not known. Spike frequency, interspike interval, and burst duration are all plausible candidates to regulate differentiation. In this project I have used an unbiased mathematical screening technique to determine what factors can distinguish different classes of neurons, by analyzing spike patterns recorded from neurons of known identity. The results indicate that neuronal classes can be sorted successfully by their spike frequencies and amplitudes, and not by interspike intervals or burst durations. This suggests that neurons use spike frequency and amplitude to specify transmitter expression. Determination of the molecular basis of transmitter specification will confirm or refute this conclusion.

**METHODS**

*Imaging*

Fluorescence of the Ca indicator fluo-4AM (Molecular Probes) was used to study elevations of [Ca]$_i$ in neurons. Neural tubes and cultured cells were incubated for 30 min to 1 hr in culture medium containing 2-5 µM dye and 0.01% Pluronic F-127 and washed in culture medium prior to imaging. Culture medium contained (in mM): 116.6 NaCl, 0.67 KCl, 1.31 MgSO$_4$, 2 CaCl$_2$ and 4.6 Tris, pH adjusted to 7.8 with HCl. The osmolarity of all solutions was held constant during ion substitutions.
Images were acquired at 0.2 Hz for 1 hr periods with a BioRad MRC 1024 or Leica SP5 laser confocal system with a 20x water immersion objective. Images were transferred to Power Macintosh computers and saved to CD-ROM.

Image stacks were imported into NIH Image 1.62 (W. Rasband, NIH) for analysis and transients were initially identified by eye when the image stack was animated. Using the line-drawing tools in the program, a region of interest (ROI) was traced around the transient and a measurement of the average pixel intensity within the ROI was acquired. This measurement was repeated for every image in the stack and the values were exported to the Microsoft Excel program for analysis, plotting these values against the time of image acquisition to yield kinetic and fluorescence intensity ratio data.

Analysis

Clustering analysis was done with an R 1.9.1 program, using the “hclust” algorithm and the “ward” method. The “hclust” algorithm is an agglomerative (bottom up) hierarchical clustering algorithm that can create a hierarchy of clusters with similar data grouped together. It starts by assigning each single data point to a group and then calculating distances between data points (now also groups). It combines the closest groups to form a new group. This clustering process is repeated until a single, all inclusive group is generated. In the end, a dendrogram of clusters is produced.

The ward agglomerative clustering method calculates the distance between groups using the following formula:
\[
d(AB,C) = \frac{(n_A+n_C) \cdot d(A,C) + (n_B+n_C) \cdot d(B,C) - n_C \cdot d(A,B)}{(n_A+n_B+n_C)}
\]

d(X, Y) calculates the distance (Euclidean distances) between two data points and \(n_X\) represents the number of data points within the X group.

**Results**

During early neural development, spontaneous calcium spike activity can be observed in embryonic spinal neurons in *Xenopus laevis* via calcium imaging (Gu & Spitzer, 1993; Borodinsky et al. 2004). Interestingly, different groups of neurons appear to have distinct patterns of calcium activity (Fig. 1A & B). We are interested in 4 groups of neurons: Rohon-Beard sensory neurons (RB), which lie along the midline of the dorsal spinal cord; dorsolateral interneurons (DLI); ventral interneurons (VI) and motorneurons (MN), which are located ventrolaterally. This mini project sought algorithms by which to identify different neurons by their patterns of calcium spike activity.

I acquired 15 *in vivo* calcium imaging recordings from stage 25-26 embryos for each of the 4 groups of neurons. All were 1 hr in duration, under the same conditions. For each calcium imaging trajectory, I measured 5 different parameters that we considered of potential importance in distinguishing the activity patterns of different classes of neurons (Fig. 2, Table 1): frequency (number of spikes per hour), number of bursts (spikes that occur adjacent to the previous one, without intensity dropping to baseline), amplitude (\(F/F_0\)), duration (spike width), and ISI (duration of interspike interval). I tested different parameters and different combinations of all
parameters in order to find the parameter(s) that best distinguish different neurons via their spiking patterns.

In the end, I found the program gave best results using frequency by itself: dorsal neurons were mostly distinguished from ventral neurons and most RB neurons were also distinguished from DLIs (Fig. 3). However, VI and MN were not well separated.

To further identify the difference of activity patterns between VI and MN, I chose amplitude as the new parameter. MNs seemed to have more large amplitude spikes than VIs. For each recording trace, there were usually no more than 15 spikes, which was not enough to generate a sensible distribution graph for each individual trace. Since all the traces were recorded in a similar condition, I combined all 15 traces for each group of neurons and plotted a distribution graph for all the spikes for the same group of neurons (Fig. 4).

From this distribution, it appeared that there exist two types of spikes, which formed two peaks of the distribution, centered at amplitudes of 130 and 190. VI and MN had different distribution patterns among these two types of spikes. VIs seemed to have more spikes in the “190 group” and MNs tended to have more spikes in the “130 group”. In order to identify statistical significance between these two types of amplitude distribution in different neurons, I arbitrarily set 170 as the break mark: every spike with an amplitude $\leq 170$ was called a “small spike” and every spike above 170 was called a “big spike”. After counting the numbers of small spikes vs. big spikes in the two groups of neurons, I performed a Chi-square test (Table 2.).
It turned out that the distributions of big and small spikes between the two groups of neurons are significantly different, with $P<0.005$. This means that the possibility that VI and MN had an equal probability of generating big spikes and small spikes was very small. Accordingly I am at least 99.5% confident in saying that MNs and VIs have a different distribution pattern between big spikes and small spikes.

However, a dendrogram cannot be calculated, because instead of using individual traces from each recording, all 15 traces were combined into a single pool of data to calculate the incidence of small vs big spikes. Even though we obtained too few spikes to analyze the distribution of spike amplitudes from a single recording, combination of several recordings (or prolonging the recording time to get more spikes) enables distinction between the two groups of neurons.

**DISCUSSION**

The four classes of neurons analyzed can be distinguished on the basis of the frequency and amplitude of the calcium spikes that they generate. It will be interesting to determine the biological and physiological basis of the differences in these parameters. Spike frequency could be specified by constellations of ion channels, local ligands, or some combination of the two. Spike amplitude may be specified by the amplitude of calcium stores in the endoplasmic reticulum. Whether the downstream effects of calcium spikes are determined solely by their frequency and amplitude or by other parameters such as spike bursts remains to be investigated.
REFERENCES


Figure 2_1 Different patterns of calcium spike activity in different neurons. Upper: Calcium imaging trajectory from a RB neuron. Bottom: Calcium imaging trajectory from a ventral interneuron.
Table 2.1 Values of 6 parameters for calcium patterning for 59 neurons analyzed.

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Figure 2.2 Dendrogram of clustering the calcium imaging trajectory of four different groups of neurons by frequency.
Figure 2-3 Amplitude distributions of spikes from VIs and motoneurons.
Table 2.2 Chi-square test results of spike amplitude distributions of ventral interneurons and motoneurons.

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CHAPTER III

MUSCLE-DEPENDENT REGULATION OF NEUROTRANSMITTER SPECIFICATION AND EMBRYONIC NEURONAL CALCIUM SPIKE ACTIVITY
Abstract

Neurotransmitter specification has been shown to depend on genetic programs and electrical activity, however target-dependent regulation also plays important roles in neuronal development. We used a neuron-muscle co-culture system to investigate the impact of muscle targets on transmitter specification in *Xenopus* spinal neurons. Neuron-muscle contact reduces expression of the non-cholinergic transmitters, GABA, glycine and glutamate, while having no effect on choline acetyltransferase (ChAT) expression. Muscle activity is necessary for target-dependent reduction of non-cholinergic transmitter expression. In addition, co-culture with muscle cells suppresses early spontaneous calcium spike activity in neurons and the presence of muscle cells abolishes activity-dependent transmitter specification. The results demonstrate that muscle-dependent regulation can be crucial in establishing neurotransmitter phenotypes and altering early neuronal excitability.

Introduction

Neurotransmitters provide the fundamental basis for neurons to communicate with their targets, and thus the expression of proper transmitters is critical for the normal functioning of the nervous system. The developmental specification of transmitter phenotype is a complicated process that involves the interplay of several different mechanisms and pathways. Selective gene expression can specify neuronal identities (Lee & Pfaff, 2001). Misexpression of the homeobox gene *MNR2* in chick embryos induces ectopic expression of choline acetyltransferase (ChAT), the gene encoding the enzyme that synthesizes acetylcholine (Tanabe et al., 1998). LMO4
induces GABAergic differentiation in V2b-interneurons and blocks Lhx3-induced glutamatergic differentiation in V2a-interneurons in mouse spinal cord (Joshi et al., 2009). Early neuronal activity also plays important roles in transmitter specification. Spontaneous brief elevations of intracellular calcium (calcium spikes) are observed in embryonic spinal neurons of *Xenopus laevis* both in culture and *in vivo* (Gu et al., 1994). Different classes of neurons express different patterns of calcium spikes (Borodinsky et al., 2004), and the frequency of calcium spikes specifies neurotransmitter expression in a homeostatic manner: when calcium spike frequency is enhanced, more neurons express inhibitory neurotransmitters (GABA and glycine); when calcium spike frequency is suppressed, more neurons express excitatory neurotransmitters (acetylcholine and glutamate; Borodinsky et al., 2004).

At embryonic stages, developmentally regulated extrinsic factors determine neural fate in combination with factors intrinsic to the neurons. Target-derived factors regulate neuronal survival, axonal and dendritic growth, and synapse formation (Zweifel et al., 2005). Neurotrophic factors can drive acetylcholine expression instead of noradrenaline in mammalian sympathetic ganglion neurons, both *in vivo* and *in vitro* (Furshpan et al., 1976; Landis & Keefe, 1983; Nawa & Patterson, 1990; Francis & Landis, 1999). Neurotrophin transcript expression peaks early in embryonic development and declines at later times (Timmusk et al., 1993), consistent with a developmental role of these factors. Muscle tissue is an important source for many trophic factors including NT-3/4/5, BDNF, NGF, GDNF, CNTF, and LIF. Muscle-derived factors have been shown to be critical for neuronal expression of ChAT (Lams et al., 1988) and acetylcholinesterase (AChE) (Jiang et al., 2003). Eliminating these
factors by axotomy or muscle removal often results in cell death or loss of ChAT and AChE expression. The loss can be rescued by application of trophic factors including NT-4/5, BDNF and GDNF (Kou et al., 1995; Fernandes et al., 1998). Muscle-derived factors are also critical for establishing mature electrophysiological properties at synapses (Liou & Fu, 1997; Nick & Ribera, 2000). These studies demonstrate the importance of muscle-derived factors in neuronal development. However the interplay between target-derived factors and early neuronal activity, as well as the mechanism associated with this muscle-dependent regulation of neuronal development, is yet to be understood.

Here we report that in culture, muscle cells suppress non-cholinergic transmitter expression in *Xenopus* spinal cord neurons that grow and make contact with them. We show that retrogradely transported signals and muscle activity are key players in target-dependent transmitter specification. Muscle cells also suppress early neuronal calcium spike activity. In addition, co-cultured muscle cells abolish activity-dependent transmitter specification without neuronal contact.

**METHODS**

*Cell Culture* Mixed cell cultures containing neurons and muscle cells were prepared from *Xenopus* neural tube (stage 20) embryos (Ribera & Spitzer, 1989; Holliday & Spitzer, 1990). Myotomes and neural tube were dissected from embryos using jewelers' forceps and tungsten needles in the presence of 1 mg/ml collagenase. Dissociated muscle cells were plated on one side of a 35 mm tissue plastic culture dish, either as a dense lawn or in a striped fashion. Neurons were plated in stripes
across the whole dish, 0 or 24 hr after plating muscle cells unless otherwise specified. Neuron-alone cultures were prepared as previously described (Gu & Spitzer, 1995; Borodinsky et al., 2004). Cultures were allowed to grow 24 hr after plating neurons before they were fixed and stained (Fig. 1A, B). Culture medium contained (in mM): 116.6 NaCl, 0.67 KCl, 1.31 MgSO$_4$, 2 CaCl$_2$ and 4.6 Tris, pH adjusted to 7.8 with HCl. Only those cultures with more than 100 neurons on each side were scored (Holliday and Spitzer, 1993).

**Conditioned Medium,** Myotomes from 6 neural tube stage embryos were dissected, dissociated, plated and allowed to grow for 48 hr in 3 ml of culture medium. 2.5 ml of conditioned medium were harvested from each dish and transferred to a new culture dish in which neurons from a single neural tube stage embryo were then plated. For the control condition, 3 ml of culture medium were incubated in a culture dish without muscle cells and harvested after 48 hr. Neurons were allowed to grow in each medium for 24 hr before they were fixed and stained.

**Immunocytochemistry** Cultures were fixed in 4% paraformaldehyde, 0.1% or 0.025% glutaraldehyde, phosphate-buffered saline (PBS; pH 7.4) for 10-15 min at 20°C. Preparations were washed in PBS for 30 min, incubated in a blocking solution of 1% fish gelatin (Sigma) for 0.5 hr at 20°C, and stained overnight at 4°C using the following antibodies: mouse IgG anti-β-tubulin (Sigma), 1:1000; guinea pig anti-GABA (Chemicon), 1:1000; rabbit anti-glycine (Chemicon), 1:50; rabbit anti-glutamate (Sigma), 1:10000; mouse IgM anti-HNK-1 (Sigma), 1:100. Fluorescent secondary antibodies (Invitrogen) were used at 1:300 for 1-2 hr at 20°C. Immunoreactivity was examined on a Zeiss Axioskop with a 40x water objective.
using a Xenon arc lamp, attenuated by neutral density filters and the appropriate excitation and emission filters for Alexa 488 and Alexa 594 fluorophores. Images were acquired and analyzed with Axiovision (Zeiss). Neurons were considered immunopositive when the average pixel intensity of the neuronal cell body was 10 times higher than that of the background tissue culture plastic dish. The average pixel intensity of immunonegative neurons was less than 5 times of that of the background.

**In situ hybridization** A 1.5 kb probe cDNA template for ChAT was a generous gift from Dr. Margaret Saha (College of William and Mary). It was cloned in a PCR4 vector and anti-sense RNA probe was transcribed using the Megascript Kit (Ambion) in the presence of digoxigenin-labeled UTP (Roche). Cell cultures were fixed in 4% PFA for 1 hr, washed for 30 min in PBS and treated with 0.2 M HCl for 10 min. Hybridization was carried out at 60°C overnight with a probe concentration of 40 ng/ml. Preparations were washed in 0.2X saline sodium citrate buffer for 1 hr at 60°C and 30 min at 20°C before staining overnight at 4°C using anti-digoxigenin antibody (coupled to alkaline phosphatase) diluted to a final concentration of 1:1000 in 10% goat serum. Cultures were then rinsed 5 times in PBT and 2 times in alkaline phosphatase buffer at 20°C and incubated in BM Purple (Roche) for 12-36 hr for signal development. Cultures were washed in PBS for 1 hr before anti-β-tubulin antibody was introduced following the protocol described above, to help visualize neurons.

**Imaging** Fluorescence of the calcium indicator Fluo-4AM (Invitrogen) was used to study elevations of [Ca] in neurons. Cultured cells were incubated for 45 min to 1 hr in culture medium containing 5 µM dye and 0.01% Pluronic F-127 detergent
(Invitrogen) and washed in culture medium prior to imaging. Images were acquired at 0.2 Hz for 30 min periods with a BioRad laser confocal system with a 20x water immersion objective. Image stacks were imported into NIH ImageJ for analysis. Transients were initially identified by eye. For each neuron of interest, the cell body was traced using the line-drawing tools in the program and the average pixel intensity was determined. This measurement was repeated for every image in the stack and the values were exported to Microsoft Excel for analysis. These data were plotted against the time of image acquisition to yield kinetics and fluorescence intensity. Calcium transients were scored as spikes when the amplitude exceeded twice the baseline variation during the previous ten minutes and the rise time was complete within 5 sec. 4-5 cultures were imaged for each experiment and the incidence of spiking neurons was calculated by dividing the total number of neurons that spiked at least once during the 30 min imaging period by the total number of neurons imaged in that culture dish.

**Pharmacology** Stock concentrations of drugs were 1 mM veratridine, 100 mM α-bungarotoxin (BgTx). Veratridine and BgTx were added immediately before plating neurons, with final concentrations of 1 µM and 250 nM, respectively.

**Channel overexpression:** The hKir2.1 construct was a generous gift of Dr. Eduardo Marban (Johns Hopkins University). The gene was subcloned into a Bluescript vector and mRNA was transcribed using the mMessage mMACHINE kit (Ambion). Capped RNA (5-10 nl of a 0.01-0.1 mg/ml RNA solution in 10% MMR, 6% Ficoll) was co-injected with a Cascade Blue or Rhodamine Red 30 kDa dextran (30 mg/ml) into both blastomeres at the 2-cell stage using a picospritzer (Picospritzer III, Parker Instr). Control injections consisted of fluorescent dextran alone (30 mg/ml in 10% MMR).
**Statistical analysis** Means and standard errors were calculated using Microsoft Excel. Statistical analyses were performed using online software provided by BrightStat (www.brighstat.com). For comparison among more than two groups, the non-parametric Kruskal-Wallis ANOVA test was used and followed by the non-parametric Conover post-hoc test. For comparison between two groups, the Mann-Whitney U test was used. Data are presented as mean ± s.e.m. Results are considered significant when p<0.05.

**Results**

**Neuron–muscle co-culture reduces the number of neurons expressing non-cholinergic neurotransmitters**

To investigate the influence of muscle cells on neurotransmitter expression, we designed a co-culture system in which muscle cells dissociated from a neural tube stage *Xenopus* embryo were plated onto one half of a culture dish and spinal neurons dissociated from another neural tube stage embryo were plated across the whole dish one day later. This stage is prior to the formation of nerve-muscle contacts *in vivo* (Kullberg et al, 1977). The procedure allowed neurons on the muscle side to form nerve-muscle contacts while neurons on the blank side grew free of contacts (Fig. 1A, B). Muscle contractions were observed on the neuron-muscle side one day after plating neurons, consistent with the formation of neuromuscular junctions (NMJs) during this period (Spitzer & Lamborghini, 1976). Contractions were not observed when muscle cells were plated alone.
The numbers of neurons expressing non-cholinergic neurotransmitters, GABA, glycine, and glutamate, were compared between the muscle side and the blank side within the same culture dish, by assaying GABA-, glycine- and glutamate-immunoreactivity (-IR). Neuronal identity was recognized by both morphology and immunostaining for neuron-specific β-tubulin (Fig. 2A). Muscle cells pick up light β-tubulin staining both in vitro and in vivo (S.B. Sann, unpublished data). However this does not affect recognition and counting of neurons because muscle cell morphology is distinct from that of neurons. Neurons expressing the three non-cholinergic neurotransmitters on the muscle side constitute a significantly smaller percentage of the total neurons scored when compared to the blank side (Fig. 2B). Similar results were obtained when neurons were plated immediately after plating muscle cells. About 10% of neurons grew axons long enough to make contacts with each other, both on the blank and the muscle side. However, scoring transmitter expression in neurons with or without neuronal contact on both sides did not reveal differences between these groups. Thus the presence of muscle cells reduces the fraction of neurons expressing non-cholinergic transmitters only on the muscle side of the culture dish. The incidence of the three non-cholinergic neurotransmitters exceeds 100%, suggesting coexpression. This result is consistent with previous observations of transmitter coexpression in Xenopus spinal neurons at early stages of development, both in vitro and in vivo (Borodinsky et al. 2004; Root et al, 2008).

The reduction in number of neurons expressing non-cholinergic neurotransmitters could result from a decrease in differentiation or a lower survival rate of neurons that are genetically encoded for non-cholinergic phenotypes. However
the total number of neurons on each side was not significantly different (109±10 neurons on the blank side and 103±15 neurons on the muscle side, n=3 cultures). We also scored the number of neurons expressing HNK-1, an intrinsic molecular marker for a subtype of embryonic neurons (Somasekhar & Nordlander, 1997). There was no difference in the percentage of HNK-1-IR neurons between the blank side (55±1%) and the muscle side (53±1%). Staining of another molecular marker, islet-1, also revealed no statistically significant difference in its expression in neurons on either side (52±1% on the blank side and 59±3% on the muscle side) (Fig. 2B). These results indicate that cell death is unlikely to play a role in reducing the percentage of non-cholinergic neurons.

To examine the impact of neuron–muscle co-culture on cholinergic neurons, we used in situ hybridization to identify neurons expressing choline acetyltransferase (ChAT) (Fig. 3A). Neuronal identity was recognized by both morphology and fluorescent immunostaining for neuron-specific β-tubulin (not shown). On both sides of the cultures, roughly 30% of the neurons express ChAT (Fig. 3B), suggesting that the number of cholinergic neurons is not affected by the presence of muscle cells. In situ signal for ChAT was also observed in muscle cells, consistent with their release of ACh (Fu et al., 1998).

**Mechanism by which muscle cells induce suppression of non-cholinergic neurotransmitter expression**

Muscle cells could exert this effect on neurons by several different means. On the muscle side, direct contact between neurons and muscle cells allows physical
interaction between surface molecules and axon–muscle contact permits retrograde transport of trophic factors that are normally taken up at the distal ends of axons (Ginty et. al. 2005). Diffusible factors released by muscle are present in the culture medium throughout the entire dish, with higher concentrations on the muscle side compared to the blank side. Extracellular matrix molecules are also released by the muscle but only act locally on neurons growing in contact with muscle cells. To determine which of these interactions drives the reduction in expression of non-cholinergic transmitters, we modified the co-culture system to produce a lattice culture in which stripes of neurons are plated on stripes, instead of a lawn, of muscle cells (Fig. 4A). These cultures generate three classes of neurons: 1) those with no contact with muscle; 2) those with both axon and soma contacts with muscle (Soma-M group); and 3) those with only axon contact with muscle (Axon-M group).

The first class, growing on the blank side, may receive a low concentration of diffusible factors but their axons and somas remain free of muscle contact; thus other forms of interaction have little if any impact on them. The second class of neurons receives retrograde signals and a higher concentration of diffusible factors. The neurons in this group can also interact with extracellular matrix and surface-bound molecules. The third class, whose somas normally lie within 20-100 µm of the muscle, also receives retrograde signals and a high concentration of diffusible factors, but their somas have little or no interaction with extracellular matrix or surface molecules.

We scored the incidence of GABA-IR, glycine-IR and glutamate-IR neurons for all three classes of neurons and found both axon-muscle and soma-axon-muscle groups contain significantly fewer non-cholinergic neurons than the no-contact group.
but are not significantly different from each other (Fig. 4B). This result suggests that physical interaction between the neuronal soma and muscle cell body and associated extracellular molecules is not involved in the reduction of expression of non-cholinergic transmitters. However, axons can also interact with extracellular matrix molecules that regulate developmental processes such as neurite elongation (Hall & Sanes, 1993; Mantych & Ferriera, 2001). Thus our results do not exclude the possibility that extracellular matrix molecules act directly on axons and activate pathways that lead to changes in transmitter expression in neuronal cell bodies.

To investigate whether a high concentration of diffusible factors contributes to the suppression of non-cholinergic transmitter expression, we further divided the no-contact group according to the distance from the neuronal soma to the nearest muscle cell. The concentration of diffusible factors that reaches neurons is expected to decline with distance from the muscle. However, no difference in the number GABA–IR, glycine–IR and glutamate–IR neurons was observed in neurons located 0-50 µm, 50-150 µm, 150-250 µm and over 250 µm from muscle cells (Fig. 4C). To further address the issue of diffusible factors, we cultured neurons in medium conditioned by muscle tissue for 48 hr, to parallel the time of neuronal exposure in neuron-muscle co-culture. Muscle cells dissected from 6 embryos densely cover 70 - 80% of the dish. It was expected that the conditioned medium would provide relatively abundant diffusible factors released by the muscle. However, no changes were identified in the number of GABA-IR, glycine-IR and glutamate-IR neurons, when the conditioned medium condition was compared with the control condition in which neurons were cultured in medium without exposure to muscle cells (Fig. 4D). These findings suggest that
diffusible factors play little role in suppressing non-cholinergic neurotransmitter expression, although the action of such factors over distances of a few microns or at very high concentrations is not excluded. Taken together, axon-muscle contact alone appears to be sufficient to cause the significant suppression of non-cholinergic transmitters on the muscle side of these cultures, and muscle-derived, retrogradely-transported trophic factors are likely to be the key players in this suppression.

**Blocking muscle electrical activity rescues non-cholinergic neurotransmitter expression.**

To determine whether electrical activity in muscle cells is necessary to suppress non-cholinergic neurotransmitter expression, we used two separate approaches to block muscle activity chronically: 1) incubating cultures in 250 nM α-bungarotoxin (BgTx) to block postsynaptic activity mediated by acetylcholine receptors (Berg & Hall, 1975); 2) exogenously expressing human Kir channels in embryos from which the muscle cells were dissociated, to hyperpolarize their membrane potential (Borodinsky et al., 2004) (Fig. 5A, B). Neither of these two methods leads to a change in non-cholinergic transmitter expression in the neurons on the blank side (Fig. 5C). On the muscle side, the percentage of GABA–IR positive neurons is fully restored to the level on the blank side when the muscle is silenced by either BgTx or Kir expression. Significant although partial rescue of glycine-IR and glutamate-IR is also achieved by both of these two methods. These results demonstrate that muscle activity plays a key role in suppressing expression of non-cholinergic neurotransmitters. However the different extents of rescue for GABA, glycine and
glutamate indicate that the expression of different neurotransmitters is regulated differently by the muscle target.

**Neuron–muscle co-culture suppresses embryonic neuronal calcium spike activity.**

We next investigated the interplay between muscle-derived regulation and spontaneous activity in transmitter specification. Spontaneous neuronal calcium spike activity is observed at early stages of development and plays an important role in neurotransmitter specification both *in vitro* and *in vivo* (Gu & Spitzer, 1995; Borodinsky et al., 2004, Dulcis & Spitzer, 2008). Calcium spikes are produced by calcium-dependent action potentials generated by voltage-dependent calcium channels, and involve calcium-induced calcium release from calcium stores. They are usually first detected at the neural tube stage (stage 20) (Spitzer & Lamborghini, 1976; Holiday & Spitzer, 1990; Gu & Spitzer, 1993; Borodinsky et al. 2004), prior to synapse formation (Hayes & Roberts, 1973), and are generated for 10-12 hr until the early larval stage (stage 32; Root et al., 2008). Later the action potential matures to a brief sodium-dependent impulse and calcium spike activity disappears. In cultures, spontaneous activity is also observed during a limited time window. It peaks from 5 to 10 hr after plating and gradually decreases afterwards. (Gu et al., 1994; Gu & Spitzer, 1995; Chang & Spitzer, 2009). To find out whether the presence of muscle cells affects this neuronal activity, we imaged intracellular calcium in neurons dissociated from neural tube stage *Xenopus* embryos and grown either on the muscle side or the blank side of co-cultures. Neurons growing in neuronal cultures were also imaged, as a further control (Fig. 6A). Neurons were imaged for a 30 min period, starting 4 to 8 hr
after plating. The incidence of spiking neurons was calculated by dividing the number of neurons exhibiting spike activity during an imaging period by the total number of neurons imaged. We observed a significantly lower percentage of spiking neurons on the muscle side of the culture dishes, when compared with those on the blank side or in neuronal cultures (5% vs 32% and 28%; Fig. 6B).

On the muscle side, neurons extend axons more rapidly and grow longer than on the blank side during the first 8 hr after plating. To determine whether those neurons mature faster and shift the period during which they generate spikes to earlier times in culture, we imaged them at 1-4 hr after plating. However, only 6% of neurons grown on the muscle side generated spikes during this period (Fig.6C). This result suggests that shifting of the spiking window toward earlier times is unlikely.

**Muscle cells abolish veratridine-induced neuronal activity-dependent neurotransmitter specification**

Manipulations of neuronal calcium activity alter neurotransmitter expression both in cultures prepared from neural plate stage embryos and *in vivo* (Gu and Spitzer, 1995; Borodinsky et al., 2004). We repeated the *in vitro* experiment with neuronal cultures dissociated from neural tube stage embryos. Enhancing activity by chronic incubation with 1 µM veratridine, a drug blocking sodium channel inactivation, leads to an increase in the number of neurons expressing the inhibitory transmitters, GABA and glycine, and a decrease in the number of neurons expressing the excitatory transmitter, glutamate (Fig. 7A, B).
To investigate whether reduced neuronal spontaneous activity is responsible for muscle-dependent transmitter specification, we performed a rescue experiment to enhance neuronal activity. Treatment of co-cultures with 1 µM veratridine increases the incidence of spiking neurons on the blank side by 100% and rescues the incidence of spiking neurons on the muscle side (Fig. 7C). Veratridine does not elicit calcium transients in muscle cells in nerve-muscle co-cultures (Borodinsky and Spitzer, 2007). However, even with complete rescue of neuronal activity, there is no difference in the percentage of neurons expressing GABA, glycine or glutamate, on either side of the dish (Fig. 7D). This result suggests that suppression of neuronal activity by muscle contact does not account for muscle-dependent non-cholinergic transmitter suppression. In addition, the surprisingly unaltered transmitter expression on the blank side shows that muscle-derived diffusible factors may be sufficient to eliminate veratridine-induced, activity-dependent neurotransmitter specification. Since presynaptic activity may be involved in stimulating the release of muscle-derived factors, the high level of neuronal activity induced by veratridine may cause muscle to dominate the regulation of transmitter expression.

**Blocking trk receptors does not rescue non-cholinergic neurotransmitter expression.**

Muscle-derived members of the neurotrophin family are important for motor neuron development, including the induction and maintenance of ChAT expression (Chiu et al. 1994; Zurn et al., 1996) and establishment of mature electrophysiological properties of the NMJ (Funakoshi et al., 1995; Liou & Fu, 1997; Xie et al., 1997; Nick
& Ribera, 2000). To determine whether neurotrophins play a role in suppressing non-cholinergic neurotransmitters, cultures were incubated with 10 nM K252a, a trk receptor blocker. K252a was applied 5 hr after plating, to minimize its effect on preventing neuronal differentiation. At this concentration no rescue of GABA, glycine or glutamate expression was achieved (Fig. 8). At higher concentrations, K252a reduces the number of morphologically distinguishable neurons while leaving the percentage of tubulin-positive cells unaltered. It causes neurons to display short neurites and abnormal hairy morphology both on the cell body and along axons. Although morphological differentiation was clearly abnormal, concentrations of K252a up to 10 µM were equally ineffective in rescuing expression of the three non-cholinergic transmitters and no concentration-dependent effect of K252a was observed (data not shown).

**Discussion**

The presence of muscle cells has a striking impact on differentiation of neurons in co-cultures. Contact with muscle cells significantly suppresses non-cholinergic transmitter expression. Further investigation of the mechanisms underlying muscle-dependent regulation reveals an important role for retrograde signals released in a manner that depends on muscle activity. Contact with muscle cells also suppresses early neuronal calcium spike activity, and even when neurons are free of contacts, muscle cells abolish activity-dependent transmitter specification in co-cultured neurons, most likely via diffusible factors. These results suggest an important multi-level, muscle-dependent regulation of neuronal differentiation.
Muscle cell-dependent neurotransmitter specification

Previous studies have demonstrated that muscle cells are critical to support neuronal survival and maintain ChAT expression in adult motor neurons (Lams et al., 1988; Kou et al., 1995). However, the initial expression of ChAT in motor neurons is thought to be target-independent, since rat thoracic spinal motor neurons express ChAT before they can be retrogradely labeled through their targets (Phelps et al., 1991). In *Xenopus laevis*, however, the onset of ChAT expression in spinal motor neurons is first detected at the tailbud stage (stage 30) by immunocytochemistry (Root et al., 2008), almost 12 hr after axons start to contact developing muscle cells at the neural tube stage (stage 21) (Kullberg et al., 1977). Despite differences between rat and *Xenopus* in the order of the onset of ChAT expression and establishment of muscle contact, we found that the number of neurons expressing ChAT transcripts is not altered by contact with muscle cells, suggesting that embryonic ChAT expression is indeed independent from muscle-derived factors.

Muscle-derived signals appear to have a profound effect on non-cholinergic transmitter expression. *In vivo*, *Xenopus* spinal neurons start to express GABA and glutamate at neural plate stages, well before synapse formation (Root et al., 2008). Thus, when *Xenopus* neurons are dissociated and subsequently cultured from stage 20 embryos, it is expected that GABA and glutamate are present while glycine is absent. After 1 d in culture, expression of all three non-cholinergic transmitters is much higher in neurons grown free of muscle contact, when compared with those grown on muscle. Thus nerve-muscle contact seems to be effective both in reducing preexisting non-
cholinergic transmitter (GABA and glutamate) expression and in preventing neurons from acquiring new non-cholinergic phenotypes (glycine). The expression of the intrinsic molecular marker HNK-1 does not differ between the two groups of neurons, suggesting that muscle-dependent transmitter specification is independent of cell death or selective support of cell differentiation.

*In vivo*, GABA and glutamate expression peak at the neural tube stage, declining thereafter, and are expressed in neurons that are positive for lim-3, a motor neuron transcription factor (Root et al., 2008). Since the reduction of GABA and glutamate expression occurs at the time when motor neurons start to contact muscle cells, muscle-derived retrograde signals may contribute to suppression of ectopic expression of GABA and glutamate in motor neurons *in vivo*. On the other hand, early tailbud stage (stage 25) neurons that are positive for lim-3 never exhibit glycine-IR. Thus, neuron-muscle contact may also help to prevent motor neurons from acquiring the wrong transmitter *in vivo*.

**Role of muscle cell activity and candidate molecules**

Many target-derived factors are released in an activity-dependent fashion (Xie et al., 1997). Synaptic activity often modulates synaptic efficiency, both at early developmental stages and in adults. Moreover, postsynaptic excitability is critical in forming functional and mature synapses. Blocking postsynaptic activity blocks factor release and thus abolishes factor-dependent regulation of development (Funakoshi et al., 1995; Nick & Ribera, 2000). We find that blocking muscle activity prevents reductions in expression of non-cholinergic transmitters. The rescue is complete for
GABA and partial for glutamate and glycine. This suggests that multiple factors or mechanisms are responsible for target-dependent regulation and that different transmitters are regulated in different ways.

Muscle activity can be spontaneous or evoked by presynaptic activity. Electrical stimulation of the rat sciatic nerve leads to increased levels of NT4 mRNA in both the soleus and gastrocnemius muscles (Funokoshi et al., 1995). BgTx blocks nicotinic acetylcholine receptors that are responsible for transmission at the NMJ and rescues expression of non-cholinergic transmitters on the muscle side of co-cultures, suggesting that presynaptic release of acetylcholine may lead to suppression of non-cholinergic transmitter expression.

Since the lattice culture and conditioned medium experiments suggest the critical role of retrogradely-transported trophic factors, it will be useful to identify the target-derived factors that are involved in regulating the changes in neuronal development we have observed. Several neurotrophins have been shown to play important roles in muscle-dependent motor neuron differentiation. BDNF can rescue ChAT expression in adult mammalian hypoglossal neurons following transaction, as well as induce ChAT expression in cultured embryonic neurons from mice (Zurn et al., 1996; Liou & Fu, 1997). Both muscle-derived NT-3 and NT-4 regulate presynaptic electrophysiological properties in an activity-dependent manner (Funakoshi et al., 1995; Nick & Ribera, 2000). Although chronic blockade of trk receptors by K252a failed to rescue expression of the non-cholinergic transmitters, we cannot exclude the possibility that different members of the neurotrophin family act differently or have
opposing effects, in which case blocking them all could mask changes elicited by each one. Knocking down individual receptors would provide a more definitive answer.

Many other factors also play important roles in motor neuron development (Zurn et al., 1996; Dryer et al., 2003) and are attractive candidates. A number of target-derived factors, including CNTF, LIF, and GDNF have been proposed as potential candidates regulating the switch of rat sympathetic neurons from a noradrenergic to a cholinergic phenotype during development (Francis & Landis, 1999; Zweifel et al., 2004). BMP-9 is effective in inducing and maintaining the neuronal cholinergic phenotype in the central nervous system (Lopez-Coviella et al., 2000). Pharmacological blockade and targeted gene knock-down or knock-out of specific receptors are expected to identify muscle-derived factors responsible for transmitter specification in neuronal development.

Muscle cell inhibition of early neuronal calcium spike activity

Spontaneous calcium-dependent spikes are characteristic of the early development of Xenopus spinal neurons, both in vivo and in vitro. During the first 8 hr after plating, calcium spikes are rare in neurons grown on muscle although they are relatively common in neurons free of muscle contact. This result shows that muscle cells prevent neurons from generating spontaneous spikes. It remains to be determined whether this result is due to the action of retrogradely transported or diffusible factors. Because veratridine not only rescues but also enhances spike incidence on the muscle side to the same extent as the blank side, calcium channels are not functionally impaired; however, the trigger mechanism may be suppressed. Spontaneous calcium
spikes disappear as synapses mature \textit{in vivo}, but this also happens in dissociated cell cultures in the absence of cell-cell interactions (Gu & Spitzer, 1995). It is not yet clear whether genetic programs or target-derived factors or both lead to termination of this spontaneous activity.

**Muscle cells override activity-dependent transmitter specification**

Spontaneous calcium spike activity plays a key role in specifying transmitter expression in a homeostatic manner. Altering calcium activity pharmacologically or by ion channel overexpression results in changes in transmitter expression that tend to counterbalance the effects of activity manipulation. Like spontaneous calcium spike activity, activity-dependent transmitter specification occurs only during a brief time window during development (Borodinsky et al., 2004; Root et al., 2008), prior to synapse formation.

In neuron-muscle co-cultures, however, even when neuronal activity is altered, no change in transmitter expression takes place. More remarkably, abolition of activity-dependent transmitter specification occurs with or without physical contact between neurons and muscle cells, as long as they are placed in the same dish, arguing for regulation by diffusible factors. As diffusible factors appear to play little or no role in reducing non-cholinergic transmitter expression, it appears that different factors and target-dependent mechanisms are responsible for these two aspects of neuronal differentiation. In addition, as target-derived factors can be released in an activity-dependent fashion, it is likely that veratridine enhances neuronal activity and in turn enhances postsynaptic muscle activity, which leads to increased release of trophic
factors that eventually abolish activity-dependent transmitter specification. These results suggest that muscle provides factors that prevent neurons from resuming activity-dependent transmitter specification even if calcium-dependent neuronal activity returns at a later time.

**Model for target-dependent regulation of transmitter expression**

We propose a model in which different combinations of intrinsic genetic coding, neuronal activity and target derived factors interact with each other at different stages to define the pattern of neurotransmitter expression. We propose that the pattern of neurotransmitter expression is initially specified genetically through the activity of transcription factors and modulated by general environmental influences through early spontaneous calcium-dependent activity (Fig. 9A). Once neurons have made contact with their targets, the influence of target-derived factors, including both retrogradely transported signals and diffusible factors, overrides that of early activity. Targets block spontaneous calcium activity and further modulate the action of transcription factors to refine the neuronal transmitter expression phenotype (Fig. 9B). Thus, several mechanisms are utilized to ensure that the correct transmitter phenotype is established and maintained throughout development.

**References**


Figure 3_1 Neuron-muscle co-culture system. A, Diagram showing the procedure for establishing neuron-muscle co-cultures. Areas where muscle cells are plated are shown in gray and areas where neurons are located are shown in red. B, Neurons growing on muscle cells. Neurons were dissociated from rhodamine dextran-injected embryos. Scale bar, 10 µm.
Figure 3.2 Muscle cells suppress non-cholinergic neurotransmitter expression. A, Immunostaining of neurons for GABA (red) and tubulin (green) on the blank side (left panels) and the muscle side (right panels). Top two panels show positive staining and the bottom two show negative staining. B, Non-cholinergic neurotransmitter expression is reduced on the muscle side (** p<0.01, ***p<0.001, in comparison to the blank side; n>8 cultures per transmitter, >200 neurons per culture). HNK-1 and islet-1 expression does not differ between the two sides of the culture; the Y-axis applies to the incidence of HNK-1, islet-1 and transmitter expression. Error bars indicate SEM. The Mann-Whitney U test was used to determine statistical significance. Scale bar, 10 µm.
Figure 3. Neuronal ChAT expression is independent of muscle regulation. A, *In situ* hybridization for ChAT on the blank side (left panels) and the muscle side (right panels; neurons circled). Top two panels show positive staining and the bottom two show negative staining. B, ChAT expression on the muscle side is not different from that on the blank side (n>5 cultures per transmitter, >100 neurons per culture). The Mann-Whitney U test was used to determine statistical significance. Error bars indicate SEM. Scale bar, 10 µm.
Figure 3_4 Different neuron-muscle interactions play different roles in muscle-dependent suppression of neurotransmitter expression. A, Schematic view of the lattice neuron-muscle co-culture and groups of neurons with different interactions with muscles. B, Neurons with or without cell body interaction with muscle show similar levels of reduction in incidence of non-cholinergic neurotransmitter expression, when compared with the no-contact group. There is no significant difference in non-cholinergic neurotransmitter expression between axon-m and soma-m groups (** p<0.01, *** p<0.001; n>5 cultures per condition per transmitter, >100 neurons per culture). C, The incidence of expression of the three non-cholinergic neurotransmitters in the no-contact group from (B) (GABA in red, glycine in yellow and glutamate in green) does not change as a function of distance from neuronal soma to muscle cells (>100 neurons scored at each distance for each neurotransmitter). D, The incidence of expression of three non-cholinergic neurotransmitters does not differ between the conditioned medium condition and the control condition (n>5 cultures per condition per transmitter, >100 neurons per culture). The Kruskal-Wallis test and Conover post-hoc test were used to determine statistical significance. Error bars indicate SEM.
Figure 3_5 Rescue of non-cholinergic neurotransmitter expression by blockade of muscle activity. 

A, Diagram illustrating hKir mRNA injection and neuron-muscle co-culture with silenced muscle and wild type neurons. 

B, Diagram showing method of pharmacological blockade of muscle activity by α-bungarotoxin. 

C, Complete rescue of GABA expression and partial rescue of glycine and glutamate expression are achieved by either BgTx incubation or Kir expression. Lower asterisks indicate a significant difference between the bar beneath and its counterpart on the blank side. Upper asterisks indicate a significant difference between the bars (n>5 cultures per condition per transmitter, >100 neurons per culture; * p<0.05, ** p<0.01, *** p<0.001). The Kruskal-Wallis test and Conover post-hoc test were used to determine statistical significance. Error bars indicate SEM.
Figure 3_6 Muscle cells suppress early neuronal spontaneous calcium spike activity. 

A. Diagram showing areas of interest where neurons were imaged. 

B. Neurons grown on muscle cells exhibit a significantly lower incidence of spiking at 4-8 hr in vitro (n=10 cultures, >50 neurons per condition; ** p<0.01). 

C. Neurons grown on muscle cells exhibit reduced incidence of spiking during each hr after plating (n>10 cultures, >50 neurons per hour). A higher percentage of neurons exhibit calcium spikes from 4 to 8 hr on the blank side of co-cultures and in neuron-alone cultures. Imaging at earlier times was not carried out with the two control groups due to lack of morphological distinction between neurons prior to axon outgrowth and the presence of other cell types. The Kruskal-Wallis test and Conover post-hoc test were used to determine statistical significance. Error bars indicate SEM.
Figure 3_7 Muscle cells abolish activity-dependent neurotransmitter specification induced by veratridine. A, Veratridine increases the incidence of spiking neurons in neuron-alone cultures. B, In neuron-alone cultures, veratridine increases the incidence of the inhibitory neurotransmitters, GABA and glycine, and reduces incidence of the excitatory neurotransmitter, glutamate. C, Veratridine increases the incidence of spiking neurons in neuron-muscle co-cultures, both on the blank side and the muscle side. D, In neuron-muscle co-cultures, veratridine fails to induce changes in either inhibitory or excitatory neurotransmitter expression (n>5 cultures per condition per transmitter, >200 neurons per culture; ** p<0.01, *** p<0.001). The Mann-Whitney U test (A–C) and the Kruskal-Wallis test (D) were used to determine statistical significance. Error bars indicate SEM.
Figure 3_8 Blockade of trk receptors does not rescue expression of non-cholinergic transmitters. 10 nM K252a were applied from 5-24 hr. No significant difference in expression of GABA, glycine or glutamate was observed between the blank and muscle sides of the dish (n>5 cultures per condition per transmitter, >100 neurons per culture). Higher concentrations (up to 10 μM) were also ineffective. The Kruskal-Wallis test was used to determine statistical significance. Error bars indicate SEM.
Figure 3.9 Model of neurotransmitter specification by transcription factors, activity and target-derived factors. **A**, Early in development, when neurons have not yet established contact with their targets, spontaneous calcium spike activity and transcription factors work together to specify their neurotransmitter expression profile. **B**, After neurons contact their targets, target-derived factors, including both retrograde factors (orange dots) and diffusible factors (green dots), suppress expression of non-cholinergic transmitters, block spontaneous calcium activity and disrupt evoked activity-dependent neurotransmitter specification. These factors work in combination with transcription factors to further refine and lock in the transmitter expression phenotype.
Chapter III is a preprint of “Xiao Q, Spitzer NC. Muscle-dependent regulation of neurotransmitter specification and embryonic neuronal calcium spike activity. (In preparation)” of which I am first author.
CHAPTER IV

SUMMARY AND FUTURE DIRECTIONS
**Muscle-dependent regulation of neurotransmitter expression**

**Summary**

I developed a muscle-neuron co-culture system in which the impact of muscle contact on neural development could be observed by comparing transmitter expression and calcium spiking activity in neurons grown on different sides of the culture dish, with or without cultured muscle cells.

Muscle contact appears to reduce non-cholinergic transmitter (GABA, glycine and glutamate) expression, but it does not change ChAT expression. Further investigation reveals that extracellular molecules, interaction between surface molecules on cell bodies with physical contact, and diffusible factors are not likely to be the cause of reduced expression of non-cholinergic transmitters, while synaptically-delivered and retrogradely-transported trophic factors appear to be sufficient to suppress non-cholinergic transmitter expression. Muscle activity is required for this suppression and blocking muscle activity leads to a partial rescue of expression of non-cholinergic transmitters.

Muscle contact also suppresses early spontaneous calcium activity in spinal neurons grown on the muscle side of the culture dish. In addition, the presence of muscle cells in culture abolishes activity-dependent transmitter expression both on the blank side and on the muscle side, arguing that diffusible factors play a role in overriding the impact of activity on transmitter regulation.

I propose that refining the pattern of neurotransmitter expression is a complicated developmental process that requires interplay among transcription factors, neuronal activity and target regulation. Once neuron-target connection is established,
target regulation can override neuronal activity and most likely work in concert with transcription factors to establish and maintain transmitter expression in neurons.

**Future Directions**

Identifying candidate molecules is probably the most interesting remaining task. It has been demonstrated that many neurotrophins play important roles in muscle-dependent motor neuron differentiation. BDNF can also induce ChAT expression in cultured embryonic neurons from mice. *In vivo*, following axotomy, application of BDNF can promote motor neuron survival and rescue transection-induced ChAT loss in axotomized neurons. (Zurn et al., 1996; Liou & Fu, 1997). Both muscle derived NT-4 and NT-3 are critical in establishing mature electrophysiological properties in developing neurons and their release is dependent on muscle activity. Blocking muscle activity leads to abnormal features in neuronal action potentials and quantal release of acetylcholine. (Funakoshi et al., 1995; Nick & Ribera, 2000). Many factors outside the neurotrophin family also play important roles in motor neuron development (Zurn et al., 1996; Dryer et al., 2003) and are suitable candidates for further investigation. BMP-9 has been shown to be a differentiating factor for cholinergic neurons in the central nervous system in mice. In cultured mammalian neurons, BMP-9 can induce the expression of ChAT and this effect is reversed by removal of BMP-9. In addition, injection of BMP-9 can increase acetylcholine levels *in vivo* (Lopez-Coviella et al., 2000). CNTF, LIF, and GDNF have been proposed as potential candidates regulating the switch of rat sympathetic neurons from a
noradrenergic to a cholinergic phenotype during development (Francis & Landis, 1999; Zweifel et al., 2004).

Although K252a application failed to rescue non-cholinergic transmitters, it is too early to conclude that members of neurotrophin families are not involved. Different members of the neurotrophin family may act differently, or even in opposite directions, and thus blocking them all together may mask the changes elicited by each one alone. Although pharmacological blockade for each receptor may not be available, it will be useful to design morpholinos specifically for each receptor to knock down their expression. Pharmacological blockade and targeted gene knock-down or knock-out of receptors for factors other than neurotrophins are also expected to help in identifying muscle-derived factors responsible for transmitter specification in neuronal development.
REFERENCES


APPENDIX

REOPENING THE SENSITIVE PERIOD FOR ACTIVITY-DEPENDENT TRANSMITTER SPECIFICATION
BACKGROUND AND SIGNIFICANCE

**Sensitive period for transmitter specification in Xenopus neural development**

Spontaneous brief elevations of intracellular calcium (calcium spikes) are observed in embryonic spinal neurons of *Xenopus laevis* both in culture and in vivo (Gu et al., 1994). Different classes of neurons express different patterns of calcium spikes. By altering the frequency of calcium spikes, neurotransmitter expression is specified in a homeostatic way: when calcium spike frequency is enhanced, more neurons express inhibitory neurotransmitters (GABA and glycine); when calcium spike frequency is suppressed, more neurons express excitatory neurotransmitters (acetylcholine and glutamate; Borodinsky et al., 2004).

However, like many other activity-dependent developmental processes (Hubel and Wiesel, 1970; Ribera and Spitzer, 1989; reviewed by Hensch, 2004), the effects of calcium spike frequency on neurotransmitter expression are observed only during a short time window during development. *In vitro*, there is a 5 hr critical period that occurs 5 hr after neural plate neurons are plated, during which calcium spikes can specify neurotransmitter expression (Borodinsky et al., 2004). *In vivo* the sensitive period for activity-dependent specification of neurotransmitter expression is 16 hr in duration (Root and Spitzer, 2004). This period extends from neural tube formation to early larval stages (19 hr to 35 hr after fertilization), during which pharmacologically enhancing or suppressing spontaneous calcium spikes leads to homeostatic regulation of expression of both excitatory and inhibitory neurotransmitters, glutamate and GABA.
The time course of the sensitive period is coincident with the time course of spontaneous calcium spike activity. Spontaneous calcium-dependent action potentials are first detected at the neural tube stage (22 hr after fertilization), and these action potentials mature into brief sodium-dependent action potentials during the following 24 hr (Spitzer & Lamborghini, 1976). The disappearance of calcium dependent action potentials is due primarily to the maturation of delayed rectifier potassium current (O’Dowd et al., 1988; Lockery and Spitzer, 1992).

However, Root and Spitzer (2004) found that reintroducing calcium spike activity to neurons after the sensitive period, by blocking voltage-gated potassium currents with TEA, was not sufficient to enable alterations in spike frequency to drive changes in neurotransmitter expression. This finding indicates that other factors playing an important role in activity-dependent neurotransmitter expression have changed, so that calcium spikes can no longer exert the effect they achieve at earlier stages. Thus the sensitive period appears to require an appropriate molecular context in addition to the presence of calcium spikes.

**Neuronal regeneration**

Neurons can undergo regeneration or apoptosis after injury. In higher vertebrates, the extent of regeneration in the adult central nervous system is usually limited, primarily because of low survival rate, nonpermissive environment and loss of intrinsic growth state (reviewed by Jacobs & Fehlings., 2003; Steeves & Tetzlaff, 1998). However anuran amphibian embryos and larvae can regenerate the spinal cord after injury (Forehand & Farel, 1982; Beattie et al., 1990).
Nerve regeneration in adults involves many processes that are similar to growth at embryonic stages, including axon extension, target finding and neural plasticity. Some embryonic states are recapitulated after neural injury (Toyoda et al., 2003; reviewed by Virera et. al., 2004) and certain genes promoting neuron growth, such as GAP-43, are activated during neural regeneration (Hoffman 1989; reviewed by Emery et al., 2003). Determining precisely which aspects of the embryonic state are recapitulated following nerve injury and how these can be manipulated to ensure proper regeneration is an important goal.

Spontaneous activity following axotomy

Nerve injury results in an increase in the excitability and spontaneous activity of axotomized neurons as well as neighboring intact neurons (Ma et al., 2003) in many organisms, including mammals (Abdulla & Smith, 2001a), reptiles (Troyer et al., 1992), insects (Goodman & Heitler, 1979) and molluscs (Clatworthy et al., 1994). Studies investigating electrophysiological properties following axotomy have revealed a reduction in the threshold for action potential initiation (Gurtu & Smith, 1988), an increase in spike width (Gallego et al., 1987), changes in sodium and chloride channel expression (Andre et al., 2003; Lai et al., 2003) and decreases in potassium and calcium currents (Abdulla & Smith, 2001b, Baccei & Kocsis, 2000). This information promotes the hypothesis that nerve injury may return neurons to an embryonic state. The presence of calcium spikes in embryonic Xenopus plus our understanding of their physiological significance makes this an attractive system in which to study the roles of spontaneous calcium spike activity following axotomy.
**Extrinsic environment and sensitive period**

In addition to changes in electrical properties of neurons, it is clear that there must be changes in molecular context to permit calcium spike frequency to alter neurotransmitter specification in following nerve injury, as I hypothesize. At embryonic stages, many developmentally regulated extrinsic factors determine neural fate in combination with factors intrinsic to the neurons. Neural tube transplantation, cellular transplantation and non-neural tissue grafting have been widely used to study many aspects of neural development, including neural crest cell migration (LeDouarin & Kalcheim, 1999), axonal projection (Nomura & Fujisawa, 2000; Nordlander & Liu, 1996), neurotransmitter plasticity (Sechrist et al., 1998) and neural regeneration (Novikova et. al., 2002, reviewed by Murray, 2004). These are very useful techniques with which to study the role of extrinsic environment in activity-dependent transmitter respecification.

I hypothesize that reinitiation of spontaneous calcium spike activity together with recapitulation of the embryonic molecular context, either by transection or by transplantation, will reopen a sensitive period for activity-dependent neurotransmitter respecification. The information acquired in these studies may help us further our understanding of plasticity in development, and has potential medical relevance in that respecification of neurotransmitters in the adult could be useful in treating spinal injury, depression and other disorders of the nervous system.

**METHODS**
Dissection and culturing of spinal cord explants

Stage 36-40 larvae are anaesthetized in 0.5% ethyl 3-aminobenzoate methanesulfonate salt (TMS), dissolved in 2 mM calcium Ringer solution (116.7 mM NaCl, 0.67 mM KCl, 1.31 mM MgSO$_4$, 2 mM CaCl$_2$, 8 mM MgCl$_2$, 4.6 mM Tris, pH=7.8). The head and tail are cut off and the dorsal region of the middle third of the larva is transferred to a Sylgard dish containing Ringer solution plus 1 mg/ml collagenase B. The skin, myotomes and notochord are removed and spinal cord is cut into small pieces roughly 0.1~0.5 mm long with a 26G5/8 gauge needle. Using a pulled glass pipet, small explants are plated in uncoated tissue culture plastic dishes containing 3 ml of 2 mM calcium Ringer solution solution, 20 µm of gentamicin and 20 µm of penicillin (Cambrex). Explants are allowed to grow for 1 day.

Transection of the spinal cord in vivo

Larvae at stages 36-38, in which the sensitive period has already ended, are anaesthetized in 0.5% TMS (dissolved in 10% MMR solution, 0.01M NaCl, 0.2 mM KCl, 0.1 mM MgSO$_4$, 0.5 mM Hepes, 0.01 mM EDTA, 0.2 mM CaCl$_2$). Larvae are placed on their side and the spinal cord is completely transected at ~500 µm behind the posterior edge of the eye with a 26G5/8 gauge needle. Injury to the notochord is carefully avoided to prevent larvae from dying and keep larvae stable in later imaging sessions. Spinally transected larvae are then transferred to 10% MMR and grown at 20°C for 1, 2 or 3 days.

Dissection of spinal cord for imaging
Control or neural tube-transected larvae are anaesthetized in 0.05% TMS, dissolved in 2 mM calcium Ringer solution. After they become immobilized, they are pinned on the side of the wall of a 5 mm x 10 mm well in a Sylgard dish with dorsal side up. A drop of 10mg/ml collagenase B is applied to the top of the embryo. Using forceps and minuten pins bent at a right angle, the dorsal skin, muscle and pigmented cells are removed and the dorsal surface of spinal cord is exposed. For ventral dissection, all the conditions are the same, but ventral tissues including skin, gut, muscle and notochord are removed to expose the ventral surface of the spinal cord.

**Imaging and analysis**

Explants are examined under a Zeiss inverted phase contrast microscope 1 day after plating, and those attaching stably to the dishes with axons extending at least twice the length of the explants are used for imaging. A plastic ring is stuck to the dish with Dow Corning grease to restrict the fluid volume. Explants are loaded with 3 µl of 0.9 µg/µl Fluo-4 AM (Molecular Probes; 50 µg Fluo-4 AM stock dissolved in 45 µl DMSO plus 10 µl 20% Pluronic solution in DMSO) for 45 min at room temperature. Before imaging, the rings are removed and explants are washed 3 times with 3 ml 2 mM calcium Ringer solution.

Dissected larvae are washed twice with 2 mM calcium Ringer solution and loaded with 2 µl per well of 0.9 µg/µl Fluo-4 AM for 45 min in room temperature and free fluorescent dye is then washed out with 2 mM calcium Ringer solution 3 to 5 times, each with 3 ml of 2 mM calcium Ringer solution. Larvae are then kept in 2 mM
calcium Ringer solution for another 45 min before imaging to let TMS diffuse away and an additional 3 washes are applied immediately before imaging.

Images of fluo-4 loaded spinal neurons are acquired at intervals of 5 s or 2.5 s for 1 hr or 0.5 hr periods, using a Bio-Rad MRC 600 laser confocal system on an Olympus microscope with 20x water immersion objective. Live videos are displayed and analyzed using Image J software.

Spiking neurons are identified by eye and confirmed by measuring fluorescence intensity and plotting the intensity as a function of time. Spikes are identified by the following criteria: 1) a rising phase shorter than 5 s; 2) an amplitude greater than twice the range of the noise, which is the peak-to-peak calcium reporter fluorescence fluctuation amplitude, averaged 1 min before and after the potential spike.

Bead implantation

Agarose beads (80 µm; Biorad) are washed in PBS for 10 min and incubated with drugs at 20°C. for 1 hr. To enhance calcium spike activity, a combination of 0.1 M TEA and 1 mM veratridine is used. TEA has been shown to block the rectifier potassium current in larvae and extend the duration of action potentials, letting voltage dependent calcium channels become fully activated (Baccaglini and Spitzer, 1977). Veratridine is a voltage-dependent sodium channel agonist and leads to activation of voltage-dependent calcium channels that increase calcium spike activity (Borodinsky et al., 2004). To suppress calcium spike activity, beads are incubated with a cocktail solution of blockers to different types of voltage-dependent calcium channels. This
solution contains 200 nM calcicludine, 10 µM GVIA ω-conotoxin, 10 µM flunarizine and 10 µg/ml tetrodotoxin.

Right after transection or 1 day later, larvae are anesthetized in 0.5% TMS dissolved in 10% MMR. With forceps and pins, each larva is implanted with a bead close to the spinal cord and 200 µm – 300 µm posterior to the transection. Larvae are transferred back into 10% MMR.

*Spinal transplantation*

An embryo at stage 22-24 is stabilized in a small well of a Sylgard dish by a pair of staples made from minuten pins, with dorsal side up. Dissection and transplantation are done in 1x MMR solution. A small region of skin (0.5 mm-1 mm) starting at the third myotome is peeled off (with one end left attached to the embryo) and the corresponding segment of myotome is removed. A small portion (0.5 mm-1 mm) of spinal cord is cut at both ends and removed.

A larva at stage 35-39 is anaesthetized in 0.05% TMS dissolved in 10% MMR solution and pinned by a pair of minuten pins in a Sylgard dish with dorsal side up. The skin is peeled off and myotomes are removed to expose the spinal cord. A 0.5 mm-1 mm length of spinal cord from 0.5 mm posterior to the edge of the eye is transected at both ends and removed and transferred to the host embryo dish by a glass pipette.

The transplant from the larva is inserted into the gap in the embryo spinal cord and an agarose bead containing drugs (as described above) is placed on top of it. The transplant and bead are re-covered by the skin attached at one end. A third staple is
added at this spot to stabilize the tissue. The embryo is allowed to sit at the dish for 30 min-60 min to allow the healing process and then transferred to 50% MMR.

For transplantation from young embryo to larva the procedure is similar, but the donor and host are exchanged. For homochronic transplantation, all the procedures are similar except that the transplantation is carried out between embryos or larva at the same stage, or in the same donor.

**Immunocytochemistry**

1 or 2 days after bead implantation, larvae are fixed for 30 min at 4° C. The fixation solution contains 4% paraformaldehyde, 0.025% glutaraldehyde, 0.1 M phosphate-buffered saline dissolved in calcium-magnesium-free phosphate-buffered saline. Fixed embryos are soaked for 2.5 hr in 30% sucrose, 10 min in 1:2 OCT/sucrose solution, 10 min in 2:1 OCT/sucrose solution at room temperature, and embedded in OCT, frozen and stored at -20° C. 10 μm thick frozen sections are cut of a roughly 500 μm long region of the spinal cord starting from 500 μm after the eyes have disappeared from sections. Slides are always checked to make sure that the bead region is included.

Slides are incubated in a blocking solution for 30 min at 21-23°C. The blocking solution contains 1% fish gelatin and 0.1% Triton-100. Slides are incubated with primary antibody overnight at 4°C (GABA is diluted 100 times in blocking solution and glutamate 10,000 times from the original stock), and incubated with secondary antibody for 2 hr at 20° C (1:300 dilution from original stock for GABA, 1:1000
dilution for glutamate). Nuclei of cells are stained with DAPI added to the mounting medium.

Immunoreactivity is examined on a Zeiss fluorescence microscope with a 20x objective and the excitation and emission filters for Cascade Blue, Alexa 488 and Alexa 594. Images are acquired and analyzed with AxioVision software.

**RESULTS**

**Spontaneous activity after neuronal axotomy**

Spontaneous oscillations of intracellular calcium have been observed in rat facial motoneurons 3 days after axotomy (Toyoda et al., 2003). These calcium oscillations resemble the long duration (10 s) calcium spikes observed in embryonic *Xenopus* spinal neurons but are longer in duration (from ~30 s to ~2 min). I hypothesize that transection of the *Xenopus* spinal cord at larval stages will induce similar spontaneous calcium spike activity in *Xenopus* spinal neurons at this stage of development and that this activity is important for transmitter respecification.

As described above, a variety of types of spontaneous activity including spikes and calcium oscillations is observed in many organisms following axotomy. However no evidence of spontaneous calcium spike activity in *Xenopus* tadpole spinal neurons after neural injury has been reported.

To identify spontaneous calcium spike activity after axotomy, I imaged intracellular calcium in explants of *Xenopus* spinal cord. I dissected the spinal cord from stage 36-40 larvae, in which the sensitive period has closed, cut them into pieces 100-500 µm in length and cultured them for 24 hr before imaging. Explants were
loaded with Fluo-4AM calcium indicator and images were acquired every 5 s for 1 hr with a confocal microscope.

11 of 16 explants from 8 larvae exhibited spiking neurons, and an average of 2 spiking neurons were observed in a single optical section imaged for a 1 hr period. The frequency of spikes varied from 1 spike $h^{-1}$ to 25 spikes $h^{-1}$, with a mean of 8.8 spikes $h^{-1}$ (Fig. 3_1). Different classes of spinal neurons have different patterns of calcium activity at embryonic stages of development (Borodinsky et al., 2004), thus neurons with different spiking frequencies may represent different classes of spinal neurons. In addition, some neurons in the explants exhibited elevations of calcium that were distinct from spikes. These events were usually broader and slower and resembled the spontaneous calcium waves identified at embryonic stages (Gu et al., 1994). These preliminary results indicate that embryonic forms of calcium-dependent excitability reappear in explants from larval stage spinal cords.

Though explants serve as a convenient and quick way to identify spontaneous activity following axotomy, I next investigated a more physiological condition by imaging intracellular calcium in the larval spinal cord in situ. Stage 36-40 (~ 3 d post fertilization) larvae were transected at the rostral end of the spinal cord, the spinal cord was exposed by dissection at 1 d, 2 d, and 3 d after transection, and spinal neurons were imaged for 1 hr to identify spiking neurons. 0.05% TMS was used to immobilize embryos during the dissection and then washed out for >45 min prior to imaging. The imaged region was 200 µm - 300 µm posterior to the transection, to avoid imaging neurons dying close to the cut, while simultaneously avoiding imaging neurons so distant from the cut that their axons were not transected.
Neurons on the dorsal surface of 6 untransected spinal cords of control larvae imaged for 1 h at stage 41 (3 days post fertilization) showed no calcium spikes. In contrast, 2 out of 3 larvae exhibited spiking neurons in the dorsal spinal cord following spinal cord transection (Fig. 3_2). One of them was imaged 1 d after transection and had 3 spiking neurons, all of which generated a single spike during the 1 h imaging period, comparable to the spiking frequency of sensory Rohon-Beard neurons at embryonic stages (Borodinsky et al., 2004). The other larva was imaged 3 d after transection and had 2 spiking neurons, firing at 1 spike h$^{-1}$ and 2 spikes h$^{-1}$, respectively.

These preliminary results suggest that spontaneous calcium spike activity occurs between 1 and 3 days after transection in the *Xenopus* spinal cord, at stages in which embryonic spontaneous calcium spike activity has disappeared. It would be useful to extend these preliminary findings and to image the ventral surface of spinal cord to identify spontaneous calcium spikes after axotomy. Ventral neurons exhibit higher frequencies of calcium spikes than dorsal neurons at embryonic stages of development (Borodinsky et al., 2004). It would be interesting to investigate whether this difference also appears in the spontaneous activity that reemerges following axotomy.

**Increasing calcium spike frequency following transection alters GABA expression at later larval stages**

As discussed above, the mature nervous system often recapitulates embryonic states after injury. Because spontaneous calcium spike activity is observed in *Xenopus* larvae following spinal cord transection, I determined whether this spontaneous
calcium activity has an effect similar to that observed earlier in development -- modulating neurotransmitter expression. During the sensitive period in early development, the number of neurons expressing the inhibitory transmitters GABA and glycine decreases when calcium spikes are suppressed and increases when the frequency of spikes is increased. The excitatory transmitters glutamate and ChAT, on the other hand, are inversely regulated by calcium spike activity, suggesting homeostasis of network excitability (Borodinsky et al., 2004). I hypothesize that suppressing and enhancing calcium spikes occurring after spinal transection will alter neurotransmitter expression in the more mature spinal cord in a homeostatic manner as it does in the embryonic spinal cord.

To investigate whether increasing calcium spike frequency following spinal cord transection can respecify neurotransmitter expression, I carried out experiments with transection plus enhancement of activity 1 day after transection. Spinal cords of stage 36-40 larvae were transected and an 80 µm agarose bead loaded with 0.1 M TEA and 1 mM veratridine for ≥1 hr was implanted 200 µm-300 µm posterior to the transected site (Borodinsky et al., 2004). Future experiments will involve bead implantation at later times if the period of peak spontaneous activity occurs at later times. 1 d, 2 d or 3 d after bead implantation, larvae were fixed and neurotransmitter expression was determined by immunostaining.

I began by investigating whether the incidence of GABAergic neurons is altered by this procedure. Experimental larvae were spinally transected and implanted with activity-enhancing beads. Three classes of negative control larvae were evaluated: 1) larvae that received no treatment or were implanted with a blank bead
(with no drug), 2) transected larvae without bead implantation or with blank bead, and 3) untransected larvae implanted with drug beads. These larvae were fixed 1 day after bead insertion and stained with anti-GABA antibody. As a positive control, larvae were implanted with drug beads at stages 16-20, during which the sensitive period is open, and subsequently fixed and stained at stage 40 to determine whether bead implantation had the expected effect. Larvae implanted with activity-enhancing beads after transection had a significantly higher number of GABA-positive neurons than the larvae with no treatment or transection alone (Fig. 3_3). These results suggest that transection followed by enhanced activity causes more neurons to express GABA, which is an inhibitory transmitter in this system at these stages.

**Summary and future directions**

**Summary**

In spinal cord explants transected and cultured from larva staged embryos, at a stage when calcium spikes are normally absent, calcium spiking activity was observed with high incidence (60%), and relatively high frequency (9 spikes/hr). Calcium spiking activity was also observed in transected spinal cords, 1 d after transection. Both *in vivo* and *in vitro* results showed that neuronal axotomy may lead to reoccurrence of calcium spiking activity, a phenomenon normally only observed at earlier embryonic stages.

I showed that following axotomy, implantation with beads loaded with enhancer drugs that boost neuronal activity causes an increase in the number of GABA-IR neurons. This suggests that by altering neuronal activity, transmitter
expression can be re-specified in the axotomized spinal cord and the sensitive period for activity-dependent transmitter specification may be reopened by neural axotomy.

**Future Directions**

More calcium imaging remains to be done to determine the profile of calcium spiking activity induced by spinal transection. As early neuronal calcium spiking activities only exist in a limited time window (Gu et al., 1994; Chang and Spitzer, 2009), it would also be interesting to determine if such a time window also exists for transection-induced calcium spike activity.

Immunocytochemistry of other neurotransmitters, including glycine, ChAT and glutamate (or VGlutI), can determine whether the reopened activity-dependent transmitter specification mechanism holds true for multiple transmitters or only for GABA. It would also be interesting to use beads loaded with activity blockers, instead of enhancers, to determine the impact of suppression of activity on transmitter expression.

Since previous work showed that axotomy can cause changes in both axotomized neurons and neighbouring intact neurons (Ma et al., 2003), it will be useful to determine if transection-induced calcium spiking activity and activity-dependent transmitter specification occur only in axotomized neurons. To this end, fluorescent tracers can be delivered to the site of transection to retrogradely label axotomized neurons. Calcium spiking activity and transmitter expression in labeled neurons alone can then be compared with neighbouring neurons, and with those in non-transected animals.
However it is worth noticing that at stg. 46, about 1.5-2 days after transections are made in early larval stage embryos, the animal enters a secondary round of neurogenesis (Wulliman et. al., 2005). It is very likely that newborn neurons will enter their own natural time window for both calcium activity and activity-dependent transmitter specification; thus experimental investigation following transection or transplantation should be done before stg. 46, to avoid newborn neurons from complicating the results. An alternative approach could be to use agents such as BrdU to label newly produced neurons and exclude them from analysis.

Here I propose a list of other experiments that will allow further investigation of the project:

1) **What is the time window during which spontaneous calcium spikes can be observed?**

   It would be interesting to investigate the time window of spontaneous activity that appears in *Xenopus* larvae following spinal cord transection, because this time period may also indicate the time window of the reopened sensitive period for activity-dependent neurotransmitter expression, in parallel with observations in early development (Root & Spitzer, 2004).

   To achieve this aim, one could carry out *in situ* imaging at various time points (from 6 hr to 6 d after transection, at 6 hr time intervals). The incidence of spiking neurons and the frequency of calcium spikes would be determined for each time point. These experiments would define the temporal profile of spontaneous calcium spike
activity level after axotomy and identify the time when the highest activity is generated. At each time point, at least 5 larvae should be imaged to determine the average incidence of spiking neurons and spike frequency; these data can be compared with those obtained at embryonic stages.

Caveats

It is possible that the loss of descending innervation may also contribute to the spontaneous calcium activity observed following spinal cord transection. To address this problem, a region that is rostral to transection should be imaged, where the descending axons from the brain are intact.

2) Does blocking calcium spikes change neurotransmitter expression at larval stages?

It would also be desirable to investigate whether suppressing spontaneous calcium spike activity following larval spinal cord transection has effects on neurotransmitter expression similar to those observed following suppression of calcium spikes during early embryonic development. The experimental design for these experiments would be the same as described above, except that the beads implanted in embryos would be loaded with a solution that blocks spontaneous calcium spike activity (Gorbunova and Spitzer, 2002; Borodinsky et al., 2004), containing 200 nM calcicludine, 10 μM GV1A ω-conotoxin, 10 μM flunarizine and 10 μg/ml tetrodotoxin. I hypothesize that transection followed by activity suppression will reduce the number of GABA-positive neurons and increase the number of
glutamate-positive neurons. At least 15 larvae should be evaluated in each control and experimental group, both for GABA and glutamate.

3) Investigate whether the embryonic environment contributes to reopening the sensitive period.

Finally it would be instructive to determine whether the embryonic environment contributes to the reopening of the sensitive period. By neural transplantation it is possible to provide immature neurons with a more mature spinal cord environment, or provide differentiated neurons with an embryonic neural tube environment, to investigate how these different environments affect neurotransmitter expression. From these experiments one could learn whether the sensitive period is regulated by the extrinsic cellular context. If extrinsic factors play a role, the results raise the possibility that the molecular basis of their effects can be identified and used to reopen the sensitive period in adult neurons.

3.1) Does transplanting a segment of the larval spinal cord to the early embryo respecify neurotransmitter expression by altering calcium spike activity?

A small region (0.5-1 mm) of neural tube will be removed from a stage 24-26 embryo, in which the sensitive period is still open, and a similar sized graft from a stage 36-40 larva will be transplanted to that region. A bead loaded with drugs to enhance or suppress spontaneous calcium spike activity will be implanted adjacent to the transplant. The embryo will be allowed to grow to stage 41 and then be fixed. The neurotransmitter expression profile of the transplants will be determined by immunostaining for GABA and glutamate as described above. I hypothesize that by
transplanting segments of the larval spinal cord to the young neural tube, mature neurons will be provided with an early embryonic environment, which may facilitate reopening of the sensitive period.

Caveats

During the process of transplantation the larval spinal cord is transected; in combination with enhancement of activity this can reopen the sensitive period, as indicated by my preliminary data. To address this issue homochronic transplantation can be used as a control (transplantation between same stage larvae or embryos) or transplantation back to the donor as host. Using these controls, it will be possible to identify the difference between axotomy-induced activity-dependent neurotransmitter expression in the early embryonic environment and in the more mature spinal environment. Embryos that have received bead implantation only and embryos that have received transplantation only will also serve as controls. The numbers of neurons that are GABA-positive and glutamate-positive will be scored as above.

3.2) Does transplanting a segment of the neural tube of the early embryo to the larva respecify neurotransmitter expression by altering calcium activity?

To study whether extrinsic factors present at early stages of embryonic development are important in opening or keeping open the sensitive period, small segments of the neural tube can be transplanted from young embryos into larvae. Neurons in the neural tube graft made from stage 22-24 embryo will still have spontaneous calcium activity and this spontaneous activity will be manipulated by
bead implantation in the stage 35-39 host larva. Immunocytochemistry will be used to determine the numbers of excitatory neurons (glutamate) and inhibitory neurons (GABA). The procedures of transplantation are not difficult and it is possible to get successful early neural tube transplants into the larval spinal cord. The effects of calcium spike suppression or enhancement with drug-loaded beads can be compared to the effect of implanting blank beads, in order to confirm the contribution of spike activity.

By transplanting a young neural tube into a more mature larva I hypothesize that closure of the sensitive period will be observed, or that activity-induced changes in neurotransmitter expression in the embryonic neural tube with a nonpermissive larval spinal environment will be reduced.

On the basis of my preliminary results I expect that after axotomy or in neural regeneration, recapitulation of early embryonic environment will reopen the sensitive period, during which neurotransmitter expression can be manipulated by altering spontaneous calcium activity. These results may be interpreted to indicate that some intrinsic or environmental molecular context, existing at early embryonic stages or regained after axotomy, is important for the existence of the sensitive period for neurotransmitter respecification. This will encourage further experiments to understand the mechanism underlying opening of the sensitive period, by screening of candidate molecules, whose expression patterns are different between early and late stages of development or between pre- and post-axotomy animals.
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Figure appendix_1 Two examples of spontaneous calcium activity of cultured spinal cord explants after spinal transaction. Imaging was performed on explants 24 hr after plating.
Figure appendix_2 Spontaneous calcium spike activity of a dorsal spinal neuron 1 d after spinal transection.
Figure appendix_3 Increase in number of GABAergic neurons in spinal cord transected, activity-enhanced larvae. For transected-only results and transected-plus-enhancement results, *, P<0.05. In each group, n>10 larvae were evaluated.

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