UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Mechanism of Neurotransmitter Specification in Embryonic *Xenopus* Spinal Cord

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Biology

by

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The Dissertation of Lin Xu is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chapter 3, is part of the content that has been published in Journal of Neuroscience, 2010 Apr 21; 30(16):5792-801. Target-dependent Regulation of Neurotransmitter Specification and Embryonic Neuronal Calcium Spike Activity. Xiao Q, Xu L, Spitzer NC. I am a co-author of this paper. I thank Qian Xiao for having me involved in a large part in her thesis project.

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PUBLICATIONS

1. **Xu L**, Spitzer NC. Non-cell-autonomous mechanism of activity-dependent neurotransmitter specification in early development. In preparation for submission.

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FIELD OF STUDY

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ABSTRACT OF THE DISSERTATION

The Mechanism of Neurotransmitter Specification in Embryonic *Xenopus* Spinal Cord

by

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Specification of neurotransmitters is a crucial and fundamental aspect of development. It allows the establishment of functional connections at synapses, which ensures normal functioning of the entire nervous system. Establishing the appropriate expression pattern of neurotransmitters in different populations of neurons is a critical challenge for neural development. It is a complicated process that involves multiple mechanisms, including intrinsic genetic coding, early neuronal activity and environmental factors.

Embryonic *Xenopus* spinal neurons generate spontaneous transient elevations of intracellular calcium. Calcium activity regulates early neuronal differentiation by

regulating neurotransmitter phenotype choice, but the mechanisms by which this activity is transduced to achieve these changes are unclear. We developed a novel practical method to manipulate activity in single neurons *in vivo*. Although we found that suppression of spike activity globally in developing spinal neurons increased the incidence of glutamatergic neurons and reduced the incidence of GABAergic neurons, we didn't find the same change when spike activity was suppressed in single isolated neurons. Our results indicate that GABAergic/glutamatergic selection is regulated by the level of activity in surrounding neurons. We found that activity-dependent brain-derived factor (BDNF) modulated GABAergic/glutamatergic switching, mimicking the effect of activity enhancement. Abolition of BDNF function by blocking its tyrosine kinase B (TrkB) receptor using K252a generated the opposite neurotransmitter switch, mimicking the effect of activity suppression. Simultaneous manipulation of BDNF function and suppression of Ca^{2+} activity phenocopied the effect on neurotransmitter phenotype of BDNF alone, indicating that BDNF works downstream of Ca^{2+} activity in regulating neurotransmitter specification. We propose that activity from neighboring neurons regulates the expression and release of BDNF, which then activates the TrkB signaling cascade leading to several physiological or genetic pathways that determine neurotransmitter specification. This mechanism provides a basis for early activity-dependent regulation of neurotransmitter phenotype in developing neurons.

In addition to the role of Ca^{2+} activity, we wanted to determine whether there is a target effect on neurotransmitter specification. For this purpose we developed a neuron-muscle co-culture system. We found that muscle contact refines transmitter

expression in cultured neurons by reducing expression of the non-cholinergic transmitters, GABA, glycine and glutamate, while having no effect on the incidence of choline acetyltransferase expression. The results indicate that muscle, as a neuronal target, plays important role in regulating transmitter specification.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Calcium-dependent Electrical Activity in Early Development

The development of techniques for electrical recording and imaging intracellular calcium ion concentrations led to the discovery of spontaneous fluctuations in intracellular calcium at early stages of neuronal development in various vertebrate and invertebrate model systems (Spitzer, 2006). These fluctuations are initiated by neurotransmitters that either depolarize neurons and activate voltage-gated Ca^{2+} channels allowing the entry of calcium, or bind to receptors that flux calcium (Gu et al., 1994; Hayes and Roberts, 1973; Holliday and Spitzer, 1990). Influx of calcium ions, resulting from activation of low-voltage-activated (LVA) and high-voltage-activated (HVA) calcium channels, triggers calcium-induced calcium-release from intracellular stores (Gu and Spitzer, 1993; Holliday et al., 1991) and generates Ca^{2+} spikes. Substantial evidence has revealed that Ca^{2+} transients regulate aspects of neuronal differentiation including proliferation, migration, ion channel expression, neurotransmitter specification, axon pathfinding, and dendrite outgrowth.

1.1.1 Properties of Calcium Spike Activity

In *Xenopus*, spontaneous Ca^{2+}-dependent spikes are characteristic of the early development of spinal neurons, both *in vitro* and *in vivo* (Gu & Spitzer, 1993; Borodinsky et al. 2004). *In vitro*, they are detected in spinal neurons plated from neural plate stage (stage 15) embryos as early as 5 hr following plating (Gu and Spitzer, 1993; Spitzer and Lamborghini, 1976). *In vivo*, they are normally first detected at the neural tube stage (stage 20) prior to synapse formation (Borodinsky et al., 2004; Hayes and Roberts, 1973). The long duration, Ca^{2+}-dependent action

potentials, which can last as long as half a minute, are only transiently expressed during development. Ca^{2+} spikes are generated 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 and Lamborghini, 1976). With further maturation, voltage-dependent delayed rectifier potassium currents are developmentally up-regulated and Ca^{2+} spikes are replaced by brief sodium-dependent impulses which last for only a few milliseconds (Lockery and Spitzer, 1992; Spitzer, 1994).

The onset and regulation of spontaneous Ca^{2+} spike activity is driven by a combination of intrinsic and extrinsic factors. The evidence that expression of the Na^{+}, K^{+}-ATPase β3 subunit is up-regulated during neurulation and down-regulated at the time Ca^{2+} spikes appear and suppression of its expression inhibits the appearance of spontaneous Ca^{2+} spikes, suggests that initiation of the cascade of events necessary for spike production requires activation of the intrinsic Na^{+}, K^{+}-ATPase (Chang and Spitzer, 2009). On the other hand, promiscuously expressed neurotransmitters, GABA and glutamate, at early developmental stages drive spontaneous Ca^{2+} spike activity via metabotropic receptors and downstream kinase signaling pathways (Root et al., 2008). Additionally, in the research project described in Chapter III, we found Ca^{2+} spikes are abolished in neurons when they contact muscle (data not included in this dissertation but in the published paper) (Xiao et al., 2010), although the mechanism remains to be determined.

1.1.2 Experimental Manipulation of Calcium Spike Activity

Experimental manipulation of spontaneous Ca^{2+} spikes can be achieved by multiple pharmacological and molecular methods. Depolarization by high extracellular KCl, incubation with drugs such as veratridine that prevent sodium channel inactivation, and misexpression of sodium channels that cause depolarization all enhance Ca^{2+} spike activity (Borodinsky et al., 2004; Demarque and Spitzer, 2010; Dulcis and Spitzer, 2008; Galli et al., 1995; Holliday and Spitzer, 1990; Spitzer, 1979). Removal of extracellular calcium ions, pharmacological blockade with antagonists of various voltage-dependent Ca^{2+} channels, and misexpression of inwardly rectifying potassium channels that cause hyperpolarization all lead to Ca^{2+} spike activity suppression (Borodinsky et al., 2004; Burrone et al., 2002; Gu and Spitzer, 1993). Recently, *Chlamydomonas reinhardtii* channelrhodopsin-2 (ChR2) and *Natronomonas pharaonis* halorhodopsin (NpHR) have been developed and widely employed as new tools to excite and inhibit neuronal spikes (Li et al., 2005; Nagel et al., 2003; Zhang et al., 2007). They can be activated by illumination on the millisecond timescale in multiple cell types. ChR2 is a light-switched cation-selective ion channel that opens rapidly after absorption of a photon to generate a high permeability for monovalent and divalent cations and thus depolarizes neurons. NpHR is a light-driven chloride pump that hyperpolarizes neurons. These ultrafast, genetically based, neural-spike-controlling technologies powerfully augment the above-mentioned tools. All these methods together provide diversified ways to interrogate neural systems.

1.2 NEUROTRANSMITTER SPECIFICATION IN EARLY EMBRYONIC DEVELOPMENT

Specification of neurotransmitters is a crucial aspect of development. The proper expression of both neurotransmitters and their matching receptors establishes the basis of neuronal communication. As neurons build up functional connections at synapses they achieve efficient communication that ensures normal functioning of the entire nervous system. This developmental process requires synergistic interplay among genetic coding, electrical activity and environmental factors.

1.2.1 INTRINSIC FACTORS

It is widely accepted that distinct expression patterns of intrinsic transcription factors determine distinct neuronal identities including neurotransmitter expression. The transcription factors either work alone or interact with each other to refine the neurotransmitter pattern. 5-hydroxytryptamine (5-HT) specification has been shown to involve a set of conserved transcription factors including Pet-1, Lmx1b, Nkx2.2, Mash1, Gata2, Gata3, and Phox2b in multiple animal systems (Alenina et al., 2006; Cordes, 2005; Demarque and Spitzer, 2010; Lillesaar et al., 2007; Scott et al., 2005). Ectopic expression of the homeobox gene *MNR2* in neural cells can cause motor neuron differentiation, including the expression of acetylcholine (Tanabe et al., 1998). *Dbx1* knockout mice exhibit an increase in GABA-immunoreactive interneurons (Pierani et al., 2001). Gain- and loss-of-function experiments in mice have shown that transcription factor Barhl2 is involved in the expression of glycine (Mo et al., 2004). Tlx3 and Lbx1 act as switches in determining glutamatergic and GABAergic phenotypes in chick, mouse and *Xenopus* (Cheng et al., 2004; Cheng et al., 2005; Hoshino, 2006; Marek et al., 2010). In the mouse spinal cord, LMO4 can induce the

appearance of 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-interneuons (Joshi et al., 2009).

However, although the mature neurotransmitter phenotype of the neuron has been determined by extrinsic influences, more and more evidence has shown that regulation of this process is governed by factors including neuronal activity and environmental signals.

1.2.2 NEURONAL ACTIVITY

Neuronal activity is critical in regulating expression of inhibitory and excitatory phenotypes in the nervous system. Chronic suppression of activity in individual hippocampal neurons resulted in enhancement of glutamatergic inputs received by these neurons (Burrone et al., 2002). In slice cultures of visual cortex (Chattopadhyaya et al., 2004), hippocampus (Marty et al., 2000), cerebellum (Seil and Drake-Baumann, 2000; Seil et al., 1994), and spinal cord (Rosato-Siri et al., 2002), as well as in dissociated cultures of neocortex (Kilman et al., 2002; Rutherford et al., 1997), blockade of activity triggers a reduction in the amount of inhibition. Activity can differentially affect regulation of the cholinergic phenotype by protein factors including choline acetyltransferase (ChAT) and recombinant cholinergic differentiation factor/leukemia inhibitory factor (Rao et al., 1992). In *Xenopus*, the incidence of cultured neurons expressing the transmitter GABA and its synthetic enzyme, glutamic acid decarboxylase (GAD), is up-regulated in cultured *Xenopus* embryonic spinal neurons by increasing the frequencies of Ca^{2+} spikes (Gu and Spitzer,

1995; Watt et al., 2000). *In vivo*, spontaneous Ca^{2+} spike activity regulates transmitter expression in embryonic spinal neurons in a homeostatic fashion (Borodinsky et al., 2004). Suppression of activity increases the incidence of neurons expressing excitatory transmitters (glutamate and acetylcholine) and decreases the incidence of neurons expressing inhibitory transmitters (GABA and glycine). Enhancing activity leads to the opposite neurotransmitter phenotypes. Therefore, neuronal activity, working cell-autonomously or non-cell-autonomously, plays an important role in neuronal differentiation during early development.

1.2.3 Environmental Factors

Environmental factors, including macro-environmental factors as well as micro-environmental factors, are heavily involved in defining neuronal identities. Macro-environmental factors such as light exposure, which changes the sensory input to the circuit controlling adaptation of skin pigmentation to background, changes the number of neurons expressing dopamine in larvae of *Xenopus laevis* in a circuit-specific and activity-dependent manner (Dulcis and Spitzer, 2008). Micro-environmental factors originate from neuron-neuron and neuron-target tissue communication. Cytokines and neurotrophic factors can drive the expression of acetylcholine over noradrenaline in rat sympathetic ganglion neurons, both in culture and *in vivo* (Furshpan et al., 1976; Landis and Keefe, 1983; Nawa and Patterson, 1990). Chronic exposure to nerve growth factor (NGF) significantly increases acetycholine and glutamate release from the cholinergic diagonal band of Broca (MS-DBB) neurons (Huh et al., 2008). Exposure to fibroblast growth factor (bFGF) causes

a significant increase in glutamate-containing neurons in the cerebral cortex, while the number of GABA- and aspartate-containing neurons is not affected (Vaccarino et al., 1995).

In Chapter II, we present evidence that Ca^{2+} activity can act through brain-derived neurotrophic factor (BDNF) to regulate neurotransmitter expression in embryonic *Xenopus* spinal neurons. Among the four neurotrophins, NGF, BDNF, neurotrophin-3 (NT3) and NT4/5, BDNF is most frequently reported as a key modulator in balancing neural network excitability (Bi and Poo, 2001; Bibel and Barde, 2000; Huang and Reichardt, 2001; Lindsay, 1996; Lu, 2003; Vicario-Abejon et al., 2002), most probably due to the fact that both its expression and release are tightly regulated by neuronal activity (Gartner and Staiger, 2002; Goodman et al., 1996; Hartmann et al., 2001; Kohara et al., 2001; Thoenen, 2000; Tongiorgi, 2008; Zafra et al., 1990). Application of exogenous BDNF increased the release of dopamine from the rabbit retina amacrine cells (Neal et al., 2003). Loss of BDNF from the midbrain-hindbrain adversely affected the amount of tyrosine hydroxylase (TH) present in the striatum and establishment of the proper number of dopamine neurons within the substantia nigra pars compacta (Baquet et al., 2005). BDNF enhanced GABAergic transmission in rat hippocampal neurons (Baldelli et al., 2002; Bolton et al., 2000; Swanwick et al., 2006), or increased the number and density of GABAergic synapses in organotypic slice cultures from rat hippocampus (Marty et al., 2000), and in solitary neurons cultured from rat visual cortex (Palizvan et al., 2004) and in hippocampus neurons *in vivo* (Shinoda et al., 2011). However, BDNF is also reported to acutely depress inhibitory synaptic transmission in hippocampal slices (Frerking et al., 1998;

Tanaka et al., 1997) and to reduce miniature inhibitory postsynaptic currents (mIPSCs) in cultured hippocampal (Brunig et al., 2001) and cerebellar granule neurons (Cheng and Yeh, 2003). Similarly, dual roles of BDNF have also been observed for regulation of glutamate-mediate neuronal excitability. Exogenous BDNF prevents the enhancement of synaptic strengths normally induced by chronic blockage of activity in cortical cultures, and preventing activation of endogenous BDNF receptors increases synaptic strengths (Rutherford et al., 1998). However, other studies have shown that blocking BDNF signaling does not prevent the decrease in amplitude of miniature excitatory postsynaptic currents (mEPSCs) achieved by chronic depolarization (Leslie et al., 2001), and application of BDNF enhances excitatory synaptic transmission by increasing the phosphorylation of NMDA receptor (Alder et al., 2005; Lin et al., 1998; Suen et al., 1997). These studies argue that enhanced BDNF release is either not essential or is scaling up synaptic strength. Those data raise the question of how BDNF-dependent regulation of neuronal plasticity might differ in terms of the receptor signaling it activates in different cell types, at different developmental stages, or in different animal models.

Establishment of correct synaptic connections is a two-way process in which both the neurons and their targets communicate extensively with each other to ensure proper alignment. The expression of target-derived neurotrophins and other factors peaks early in neuronal development and declines at later times (Ibanez et al., 1993; Timmusk et al., 1993), suggesting that they have critical roles in refining neuronal development (Zweifel et al., 2005). The role of target-derived factors in neurotransmitter choice was first reported in a subset of mammalian sympathetic

ganglion neurons. They switched from a noradrenergic to a cholinergic phenotype after they innervated sweat glands, and transplantation experiments showed that this developmental change is triggered and regulated by targets (Francis and Landis, 1999; Furshpan et al., 1976; Landis and Keefe, 1983; Nawa and Patterson, 1990). In Chapter III, we demonstrated the role of muscle targets on transmitter specification. We found that muscle-derived factors seem to prevent neurons from expressing non-cholinergic neurotransmitters. Muscle tissue expresses many important trophic factors during development, including NT-3/4/5, BDNF, NGF, glia cell line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), and insulin like factor (ILF). Survival of motoneurons and establishment of the cholinergic phenotype depend on muscle factors (Bennett and Nurcombe, 1979; Flanigan et al., 1985; Lams et al., 1988; Petruzzelli and Hughes, 1989; Smith and Appel, 1983). Application of trophic factors including NT-4/5, BDNF and GDNF can partially rescue losses of muscle factors (Fernandes et al., 1998; Kou et al., 1995). In contrast, in *Xenopus*, muscle-derived signals appear to have a profound effect on noncholinergic transmitter expression. Cholinergic transmitter expression is independent of muscle regulation.

Overall, cell-specific transcription factor expression, perturbations of early neuronal activity and action of environmental cues interact with each other to provide feedback loops to validate or fine-tune the mature neurotransmitter phenotype.

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CHAPTER 2

NON-CELL-AUTONOMOUS MECHANISM OF ACTIVITY-DEPENDENT NEUROTRANSMITTER SPECIFICATION IN EARLY DEVELOPMENT

ABSTRACT

Calcium spike activity regulates respecification of neurotransmitters expressed in the developing *Xenopus* nervous system, but the mechanism by which this activity is transduced to achieve these changes is unknown. We have developed a novel practical method to manipulate activity in single neurons *in vivo*, to determine whether or not the process is cell-autonomous. Although global suppression of spike activity in developing spinal neurons increases the incidence of glutamatergic neurons and reduces the incidence of GABAergic neurons, these changes did not occur when spike activity was suppressed in single neurons. Our results indicate that GABAergic/glutamatergic selection is homeostatically regulated non-cell-autonomously by the level of activity in surrounding neurons. Application of activity-dependent brain-derived factor (BDNF) to the spinal cord decreased the number of glutamatergic neurons and increased the number of GABAergic neurons, mimicking the effect of activity enhancement. Abolition of BDNF function by blockade of TrkB receptors with K252a generated the opposite transmitter change, mimicking the effect of activity suppression. Simultaneous manipulation of BDNF function and suppression of Ca^{2+} activity phenocopied the effect on neurotransmitter phenotype of BDNF alone, indicating that BDNF acts downstream of Ca^{2+} activity in neurotransmitter specification. We propose that activity from neighboring neurons regulates the expression and release of BDNF, which most likely activates the TrkB signaling cascade to determine neurotransmitter specification. This mechanism provides a basis for

early activity-dependent specification of neurotransmitter phenotype in developing neurons.

Key words: Calcium signaling, neurotransmitter specification, BDNF, TrkB, neuronal development, *Xenopus*

INTRODUCTION

Specification of neurotransmitters is a fundamental aspect of neuronal development, allowing the establishment of functional connections at synapses. Efficient communication among neurons via synapses ensures normal functioning of the entire nervous system. Expression of an inappropriate transmitter would isolate the neuron from its normal network and reduce the trophic support from its postsynaptic targets that may be required for survival. Distinct expression patterns of neurotransmitters are determined both by intrinsic transcription factors (Cheng et al., 2004; Lee and Pfaff, 2001; Mizuguchi et al., 2006; Mo et al., 2004; Pierani et al., 2001; Pillai et al., 2007; Tanabe et al., 1998; Xu et al., 2008) and by electrical activity. In *Xenopus*, spontaneous Ca^{2+} spike activity is critical in regulating inhibitory and excitatory transmitter phenotypes homeostatically in embryonic spinal neurons (Borodinsky et al., 2004). When Ca^{2+} spikes are suppressed using molecular or pharmacological approaches, more neurons express the excitatory neurotransmitters glutamate and acetylcholine while fewer neurons express the inhibitory neurotransmitters GABA and glycine. In contrast, when Ca^{2+} spiking is increased, more neurons express the inhibitory neurotransmitters GABA and glycine while fewer neurons express the excitatory neurotransmitters glutamate and acetylcholine. However, the signal transduction mechanism underlying this phenomenon is unclear.

Generation of electrical activity leads to a wide range of elevations of intracellular Ca^{2+} (Berridge et al., 2003) that could regulate expression of genes determining excitatory or inhibitory phenotype in a cell-autonomous manner. However, these transient elevations of intracellular Ca^{2+} could also regulate cellular

secretion enabling inductive interactions among cells to specify neurotransmitter via a non-cell-autonomous mechanism (Spitzer, 2006).

The role of cell-autonomous versus non-cell autonomous mechanisms is often examined in purified and sparsely plated cultures (Kenichi et al., 2006; Tong et al., 2010; William et al., 2010). Here we address this question *in vivo* by developing a novel single-neuron targeting method. No neurotransmitter change was observed in single neurons in which Ca^{2+} spikes have been suppressed in the spinal cord of developing *Xenopus* embryos, indicating that a non-cell-autonomous mechanism is involved.

The neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT4/5, were initially identified as survival factors for sensory and sympathetic neurons but were subsequently found to regulate differentiation and maintenance of function in different populations of neurons. BDNF is an attractive candidate to regulate activity-dependent transmitter specification because its expression and secretion depend on neuronal activity (Balkowiec and Katz, 2002; Tabuchi et al., 2000). Furthermore, BDNF plays a critical role in synaptic plasticity. Application or overexpression of BDNF promotes development of inhibition (Huang et al., 1999; Marty et al., 2000; Mizuno et al., 1994; Ohba et al., 2005; Yamada et al., 2002), whereas decreased expression or disruption of the function of BDNF impairs development of inhibitory synapses (Hong et al., 2008; Kohara et al., 2007; Shinoda et al., 2011). On the other hand, application of BDNF leads to suppression of excitatory synaptic transmission (Rutherford et al., 1998; Yang et al., 2002). In present study we provide evidence that BDNF can mediate the non-

cell-autonomous mechanism of Ca^{2+} activity-dependent respecification of GABA and glutamate in spinal neurons via activation of TrkB receptor signaling. Thus, the mature transmitter phenotype is determined by genetic programs and collective electrical activity that drives neurotransmitter induction with BDNF as a likely mediator.

MATERIALS AND METHODS

Generation and staging of embryos.

Adult *Xenopus laevis* females were primed and injected with human chorionic gonadotropin (Sigma-Aldrich, St. Louis, MO) to induce ovulation. Eggs were fertilized *in vitro*. Embryos were grown at 22^{o}C in 0.1 × Marc's modified Ringer's solution (MMR) (1× MMR contains 100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 5 mM HEPES, 0.1 mM EDTA, 2 mM $CaCl_2$, pH adjusted to 7.8) and staged according to Nieuwkoop and Faber (1967).

DNA constructs.

The hKir2.1-pBluescript construct was a generous gift from Dr. Eduardo Marban (Johns Hopkins University, Baltimore, MD). The hKir2.1-mCherry fusion gene was subcloned into a pcDNA3.1 vector by *Nhe*I and *Not*I restriction sites, resulting in a recombinant construct hKir2.1-mCherry-pcDNA3.1. mCherry-pcDNA3.1 was constructed as a control plasmid.

Cell injection.

DNA injection was performed at the 16-cell stage. Specific cells for injection were identified according to Moody (1989). RNA injection was performed at the 2 cell stage. Injections were accomplished using a picospritzer (Picospritzer III, Parker Instrumentations, Cleveland, OH). hKir2.1-mCherry-pcDNA3.1 or mCherry-pcDNA3.1 (control) DNA was amplified by the Qiagen midiprep kit (QIAGEN, Venlo, The Netherlands) and dissolved in distilled water. A range of different amounts of

DNA (from 100 pg to 800 pg) was injected into 16-cell blastulae and 200 pg DNA was determined to be the optimal amount for labeling single neurons. Thus, DNA was made up at 0.2 mg/ml and 1 nl was injected. hKir2.1 mRNA was transcribed using the Ambion mMessage mMachine kit (Ambion, Austin, TX). 5-10 nl of a 0.01-0.1 mg/ml capped mRNA solution in nuclease-free water were coinjected with 10,000 MW Cascade Blue dextran (20 mg/ml, Invitrogen, Carlsbad, CA). Healthy embryos were transferred to Petri dishes containing 0.1× MMR after injections and incubated in the dark.

Embryo dissections for in vivo studies.

Stage 23-25 embryos were dissected in wells of Sylgard-coated (Dow Corning, Midland, MI) Petri dishes containing 1 mg/ml collagenase B (Roche, Basel, Switzerland) in MMR. Briefly, the gut and epidermis surrounding the presumptive spinal cord were cut away, the endodermal yolk layer was removed, and embryos were secured to Sylgard-lined wells ventral side up with 0.10-mm-diameter stainless-steel Austerlitz Minutien pins (Fine Science Tools, Vancouver, Canada). After pinning, myotomes and notochord were gently removed with sharp forceps and the solution was replaced with 2 mM Ca^{2+} saline (116.6 mM NaCl, 0.67 mM KCl, 1.31 mM $MgSO_4$, 2 mM $CaCl_2$, and 4.6 mM Tris, pH 7.8).

Confocal imaging.

Neural tubes of stage 23-25 *Xenopus laevis* embryos were dissected to expose the ventral surface and loaded for 45 min with 5 μM of the Fluo-4 AM Ca^{2+} indicator

(Invitrogen, Carlsbad, CA) and 0.01% Pluronic F-127 detergent (Invitrogen, Carlsbad, CA) in 2 mM Ca^{2+} saline. Neural tubes were washed in Ca^{2+} saline for 5 min prior to imaging to minimize background fluorescence. Images were acquired at 0.2 Hz for 1 hr periods with a Leica SP5 confocal system (Nussloch, Germany) with a 40× water immersion objective. An argon laser line (488 nm) was used to detect Fluo-4-loaded neurons and monitor Ca^{2+} spike activity. A DPSS laser line (561 nm) was used to visualize mCherry-labeled neurons. Image movies were imported into NIH Image J for analysis and the cell bodies of neurons of interest were outlined using the tracing tools. The average pixel intensity was determined and exported to Microsoft Excel for analysis. These data were plotted against the time of image acquisition to yield fluorescence kinetics and intensity. Ca^{2+} transients were scored as spikes when the amplitude exceeded twice the amplitude of baseline fluctuations during the previous 10 min and the rise time was complete within 5 sec. 7-10 embryos were imaged for each experiment. All hKir2.1-mCherry- or mCherry-expressing single neurons from these embryos were analyzed. Neurons not expressing hKir2.1-mCherry or mCherry in the same embryos were internal controls. The incidence of spiking neurons from each experimental group was calculated by dividing the number of neurons that spiked at least once during the 1 hr imaging period by the total number of neurons analyzed in that group.

Immunocytochemistry.

Three day old (stage 41) embryos were fixed in 4% paraformaldehyde (PFA) with or without 0.1% glutaraldehyde in phosphate-buffered saline (PBS) at pH 7.4 for

30 min to 2 hr at 4°C, incubated in sucrose for 2.5 hr, and embedded in OCT. Cryostat sections 20 μm in thickness were made over a length of 400 μm starting from the rostral end of the spinal cord. All sections were blocked with 1% fish gelatin (Sigma-Aldrich, St. Louis, MO) in 0.1% Triton X-100 in PBS for 0.5 hr before staining overnight at 4°C with single primary antibodies at the following concentrations: rabbit anti-vesicular glutamate transporter 1 (VGluT1; Sigma-Aldrich, St. Louis, MO) 1:5000; rabbit anti-glutamate (Sigma-Aldrich, St. Louis, MO) 1:10, 000; guinea pig anti-GABA (Millipore Bioscience Research Reagents, Temecula, CA) 1:300. Fluorescently tagged secondary antibodies were used at 1:300 for 2 hr at 22°C. Sections were mounted in Vectashield mounting medium (DAPI; Vector Laboratories, Burlingame, CA) to track cell numbers. Labeling was examined on an Axioskop with a 40× water-immersion objective or an Olympus confocal microscope (Olympus IY81, Center Valley, PA) with a 20× air objective, using the appropriate excitation and emission filters for Alexa 488, Alexa 594, and DAPI.

Whole mount in situ hybridization

Whole mount *in situ* hybridization was performed following the protocol of Harland (Harland, 1991). Antisense and sense probes were generated from pX112-13 containing a partial *Xenopus* BDNF cDNA (a generous gift from Dr. Susana Cohen-Cory, UC Irvine). Briefly, embryos of chosen stages were fixed in MEMPFA (3.7% PFA, 0.1 M MOPS pH 7.4, 1 mM $MgSO_4$, 2 mM EGTA) and stored in methanol at -20°C until use. They were then rehydrated, washed with PBST (0.1%Tween-100 in PBS), and hybridized in hybridization buffer (50% formamide, 5× SSC, 1 mg/ml

Torula RNA, 100 μg/ml heparin, 1× Denhart's, 0.1% Tween-20, 0.1% CHAPS, 5 mM EDTA) at 60°C overnight. Embryos were washed with maleic acid buffer (MAB), incubated in 20% heat-inactivated lamb serum for 2 hr at 22°C, and then incubated overnight with anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche, Basel, Switzerland) at 1:2000. After the antibody solution was removed and the embryos were washed in MAB 5 times for 1 hr each at 22°C, the chromogenic reaction was started by replacing MAB with BM Purple AP substrate (Roche, Basel, Switzerland). Embryos were examined periodically and the chromogenic reaction was stopped by replacing the staining solution with MEMPFA. Embryos were dehydrated and mounted with 2:1 benzyl benzoate/benzyl alcohol for observation.

Pharmacology.

Stock concentrations of drugs were 1 mM K252a (Sigma-Aldrich, St. Louis, MO) and 1 mM K252b (Sigma-Aldrich, St. Louis, MO) in DMSO, and 1 mg/ml recombinant BDNF (Millipore, Billerica, MA) in distilled water. Agarose (100–200 mesh, Bio-Rad, Hercules, CA) beads were loaded overnight with drugs (50 μM K252a, K252b; 100 ng/μl BDNF) or DMSO/H_2O (control) and inserted between the neural tube and myotomes beside the neural tube at 20 hr of development (stage 18). Sections of bead-implanted embryos were collected 2 days later (stage 41).

Statistics.

Means and SEMs were calculated using Microsoft Excel. Statistical analyses were performed using STATA. Significance was assessed with the Mann-Whitney U test. Values are considered different when p is < 0.05.

RESULTS

Injection of DNA into 16-cell blastomeres labels single neurons in vivo

Previous work demonstrated that Ca^{2+}-dependent electrical activity in embryonic *Xenopus* spinal neurons homeostatically regulates specification of the transmitters that neurons express without affecting cell identity (Borodinsky et al., 2004). Misexpression of human inward rectifier K^+ channels (hKir2.1) by injection of hKir2.1 mRNA caused more neurons to express the excitatory neurotransmitters glutamate and acetylcholine while fewer neurons expressed the inhibitory neurotransmitters GABA and glycine in the spinal cord. To determine whether activity-dependent neurotransmitter specification is cell-autonomous, a single-neuron targeting system was developed utilizing two strategies (Figure 2.1). First, blastomeres were injected with DNA instead of mRNA. Microinjection of mRNA into the 1- or 2-cell stage embryo has the advantage of allowing uniform expression over a large region later in development. In contrast, expression from DNA constructs is mosaic. A few cells express large amounts of transcript while most cells express none, for unknown reasons (Kroll and Amaya, 1996; Sargent and Mathers, 1991; Vize et al., 1991). Although mosaic expression from DNA constructs has been regarded as a nuisance, we have used it to advantage. Second, injection was performed at the 16-cell stage rather than at the 2-cell stage. Later stage injection further limits the number of cells expressing the DNA of interest. Additionally, neuronal lineage has been determined at the 16-cell stage (Hartenstein, 1993; Lamborghini, 1980; Moody, 1989), enabling more specific manipulation of Ca^{2+} spike activity in spinal neurons. To label

cells expressing hKir2.1 by hKir2.1 DNA injection, a hKir2.1-mCherry fusion plasmid was constructed. As a result, mCherry-labeled neurons misexpress hKir2.1.

Employing these strategies, we injected 200 pg of hKir2.1-mCherry-pcDNA3.1 DNA into the D1.1 or D1.2 cell of the 16-cell blastula (Figure 2.2 A), both of which make a major contribution to neurons in the ventral spinal cord (Xenbase). The survival rate was 70-80% and fluorescence was detected before stage 15 and persisted for at least three days. When embryos reached the tailbud stage (stage 41), mCherry was typically observed in several separated neurons along the spinal cord (Figure 2.2 B), indicating the success of targeting single neurons by this method. Further analysis of these mCherry-labeled neurons showed that 80% of them are neurons on the ventral side of the neural tube and include motoneurons (MN), ascending interneurons (AI), and descending interneurons (DI) whereas 20% of them are neurons on the dorsal side of the neural tube and include Rohon-Beard (RB) sensory neurons and dorsolateral ascending (DLA) interneurons (Figure 2.2 C). When DNA was injected into V1.2 cells of the 16-cell blastula, the survival rate was again 70-80%. Since V1.2 cells make a major contribution to the dorsal spinal cord (Xenbase), RB sensory neurons and DLA interneurons are successfully targeted (data not shown).

Misexpression of Kir-mCherry suppresses Ca^{2+} spike in single neurons, but does not change neurotransmitter expression

hKir2.1 has been used to suppress excitability in mammalian superior cervical ganglion cells, hippocampal neurons, pontine neurons and chick hair cells *in vitro*

(Burrone et al., 2002; Holt et al., 1999; Howorth et al., 2009; Johns et al., 1999) as well as to suppress neuronal activity *in vivo* in multiple animal models (Borodinsky et al., 2004; Duale et al., 2007; Mizuno et al., 2007; Yu et al., 2004). To determine whether *in vivo* misexpression of hKir2.1 from DNA injection in single neurons suppresses Ca^{2+} spikes, we assessed Ca^{2+} activity in these neurons by confocal imaging. Digital traces of fluorescence levels in each neuron of interest over time were analyzed and scored for spikes. Although neurons located on both dorsal and ventral surfaces spike *in vivo* in *X. laevis*, those positioned on the dorsal surface spike at lower frequencies at early stages of development (Belgacem and Borodinsky, 2011; Borodinsky et al., 2004; Gu et al., 1994; Root et al., 2008b). We thus imaged the intact ventral spinal cord at stage 23-25 when most classes of spinal neurons exhibit a high incidence and frequency of Ca^{2+} spikes (Borodinsky et al., 2004). hKir2.1-mCherry DNA was injected to achieve expression in single neurons in the neural tube. Neurons without hKir2.1 expression in the same embryos served as internal controls. Reflecting loading of the dissected neural tube with the Ca^{2+} indicator, Fluo 4-AM, internal control neurons are shown in green while hKir2.1-mCherry-expressing neurons are shown in yellow/red. (Figure 2.3 A). To determine whether mCherry expression alters Ca^{2+} activity, we imaged neurons expressing mCherry alone for comparison to neurons without mCherry expression in the same embryos.

We examined 7 embryos injected in single 16-cell stage blastomeres with hKir2.1-mCherry DNA and found 37 single neurons expressing mCherry fluorescence. Among them, only 2 (5%) generated Ca^{2+} spikes during the 1 hr imaging period and no Ca^{2+} transients were observed in 35 (95%) neurons. In contrast, of the 59 internal

control neurons from the same 7 embryos, 28 of them (47%) generated Ca^{2+} spikes during the 1 hr imaging period (Figure 2.3 B, C). Furthermore, in both of the spiking hKir2.1-mCherry-expressing neurons, the frequency was 1 or 2 hr^{-1} with lower amplitudes (124±9% of baseline), while among all the spiking internal control neurons the frequency ranged from 1-12 hr^{-1} with higher amplitudes (158±49% of baseline) (Figure 2.3 D). These data demonstrate that misexpression of hKir2.1-mCherry significantly decreases both spike incidence and frequency in single neurons. On the other hand, of the 63 neurons in 10 imaged embryos injected with mCherry DNA alone, 32 (51%) generated Ca^{2+} spikes during the 1 hr imaging period, with a frequency that ranged from 1-7 hr^{-1}. Of the 56 internal control neurons, 29 (52%) generated Ca^{2+} spikes during the 1 hr imaging period, with a frequency that ranged from 1-10 hr^{-1} (Figure 2.3 C, D). Both the incidence and frequency of spiking in mCherry-expressing single neurons were similar to those in internal controls, suggesting that mCherry has no effect on Ca^{2+} spike activity and allows hKir2.1 to effectively exert its hyperpolarizing function.

We then determined whether homeostatic neurotransmitter switching occurred in these single neurons in which Ca^{2+} spike activity had been suppressed. Neurons expressing mCherry-alone served as controls. Neurons on the ventral side of the neural tube are mostly cholinergic MN, GABAergic AI, and cholinergic/glutamatergic DI (Li et al., 2004; Perrins and Roberts, 1995; Roberts et al., 1987). Neurons on the dorsal side of the neural tube are mostly glutamatergic RB sensory neurons and GABAergic/glycinergic DLA interneurons (Roberts et al., 1987; Roberts et al., 1988; Sillar and Roberts, 1988). If Ca^{2+} spikes acted cell-autonomously, we expected to see

single neurons on the ventral side of the neural tube acquire a glutamatergic phenotype while single neurons on the dorsal side of the neural tube lose the GABAergic phenotype. We targeted these different groups of neurons by injection of D1.1/D1.2 or V1.2 cells of 16-cell-stage blastulae, respectively, as described above. VGlut1 and GABA antibodies were used to assess glutamatergic and GABAergic phenotypes. Strikingly, the incidence of VGluT1-immunoreactive neurons among hKir2.1-mCherry-labeled ventral neurons and among mCherry-alone-labeled ventral neurons did not differ from each other (45% vs 48%). This result indicates that the glutamatergic phenotype was not acquired in single neurons in which Ca^{2+} spikes had been suppressed (Figure 2.4 A). No difference was observed in the incidence of GABA-immunoreactive hKir2.1-mCherry labeled neurons and mCherry-alone-labeled dorsal neurons (53% vs 50%), indicating that there is no decrease in the incidence of GABA-immunoreactive cells among the single neurons in which Ca^{2+} spikes have been suppressed (Figure 2. 4 B). These data suggest that the mechanism for Ca^{2+} spike activity-dependent neurotransmitter respecification in the embryonic spinal cord is non-cell-autonomous.

BDNF regulates neurotransmitter phenotype hemostatically via Trk receptors

Neurotrophins are important regulators of neural development, survival, function, and plasticity (Bernd, 2008; Eide et al., 1993; Korsching, 1993; Lewin and Barde, 1996; McAllister, 1999; Segal and Greenberg, 1996; Sofroniew et al., 2001). Among the neurotrophins, only NT-3 and BDNF and their corresponding Trk receptors are expressed at early developmental stages in avian embryos (Bernd, 2008),

suggesting their specific roles in early development. More intriguingly, the expression and release of BDNF are regulated by neuronal activity (Castren et al., 1992; Gartner and Staiger, 2002; Tabuchi et al., 2000) and it plays a key role in regulating homeostatic plasticity in neuronal networks (Hong et al., 2008; Huang et al., 1999; Kohara et al., 2007; Marty et al., 2000; Mizuno et al., 1994; Ohba et al., 2005; Rutherford et al., 1998; Shinoda et al., 2011; Yamada et al., 2002; Yang et al., 2002). Neurotransmitter specification in *Xenopus* embryos is most responsive to changes in Ca^{2+} spike activity during the critical period between neural tube formation (stage 20) and an early larval stage (stage 28); sensitivity decreases during early tailbud stages and disappears when the embryo approaches stage 35 (Spitzer et al., 2004). BDNF expression has been detected in the neural plate in *Xenopus* embryos at stage 18 (Huang et al., 2007). *In situ* hybridization with a 387 bp fragment of BDNF revealed its expression in the neural plate and neural tube at stages 18, 24 and 28, throughout the critical period for neurotransmitter specification when spontaneous Ca^{2+} spike activity is present (Figure 2.5 A,B,C). Based on these observations, we hypothesized that BDNF might regulate activity-dependent transmitter specification during early development.

To examine the role of BDNF in the non-cell-autonomous mechanism of neurotransmitter specification *in vivo*, we implanted agarose beads adjacent to the spinal cord to locally and chronically release BDNF or a Trk receptor inhibitor (K252a). This approach has previously been shown to be effective in delivering drugs (Borodinsky et al., 2004; Root et al., 2008b) and allowed us to restrict the perturbation to specific stages of development. Beads were loaded with BDNF or vehicle,

implanted at the time of neural tube closure (stage 18) and larvae were fixed at 3 d of age (stage 41), sectioned and immunostained to examine transmitter expression (Figure 2.6 A). Only embryos with beads located adjacent to the first 100 μm of the spinal cord were analyzed and neurons along the most rostral 400 μm of the spinal cord were scored.

Embryos implanted with BDNF-loaded beads showed an increase in number of GABAergic neurons and a decrease in number of glutamatergic neurons (Figure 2.6 B), mirroring the effect of rat brain sodium channel II (Nav1.2a/b) misexpression in *Xenopus* spinal neurons on transmitter specification when the activity is enhanced (Borodinsky et al., 2004). Changes in both GABA and glutamate expression in spinal neurons were significant over the most rostral 200 μm of the spinal cord. The effect of BDNF on both GABA and glutamate expression became smaller and was not significant for neurons more than 100 μm away from the site at which the BDNF-loaded beads were implanted. The large size of recombinant BDNF (27 kD) may limit its diffusion. Furthermore, the action of BDNF is spatially restricted, as indicated by the observation that potentiation of both the mean excitatory postsynaptic current (mEPSC) amplitude and frequency was found only for BDNF-bead contact sites within a distance of ~60 μm from the synapse (Zhang and Poo, 2002).

Activation of the TrkB receptor tyrosine kinase is the principal pathway by which BDNF initiates downstream signaling. To determine whether the observed effect of BDNF on neurotransmitter specification was mediated by Trk receptors, K252a, a membrane permeable tyrosine kinase inhibitor with relatively high affinity for TrkB receptors (Tapley et al., 1992), was loaded in beads that were implanted in

embryos as described above. The opposite outcome of neurotransmitter expression was observed: the number of glutamatergic neurons was increased and the number of GABAergic neurons was decreased (Figure 2.6 C), mirroring the effect of hKir2.1 misexpression in *Xenopus* spinal neurons on transmitter specification when Ca^{2+} spike activity is blocked (Borodinsky et al., 2004). The change in both GABA and glutamate expression in spinal neurons was significant along all 400 μm of the rostral spinal cord, although the number of neurons exhibiting changes in transmitter specification diminished from 100 μm to 400 μm. The smaller size of K252a (0.4 kD) is expected to enable greater diffusion. As a negative control, we implanted embryos with beads containing K252b, the non-functional analogue of K252a (Knusel and Hefti, 1992). The number of GABAergic neurons and glutamatergic neurons in these embryos were similar to those in controls (Figure 2.6 D). These results suggest that BDNF can regulate neurotransmitter phenotype homeostatically through a K252a-sensitive pathway, most likely via TrkB signaling.

Activity and BNDF interact to specify neurotransmitter fate

To determine whether BDNF is an integration point for Ca^{2+} spike activity-dependent specification of neurotransmitters, we performed simultaneous manipulations of Ca^{2+} activity and BDNF function. Misexpression of hKir2.1 mRNA to suppress Ca^{2+} spike activity generated an increase in the number of glutamatergic neurons and a decrease in the number of GABAergic neurons in the spinal cord. Release of BDNF from beads implanted adjacent to the spinal cord produced the opposite phenotype. Simultaneous suppression of Ca^{2+} activity and application of

BDNF in the spinal cord phenocopied the result obtained by application of BDNF alone (Figure 2.7 A,B). These results indicate that Ca^{2+} spike activity regulates neurotransmitter specification upstream of BDNF function. Given the low frequency and apparently stochastic generation of Ca^{2+} spikes, the non-cell-autonomous coordination of Ca^{2+} spike activity and factors derived from neighboring neurons can drive neurotransmitter specification more reliably than cell-autonomous Ca^{2+} spike activity alone.

DISCUSSION

Increases or decreases in Ca^{2+} spike activity during development of the *Xenopus* nervous system lead to changes in neurotransmitter specification that are generally compensatory or homeostatic. Here we have made several advances in understanding the signal transduction mechanism of transmitter respecification triggered by manipulations of this activity. First, we developed a practical new method to manipulate Ca^{2+} activity in single neurons *in vivo* to determine whether changes are triggered by alteration of activity in single neurons. Second, we found that suppression of activity in single spinal neurons is not sufficient to trigger changes in neurotransmitters, indicating the role of a non-cell-autonomous mechanism. Third, we demonstrated that Ca^{2+} spike activity generated prior to synapse formation may act via the BDNF-TrkB signaling pathway to respecify transmitter phenotype.

Targeting single neurons in vivo

Although delivery of genes to single cells *in vivo* is conceptually and experimentally attractive, it has been challenging to implement due to technical difficulties. Single cell gene targeting is useful to determine whether the effects induced by exogenous or endogenous gene expression in the targeted cell are cell-autonomous or due to interactions with neighboring cells. Ca^{2+} spike frequency homeostatically regulates specification of neurotransmitters in the spinal cord during a brief critical period (Borodinsky et al., 2004; Root et al., 2008a). Here we determined whether the effect on neurotransmitter respecification induced by Ca^{2+} spike activity occurs via a cell-autonomous mechanism.

Several methods have been developed to label small populations of neurons *in vivo*. In *Drosophila* homozygous mutant cells have been targeted by placing a repressible cell marker (MARCM) in trans to a mutant gene of interest using FLP/FRT-mediated mitotic recombination in a Gal4/UAS binary expression system (Lee and Luo, 1999). In mice the Cre/loxP system was applied with double markers (MADM) for simultaneous labeling and mutation of neurons (Zong et al., 2005). Those genetic methods are efficient in labeling small populations of neurons and even single cells in small or inaccessible embryos when blastomere injection is not an option. In *Xenopus* electroporation was used to deliver genes to single neurons in the brain (Hass, 2002), but the transfection efficiency was variable.

We developed a novel practical method to target single neurons efficiently with foreign gene (hKir2.1) expression in *Xenopus* at early developmental stages. Injection of DNA into specific cells of 16-cell-stage blastula effectively drives the expression of that DNA in a mosaic pattern in spinal neurons. mCherry was fused to hKir2.1 to label neurons in which the ion channels are misexpressed. This method is technically convenient for labeling neurons at the single-cell level. The only parameter to be adjusted is the amount of DNA injected into the blastomere, which should be non-toxic as well as sufficient to label single neurons.

Manipulation of Ca^{2+} spikes in single neurons

Using this method, we suppressed Ca^{2+} spikes in single neurons by overexpressing hKir2.1-mCherry. hKir2.1, which has been used to suppress excitability in mammalian superior cervical ganglion cells, hippocampal neurons,

pontine neurons and chick hair cells *in vitro* (Burrone et al., 2002; Holt et al., 1999; Howorth et al., 2009; Johns et al., 1999) as well as suppress neuronal activity *in vivo* in multiple animal models (Borodinsky et al., 2004; Duale et al., 2007; Mizuno et al., 2007; Yu et al., 2004). Overexpression of this channel hyperpolarizes the neuron and decreases its resting membrane resistance. Once the spike threshold is reached, the hKir2.1 current contributes negligibly to the spike waveform. In present study, on the basis of the incidence of Ca^{2+} spiking in internal controls, ~17 of 37 normal neurons are expected to spike during 1 hr of imaging. However, in 37 hKir2.1-mCherry expressing neurons, we observed only 2 such neurons generating spikes during the imaging period, indicating that spikes have been abolished in ~15 neurons (88%) because of the expression of hKir2.1. These data demonstrate that hKir2.1 is highly effective in suppressing Ca^{2+} spikes in single neurons. The residual incidence in 2 neurons may be due to subthreshold expression of hKir2.1. The frequency of spikes in those 2 neurons was much lower than that in internal control neurons and the amplitudes were much smaller. This result suggests that although Ca^{2+} spikes persist in a few of these hKir2.1 expressing neurons, the neurons are still hyperpolarized to some extent. There is no effect on Ca^{2+} spikes from mCherry since mCherry alone expressing neurons showed similar spike pattern both on incidence and frequency.

Role of BDNF in balancing neurotransmitter expression

Suppression of Ca^{2+} spikes in single neurons did not result in an increase in incidence of glutamate expression or a decrease in the incidence of GABA expression, indicating that the transmitter switches observed following global spike suppression in

the entire spinal cord are due to a non-cell-autonomous process. A key determinant appears to be the activity of surrounding neurons, rather than the activity level of the target neuron itself. What is the signal linking activity to the regulation of neurotransmitter expression? BDNF is an attractive candidate. It is expressed early in development. Both its expression and release are tightly regulated by neuronal activity (Gartner and Staiger, 2002; Goodman et al., 1996; Hartmann et al., 2001; Kohara et al., 2001; Thoenen, 2000; Tongiorgi, 2008; Zafra et al., 1990). Furthermore, it has been implicated in a large number of mechanisms that optimize neuronal differentiation and neuronal plasticity (Bi and Poo, 2001; Bibel and Barde, 2000; Huang and Reichardt, 2001; Lindsay, 1996; Lu, 2003; Vicario-Abejon et al., 2002). BDNF enhances the function of inhibitory dopaminergic (DA) synapses. It increases the release of dopamine from amacrine cells in the rabbit retina (Neal et al., 2003). Loss of BDNF from the midbrain-hindbrain adversely affects the amount of tyrosine-hydroxylase present in the striatum and establishment of the proper number of DA neurons in the substantia nigra pars compacta (Baquet et al., 2005). The effects of BDNF on formation and maturation of inhibitory GABAergic neurons or synapses have also been well documented. BDNF enhances GABAergic transmission through presynaptic actions on rat hippocampal neurons (Baldelli et al., 2002; Bolton et al., 2000; Swanwick et al., 2006) and increases the number and density of GABAergic synapses in organotypic slice cultures from rat hippocampus (Marty et al., 2000), in solitary neurons cultured from rat visual cortex (Palizvan et al., 2004) and in hippocampal neurons *in vivo* (Shinoda et al., 2011). BDNF promotes the development of GABAergic neurons in the hippocampus and induces the expression of the GABA

synthetic protein, GAD67 (Yamada et al., 2002). Cultured adult glutamatergic hippocampal granule cells co-expressed GABAergic phenotypes when they were incubated in the presence of BDNF (Gomez-Lira et al., 2005).

Our results show that BDNF can increase the incidence of GABAergic neurons in the spinal cord, in agreement with demonstrations that BDNF scales up inhibitory neuronal networks. Our results also show that BDNF scales down excitatory neuronal networks, decreasing the number of glutamatergic neurons. Exogenous BDNF prevented the enhancement of excitatory synaptic strength normally induced by chronic blockade of activity in cortical cultures (Rutherford et al., 1998). Further, preventing activation of endogenous BDNF receptors mimicked the effects of activity blockade and increased excitatory synaptic strengths. BDNF depressed glutamatergic synaptic transmission in 10% of cultured hippocampal neurons (Lessmann and Heumann, 1998), suppressed spontaneous synaptic activity via non-NMDA receptors in hippocampal cultures (Song et al., 1998), and inhibited AMPA-mediated currents in developing sensory relay neurons (Balkowiec et al., 2000). Thus BDNF scales down the excitability of neural networks. However, chronic treatment with BDNF did not decrease the amplitude of mEPSCs in cultured cortical pyramidal neurons (Leslie et al., 2001), or lead to the enhancement of glutamate-induced current in the postsynaptic cells (Alder et al., 2005; Crozier et al., 1999; Lin et al., 1998; Suen et al., 1997), indicating that in these cases BDNF release either is not essential or enhances excitatory synaptic transmission. These data raise questions about how BDNF-dependent regulation of neuronal plasticity may differ among cell types, developmental stages, and animal models.

We find that shifts in neurotransmitter specification in response to decreases or increases in spike activity can be generated via a BDNF-dependent signaling pathway, most likely via activation of TrkB receptor. However, K252a also blocks TrkA and TrkC, and the extent of effects on the incidence of glutamatergic and GABAergic neurons exerted by K252a is bigger than that exerted by BDNF; thus roles for other neurotrophins are not excluded.

The intracellular pathway responsible for this effect, however, is not revealed by our data. BDNF can induce a transient elevation in intracellular Ca^{2+} concentration by activation of TrkB signaling, dependent on Ca^{2+} release from internal stores (Berninger et al., 1993; Kafitz et al., 1999; Lang et al., 2007) and on Ca^{2+} influx from the extracellular space (Marsh and Palfrey, 1996). Ca^{2+} influx can trigger the expression of GAD and GABA (Gu and Spitzer, 1995; Spitzer et al., 1993; Watt et al., 2000). BDNF upregulates GAD65/67 expression via TrkB to promote GABA expression and appearance of GABAergic neurons (Ohba et al., 2005; Patz et al., 2003; Yamada et al., 2002). Therefore, this mechanism seems likely to contribute to activity-dependent BDNF-mediated transmitter specification. BDNF can also stimulate pathways regulated by MAP kinase, PI 3-kinase and phospholipase-C-gamma by activation of TrkB receptors (Huang and Reichardt, 2003; Segal and Greenberg, 1996). The MAP kinase pathway regulates the phosphorylation of cJun (Pulverer et al., 1991; Whitmarsh and Davis, 2000). cJun acts as a Ca^{2+} spike-dependent repressor of the homeobox gene *tlx3* that functions as a switch specifying the glutamatergic over GABAergic phenotype in *Xenopus* (Marek et al., 2010). BDNF activation of a TrkB signaling cascade may increase cJun phosphorylation and thus suppress *tlx3*

transcription to generate increased numbers of GABAergic neurons and decreased numbers of glutamatergic neurons in the *Xenopus* spinal cord.

Model for non-cell-autonomous regulation of neurotransmitter expression

Neuronal activity plays an important role in regulating many important properties of neurons, such as neuronal migration and synaptic plasticity, either cell-autonomously (Burrone et al., 2002; Tam et al., 2000; Tong et al., 2009) or non-cell-autonomously (Bolteus and Bordey, 2004; Hao et al., 2010; Hartman et al., 2006; Wright and Ribera, 2010). Our study has revealed a non-cell-autonomous mechanism for Ca^{2+} spike activity-mediated homeostatic neurotransmitter respecification in *Xenopus* spinal neurons. We propose a model by which activity determines the transmitter respecification during early stages of development. The pattern is initially specified genetically and then modulated by environmental influences through changes in spontaneous Ca^{2+}-dependent activity. Ca^{2+} spikes regulate the expression and release of BDNF, which initiates the TrkB signaling cascade. Activated TrkB stimulates Ca^{2+} elevations that in turn drive downstream genetic pathways that refine neurotransmitter expression. Parallel pathways may be involved to ensure that the correct transmitter phenotype is established and maintained throughout development.

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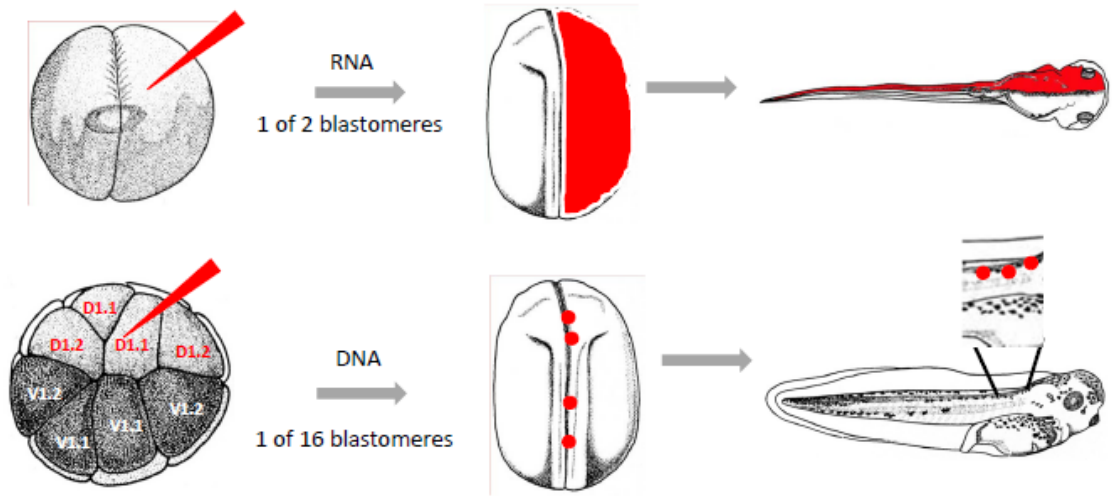

Figure 2.1 Targeting single neurons *in vivo*. Diagram illustrating the two strategies used to target single neurons *in vivo*. Red narrow wedge indicates needles filled with RNA or DNA. Red area and red dots indicate the fluorescent labeled cells expressing desired protein.

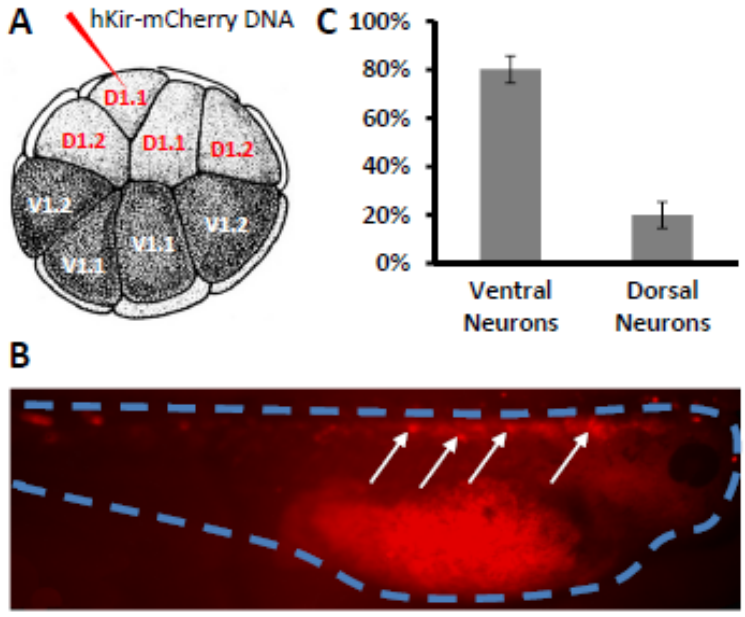

Figure 2.2 Injection of hKir-mCherry DNA into the D1.1 blastomere at the 16-cell stage labels single neurons in a stage 41 embryo. **A.** Diagram illustrating the injection. **B.** Arrows identify some of the labeled spinal neurons of a stage 41 embryo. **C.** Distribution of targeted neurons on ventral side and dorsal side of the spinal cord.

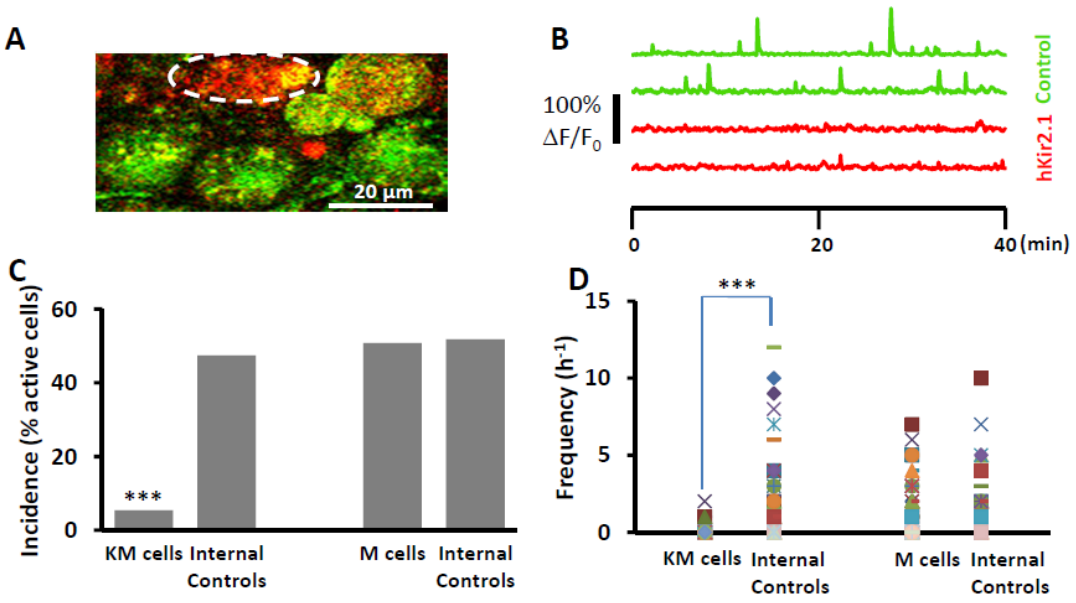

Figure 2.3 Misexpression of hKir-mCherry suppresses Ca^{2+} spike generation in single neurons. A. Confocal image of the ventral spinal cord. Dashed oval identifies a mCherry/Fluo-4 AM-expressing neuron (red and yellow). Neurons loaded with Fluo-4 AM (green) are internal controls. Stage 25. B. Spike activity in hKir2.1-mCherry-expressing neurons and internal controls. Stage 25. C. Spike incidence during one hour imaging periods. Stages 23-25. n≥7 embryos per group. D. Spike frequency during one hour imaging periods. Each symbol is the spike frequency for a single neuron. Stages 23-25. n≥7 embryos per group. KM cells, hKir2.1-mCherry-expressing neurons; M cells, mCherry alone-expressing neurons. ***, $p<0.001$.

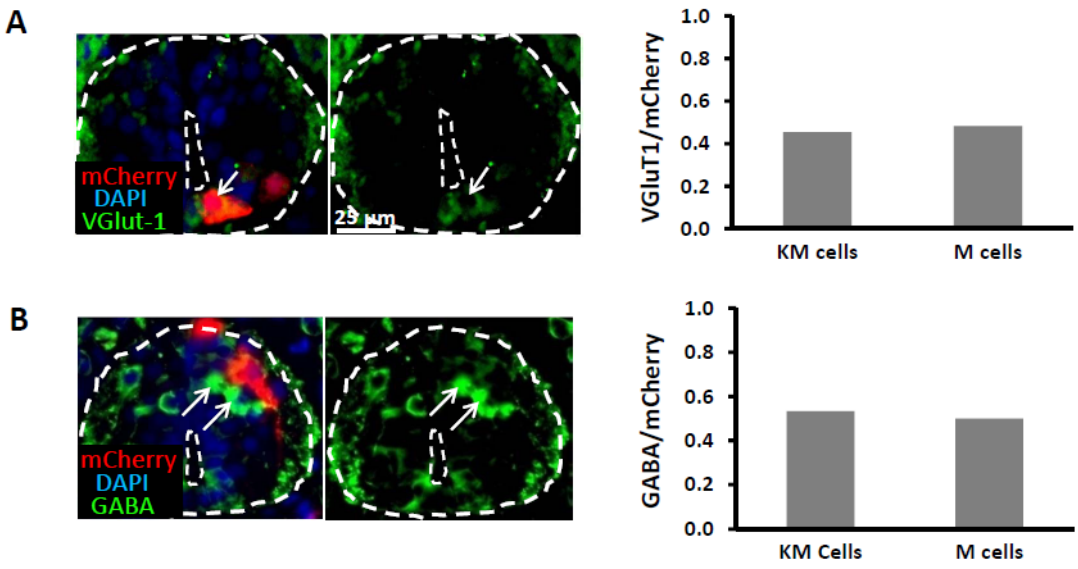

Figure 2.4 Spike suppression in single neurons does not change the identity of their neurotransmitter. **A.** VGlut-1 staining and quantitative analysis of stage 41 larvae from Kir-mCherry DNA- and mCherry DNA-injected embryos. Arrow identifies a VGluT1-immunoreactive neuron. n≥5 embryos per group. **B.** GABA staining and quantitative analysis of stage 41 larvae from Kir-mCherry DNA- and mCherry DNA-injected embryos. Arrows identify GABA-immunoreactive neurons. n≥5 embryos per group. KM cells, hKir2.1-mCherry-expressing neurons; M cells, mCherry alone-expressing neurons. VGluT1/mCherry represents the fraction of mCherry-labeled neurons that are VGluT1-immunoreactive. GABA/mCherry represents the fraction of mCherry-labeled neurons that are GABA-immunoreactive.

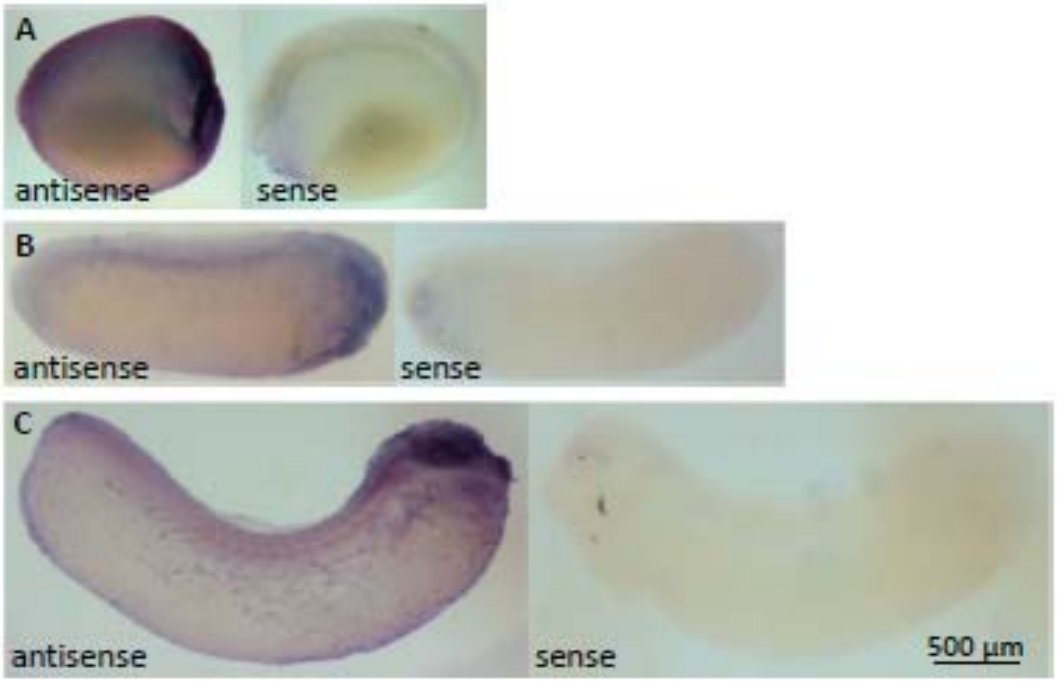

Figure 2.5 *In situ* hybridization using BDNF probe reveals its expression in *Xenopus* developing neural tube. **A.** BDNF expression in embryos of stage18. **B.** BDNF expression in embryos of stage 24. **C.** BDNF expression in embryos of stage 28.

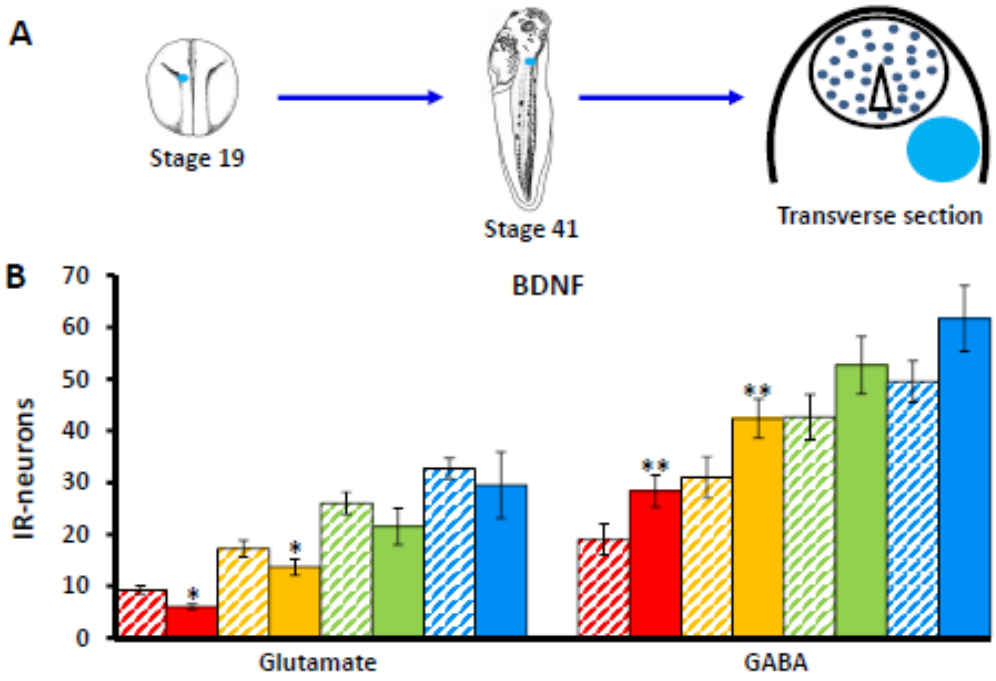

Figure 2.6 BDNF regulates neurotransmitter phenotype via Trk receptors. **A.** Experimental design. Single agarose beads loaded with BDNF, K252a or K252b were implanted adjacent to the nascent neural tube at stage 19 and stage 41 larvae were sectioned for immunocytochemistry. **B.** Glutamate and GABA staining following implantation of beads containing BDNF. Solid columns, beads with 100 ng/ml BDNF. n≥5 embryos per condition. Red, 100 µm from the beginning of the spinal cord; yellow, 200 µm from the beginning of the spinal cord; green, 300 µm from the beginning of the spinal cord; blue, 400 µm from the beginning of the spinal cord. Beads were located within the first 100 µm of the spinal cord. IR, immunoreactive. *, $p< 0.5$; **, $p< 0.01$.

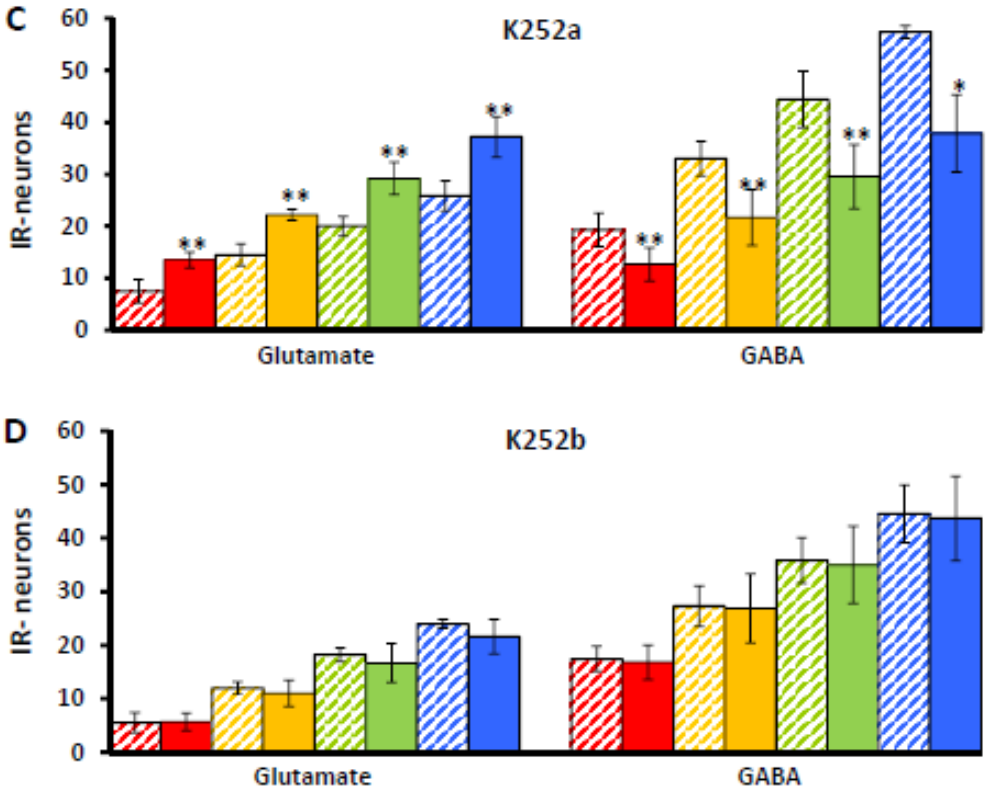

Figure 2.6, continued. BDNF regulates neurotransmitter phenotype via Trk receptors. **C.** Glutamate and GABA staining following implantation of beads containing K252a. **D.** Glutamate and GABA staining following implantation of beads containing K252b, a membrane impermeable analog of K252a. Striped columns, control. Solid columns, beads with 50 μM K252a/K252b. n≥5 embryos per condition. Red, 100 μm from the beginning of the spinal cord; yellow, 200 μm from the beginning of the spinal cord; green, 300 μm from the beginning of the spinal cord; blue, 400 μm from the beginning of the spinal cord. Beads were located within the first 100 μm of the spinal cord. IR, immunoreactive. *, $p< 0.5$; **, $p< 0.01$.

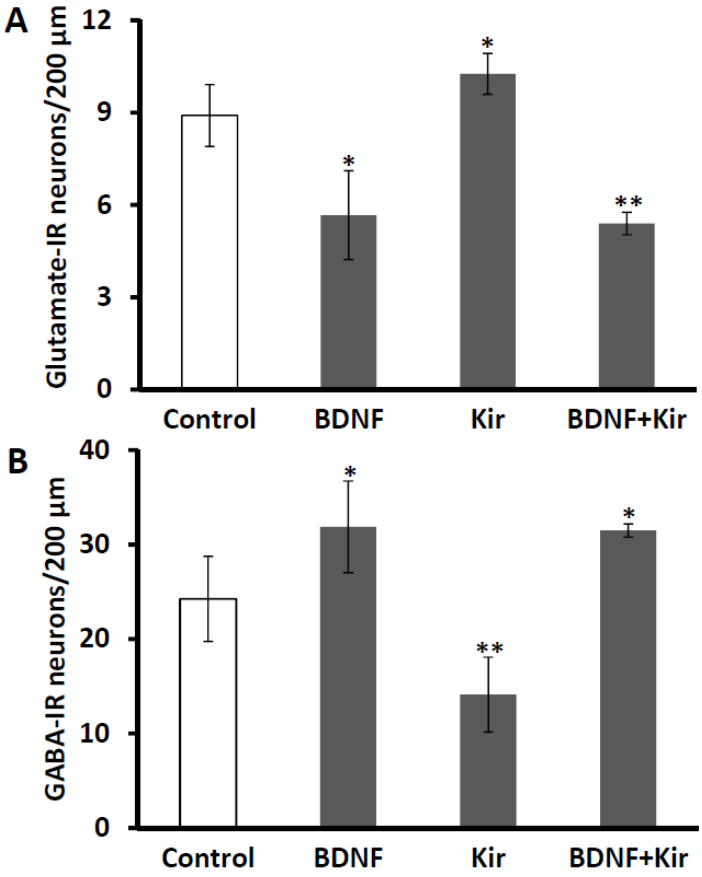

Figure 2.7 BNDF is downstream of activity in the specification of neurotransmitter fate. Combining BDNF delivery and hKir2.1 misexpression (BDNF+Kir) yields the BDNF phenotypes. **A.** Glutamate staining of embryos in presence of BDNF, embryos misexpressing hKir2.1, and embryos misxpressing hKir2.1 in the presence of BDNF. n≥5 embryos per condition. **B.** GABA staining of embryos in presence of BDNF, embryos misexpressing hKir2.1, and embryos misexpressing hKir2.1 in the presence of BDNF. n≥5 embryos per condition. IR, immunoreactive. *, $p<0.5$; **, $p<0.001$.

Chapter 2 is currently being prepared for submission for publication. Non-Cell-Autonomous Mechanism of Activity-Dependent Neurotransmitter Specification in Early Development. Lin X, Spitzer NC. I am the primary investigator and author of this paper.

CHAPTER 3

MUSCLE TARGET REGULATION OF TRANSMITTER EXPRESSION

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 have investigated the impact of muscle targets on transmitter specification in *Xenopus* spinal neurons using a neuron–muscle co-culture system. We find that neuron-muscle contact reduces the number of neurons expressing the non-cholinergic transmitters GABA, glycine, and glutamate, while having no effect on the incidence of ChAT expression. The results indicate that target-dependent regulation can be crucial in establishing neurotransmitter phenotypes.

INTRODUCTION

Establishment of the correct connections is a two way process in which both neurons and their targets communicate extensively with each other to ensure proper alignment. The expression of target-derived neurotrophins and other factors peaks early in neuronal development and declines at later times (Ibanez et al., 1993; Timmusk et al., 1993), suggesting their critical roles in refining neuronal development (Zweifel et al., 2005). Early and influential studies demonstrated that target-derived factors regulate neurotransmitter choice in a subset of mammalian sympathetic ganglion neurons. These neurons switch from a noradrenergic to a cholinergic phenotype after they innervate sweat glands; transplantation experiments showed that this developmental change is triggered and regulated by targets (Francis and Landis, 1999; Furshpan et al., 1976; Landis and Keefe, 1983; Nawa and Patterson, 1990).

Muscle tissue expresses many important trophic factors during development, including NT-3/4/5, BDNF, NGF, glia cell line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), and insulin like factor (ILF), which can act as target-derived factors to refine neuronal development. Survival of motoneurons and establishment of the cholinergic phenotype depend on muscle factors (Bennett and Nurcombe, 1979; Flanigan et al., 1985; Lams et al., 1988; Petruzzelli and Hughes, 1989; Smith and Appel, 1983). Application of trophic factors including NT-4/5, BDNF and GDNF can partially rescue such losses (Fernandes et al., 1998; Kou et al., 1995). Muscle-derived factors are also critical for establishing mature properties of synapses (Liou and Fu, 1997; Nick and Ribera, 2000). These studies demonstrate the importance of target-derived factors in neuronal development.

In this study, we developed a neuron-muscle co-culture system and demonstrated the role of muscle, acting as targets, on neurotransmitter specification. We found that muscle cells suppress non-cholinergic transmitter expression in *Xenopus* spinal cord neurons that grow and make contact with them. In contrast, cholinergic transmitter expression is independent of muscle contact.

MATERIALS AND METHODS

Cell Culture.

Tissue culture plastic dishes (35 mm) were prepared with a ring cut from the mouth of 14 ml polypropylene round-bottom tubes. The area within the ring was divided in half and muscle cells were plated only on one half of the ring area. Mixed cell cultures containing neurons and muscle cells were prepared from *Xenopus laevis* neural tube (stage 20) embryos (Holliday and Spitzer, 1990; Ribera and Spitzer, 1989). Myotomes and neural tube were dissected from embryos using jewelers' forceps and tungsten needles in the presence of 1 mg/ml collagenase (Roche, Basel, Switzerland) and then dissociated in calcium-free medium for 1 hr. Dissociated muscle cells were plated on one side of the dish as a dense lawn. Neurons were plated in stripes across the whole dish, 24 hr after plating muscle cells. Co-cultures were incubated at 22°C and allowed to grow 24 hr after plating neurons before they were fixed and stained. Only cultures with more than 100 neurons on each side were scored (Holliday and Spitzer, 1993). Neuron-alone cultures were prepared as previously described (Borodinsky et al., 2004; Gu and Spitzer, 1995) and incubated for 6 hr and 24 hr.

Immunocytochemistry

Cultures were fixed in 4% PFA, 0.1% or 0.025% glutaraldehyde for 10-15 min at 22°C. Preparations were washed in PBS for 30 min, incubated in a blocking solution of 1% fish gelatin (Sigma-Aldrich, St. Louis, MO) for 0.5 hr at RT, and stained overnight at 4°C using the following antibodies: mouse IgG anti-β-tubulin (Sigma-Aldrich, St. Louis, MO), 1:1000; guinea pig anti-GABA (Millipore Bioscience

Research Reagents, Temecula, CA), 1:1000; rabbit anti-glycine (Millipore Bioscience Research Reagents, Temecula, CA), 1:50; rabbit anti-glutamate (Sigma-Aldrich, St. Louis, MO), 1:10, 000; mouse IgM anti-HNK-1 (Sigma-Aldrich, St. Louis, MO), 1:100. Fluorescent secondary antibodies (Invitrogen, Carlsbad, CA) were used at 1:300 for 1-2 hr at 22°C. Immunoreactivity was examined on a Zeiss Axioskop 40 with a 40× 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 in cell culture.

A 1.5 kb probe cDNA template for ChAT was a generous gift from Dr. Margaret Saha (College of William and Mary, Williamsburg, VA). It was cloned in a PCR4 vector and antisense RNA probe was transcribed using the Megascript Kit (Ambion, Austin, TX) in the presence of digoxigenin-labeled UTP (Roche, Basel, Switzerland). 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 performed at 60°C overnight with a probe concentration of 40 ng/ml. Preparations were washed in 0.2× 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 five times in 0.5% Triton X-100 in PBS and two times in alkaline phosphatase buffer at 22°C and incubated in BM Purple (Roche, Basel, Switzerland) 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.

Statistical analysis.

Means and SEMs were calculated using Microsoft Excel. Statistical analyses were performed using STATA8.0. For comparison between two groups, the Mann-Whitney U test was used. Data are presented as mean±SEM. Results are considered significant when p is <0.05.

RESULTS

Neuron-muscle contact reduces the incidence of noncholinergic neurons.

To investigate the influence of muscle cells on neurotransmitter expression, we designed a co-culture system in which muscle cells dissociated from *Xenopus* embryo of stage 20 were plated onto one half of a culture dish as a dense lawn and spinal neurons dissociated embryos of the same stage were plated across the whole dish in a striped fashion one day later. This stage is prior to the formation of nerve-muscle contacts *in vivo* (Kullberg et al., 1977). This system allowed neurons on the muscle side to form nerve-muscle contacts while neurons on the blank side were free of contacts (Figure 3.1).

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 morphology or by tubulin staining. Neurons expressing the three non-cholinergic neurotransmitters on the muscle side constitute a significantly smaller percentage of the total number of neurons scored when compared to the blank side (Figure 3.2 A, B). Similar results were obtained when neurons were plated immediately after plating muscle cells (data not shown). 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. Because the culture medium does not contain any amino acids, if neurons release these compounds they may be unable to reload their

transmitter pool. Accordingly, we examined the effect of muscle cells on the expression of transmitter-related proteins glutamic acid decarboxylase (GAD) and vesicular glutamate transporter I (VGluT1). The results demonstrate decreased incidence of staining for GAD and VGluT1 on the muscle side of culture dishes, consistent with decreased staining for GABA and glutamate (Figure 3.2 C, D).

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 co-expression. This result is consistent with previous observations of transmitter co-expression 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 scored the number of neurons expressing HNK-1, an intrinsic molecular marker for a subtype of embryonic neurons (Somasekhar and Nordlander, 1997). There was no difference in the percentage of HNK-1-IR neurons between the blank side (55$\pm$1%) and the muscle side (53$\pm$1%). Staining of another molecular marker, islet-1, also revealed no statistically significant difference in its expression in neurons on either side (52$\pm$1% on the blank side and 59$\pm$3% on the muscle side) (Figure. 3.2 B). These results indicate that cell death is unlikely to play a role in reducing the percentage of

non-cholinergic neurons. Comparison of the expression of GABA, glutamate and glycine between neurons cultured 6 hr after being plated and 24 hr after being plated also revealed no difference, demonstrating that muscle cells do not increase the proportion of the GABA-, glycine-, and glutamate-IR cells over the 24 hr period (Figure 3.3).

Neuron-muscle contact has no effect on the incidence of 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. 3.4). Neuronal identity was recognized by morphology and fluorescent immunostaining for neuron specific β-tubulin (data not shown). On both sides of the cultures, ~30% of the neurons express ChAT (28$\pm$2% on the blank side and 31$\pm$1% on the muscle side; not statistically different), suggesting that the number of cholinergic neurons is not affected by the presence of muscle cells. *In situ* signal for ChAT was observed in muscle cells as well, consistent with their release of ACh (Fu et al., 1998).

DISCUSSION

The presence of muscle cells has a striking impact on differentiation of neurons in this co-culture system. Contact with muscle cells significantly suppresses non-cholinergic transmitter expression. Target-derived factors may be playing important roles in this process.

Spinal neurons start to express GABA and glutamate at the neural plate stage in *Xenopus laevis* embryos (Root et al., 2008), well before synapse formation. Accordingly, when spinal neurons are dissociated and subsequently cultured from stage 20 embryos, it is expected that GABA and glutamate are present and that glycine is absent as a transmitter. 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 cells. 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 a new non-cholinergic transmitter (glycine). The expression of the intrinsic molecular markers HNK-1 and Islet-1 does not differ between the two groups of neurons, suggesting that muscle-dependent transmitter specification is independent of cell death. Data obtained at 6 hr after plating demonstrated that muscle cells do not exert their effects by preventing increases in proportion of aminoacidergic neurons.

In vivo, GABA and glutamate expression peaks at the neural tube stage, declining thereafter, and both transmitters 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*. In addition, early tailbud stage (stage 25) neurons that are positive for lim-3 never exhibit glycine-IR. Consequently, neuron–muscle contact may also help to prevent motor neurons from acquiring the wrong transmitter *in vivo*.

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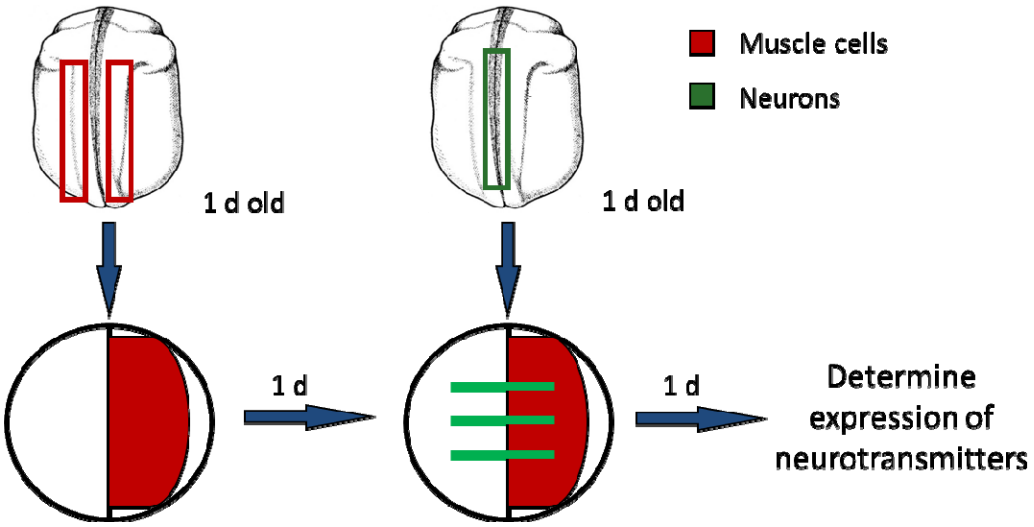

Figure 3.1 Neuron-muscle coculture system. Diagram illustrating the procedure for establishing neuron-muscle cocultures. Areas in which muscle cells are plated are shown in red and areas in which neurons are located are shown in green.

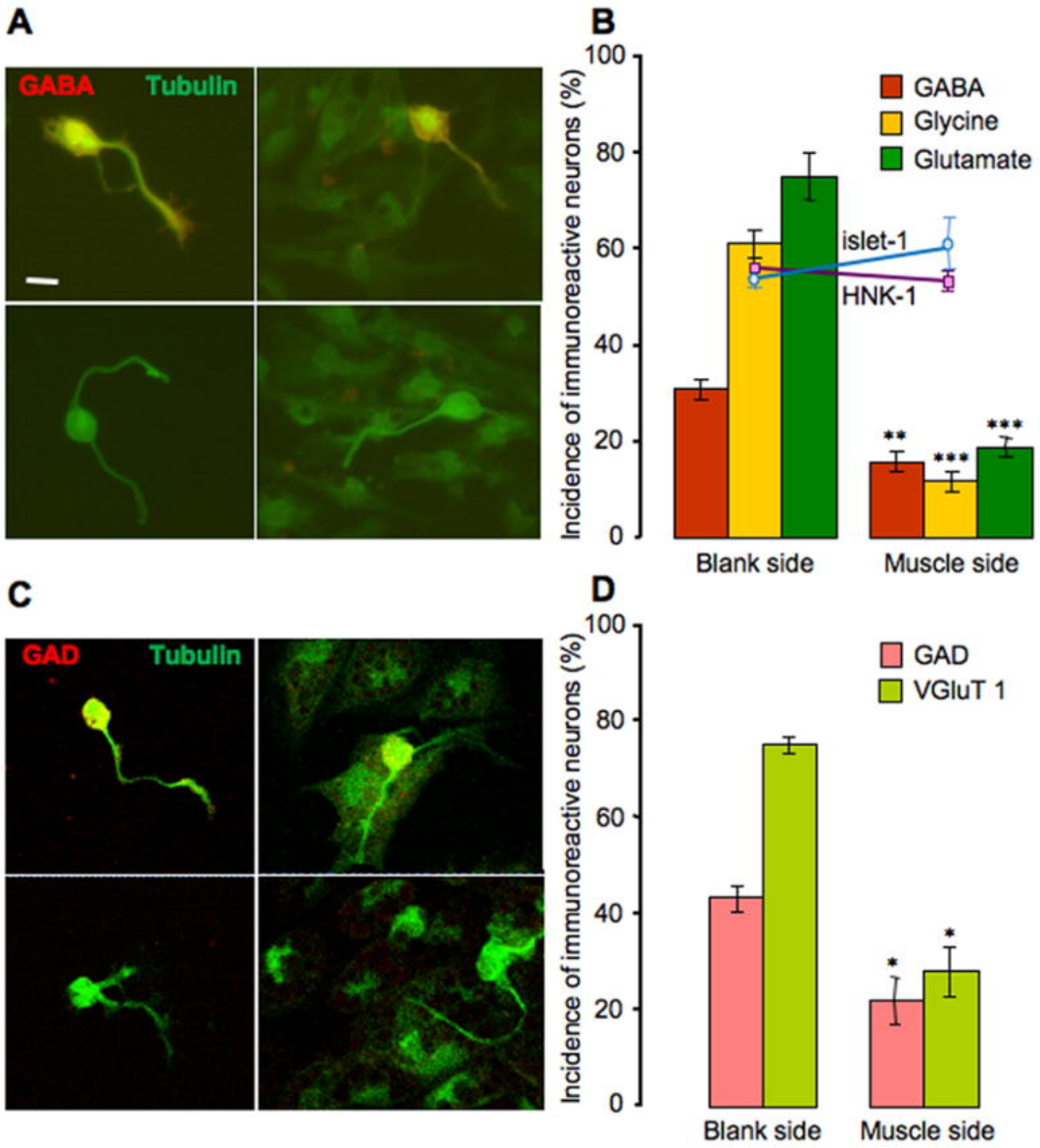

Figure 3.2 Muscle cells suppress noncholinergic neurotransmitter expression. A. Immunostaing of neurons for GABA (red) and tubulin (green) on the blank side (left) and the muscle side (right). Top, positive staining for GABA; bottom, negative staining. B. Noncholinergic transmitter expression is reduced on the muscle side (**p < 0.01, ***p< 0.001, compared with the blank side; n > 8 cultures per transmitter, > 100 neurons per culture). HNK-1 and islet-1 expression do not differ between the two sides of the culture; the y-axis applies to the incidence of HNK-1, islet-1, and transmitter expression. C. Immunostaining of neurons for GAD (red) and tubulin (green) on the blank side (left) and the muscle side (right). Top, positive staining for GAD; bottom, negative staining. D. GAD and VGluT1 expression is reduced on the muscle side (*p < 0.05, compared with the blank side; n > 5 cultures per transmitter marker, > 100 neurons per culture). For A.–D., error bars indicate SEM; scale bar, 10 µm; the Mann-Whiteney *U* test was used to determine statistical significance.

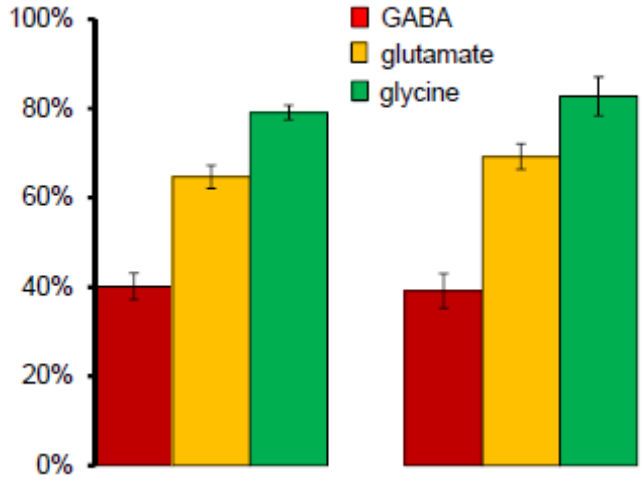

Figure 3.3 Muscle cells do not prevent increases in proportion of non-cholinergic neurons over 24 hr. Dissociated neurons from embryos of stage 20 were scored for GABA, glutamate, glycine-immunoreactive phenotype after being plated for 6 hr (left) and 24 hr (right).

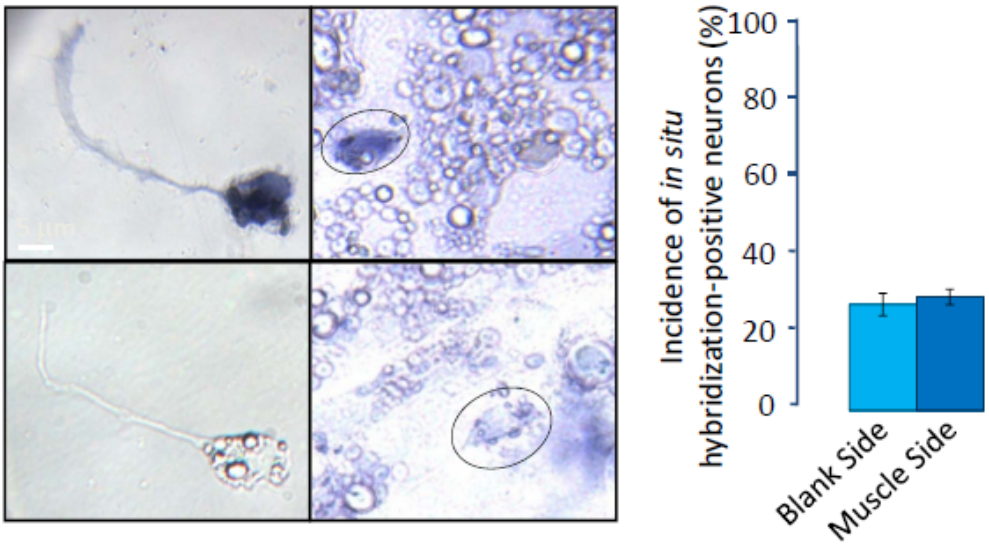

Figure 3.4 Neuronal ChAT expression is independent of muscle regulation. *In situ* hybridization for ChAT on the blank side (left) and the muscle side (right; neurons circled). Top, Positive staining; bottom, negative staining. Scale bar, 10 μm.

Chapter 3, is part of the content that has been published in Journal of Neuroscience, 2010 Apr 21; 30(16):5792-801. Target-dependent Regulation of Neurotransmitter Specification and Embryonic Neuronal Calcium Spike Activity. Xiao Q, Xu L, Spitzer NC. I am a co-author of this paper. I thank Qian Xiao for having me involved in a large part in her thesis project.

CHAPTER 4

SUMMARY AND FUTURE DIRECTIONS

Non-Cell-Autonomous Mechanism of Activity-Dependent Neurotransmitter Specification in Early Development

SUMMARY

I developed a novel, practical single-neuron targeting system to drive expression of human inwardly rectifying potassium channels in single isolated neurons in the *Xenopus* spinal cord *in vivo*, by injecting ion channel DNA into either D1.1/1.2 or V1.2 cells of the 16-cell-blastula. mCherry was fused to the potassium channel gene to label the targeted neurons.

Fusion with mCherry did not affect the ability of potassium channels to suppress calcium spike activity in targeted single neurons. However, neurotransmitter re-specification was not observed in those single neurons, which suggests that neurotransmitter respecification in the spinal cord following global alterations of activity involves a secondary effect from neighboring neurons.

Among all the neurotrophic factors, BDNF and NT-3 and their Trk receptors have been shown to be expressed early in the development of avian embryos, suggesting roles in early neuronal differentiation. More intriguingly, the expression and release of BDNF are regulated by neuronal activity, providing a likely mechanism by which activity can regulate neurotransmitter specification. *In situ* data showed that BDNF is expressed in the spinal cord during the critical period when neurotransmitter expression is most sensitive to changes in Ca^{2+} spike activity. Application of BDNF at the neural plate stage induced changes in neurotransmitter expression similar to those achieved by enhancement of activity. Blockade of BDNF action by blocking its TrkB receptor with K252a induced changes in transmitter expression similar to those

resulting from suppression of activity. Simultaneous application of BDNF and misexpression of inwardly rectifying potassium channels phenocopied the effect of BDNF alone on neurotransmitter expression, suggesting that BDNF signaling works downstream of Ca^{2+} activity to regulate neurotransmitter expression in early development of the *Xenopus* spinal cord.

I conclude that refining the pattern of neurotransmitter expression by Ca^{2+} activity is a non-cell-autonomous process. I propose that activity in surrounding neurons regulates BDNF expression and release that activates a TrkB signaling cascade. Several pathways downstream of TrkB signaling may play parallel roles that converge to activate the expression of neurotransmitter or neurotransmitter-related genes to establish and maintain neurotransmitter specification.

FUTURE DIRECTIONS

Targeting Single Neurons of Specific Classes

Our method of targeting single neurons can roughly aim for neurons on the dorsal or ventral side of the spinal cord by injecting different blastomeres at the 16-cell-stage, but it is not sufficiently accurate to selectively target specific classes of neurons. Different classes of embryonic *Xenopus* spinal neurons generate spontaneous Ca^{2+} spikes at different frequencies (Borodinsky and Spitzer, 2007). Therefore, different classes of neurons may have different sensitivities to perturbation of Ca^{2+} spike activity. Some classes could acquire or change neurotransmitters more readily than others. In mammalian hippocampal neurons, blockade of activity caused GABAergic neurons to lose GAD65 expression and may have reduced the number of

detectable GABAergic boutons (Hartman et al., 2006). In *Xenopus* embryonic dorsal sensory neurons, activity enhancement caused down-regulation of the *tlx3* gene, which decreases the number of glutamatergic neurons and increases the number of GABAergic neurons (Marek et al., 2010). However in *Xenopus* motor neurons, staining for the vesicular ACh transporter (VAChT) endured after activity enhancement. Some axons and presynaptic boutons were strongly stained for GABA, indicating that those neurons acquire an additional neurotransmitter phenotype without losing their original neurotransmitter (Borodinsky and Spitzer, 2007). Some classes of neurons may specify transmitters cell-autonomously while others do so through a non-cell-autonomous mechanism. Some classes of neurons may specify certain types of transmitter cell-autonomously, but specify other types of transmitter non-cell-autonomously. Our data suggest that neurotransmitter specification in spinal neurons involves a non-cell-autonomous mechanism. But the limitation of accuracy of this method cannot exclude the possibility that some small-scale cell-autonomous processes have gone undetected. Manipulation of activity in isolated neurons of specific classes will further elucidate the activity-dependent mechanism that finally defines the neurotransmitter expression pattern in that class.

Based on the single neuron targeting method we developed in this study, there are many ways to further label different subgroups of neurons. On the one hand, molecular markers provide a more objective way of identification and lead to less ambiguity than classifying neurons by position and firing properties. HNK-1 is a membrane glycoprotein specifically expressed on Rohon–Beard (RB) sensory neuron cell bodies and processes (Somasekhar and Nordlander, 1997). Lim-3 is a

transcription factor used as a generic motoneuron (MN) marker in *Xenopus* (Borodinsky et al., 2004), while zebrafish GABAergic interneurons also express lim-3 (Appel et al., 1995; Bernhardt et al., 1992). The Ca^{2+}-binding proteins calretinin (CR) and calbindin (CB) were used to label GABAergic interneurons in the *Xenopus* olfactory bulb (Moreno et al., 2008). Whether they are effective in labeling interneurons in the *Xenopus* spinal cord will have to be determined. On the other hand, the ease of injection of *Xenopus* embryos makes them an ideal system in which to use specific promoters to control the locolization of gene expression. Despite the fact that problems arise because injected DNA is not efficiently incorporated into the genome and, for unknown reasons, promoters on episomes are poorly regulated (Krieg and Melton, 1987), the use of neuron-type-specific promoters to drive the expression of injected DNA is still expected to gain ground. Hb9 works efficiently in driving gene expression selectively in spinal motorneurons in mammalian species and in zebrafish (Arber et al., 1999; Boon et al., 2009; Thaler et al., 1999; Wichterle et al., 2002). Islet-2 facilitates identification of sensory neurons in zebrafish (Pittman et al., 2008). SSICP, an enhancer-promoter fragment from the islet-1 gene, was able to label RB neurons in zebrafish (Higashijima et al., 2000; Jontes et al., 2004). A DNA segment containing a 10 kb region of the GAD67 promoter drives gene expression selectively in basket interneurons in rodent primary visual cortex (Chattopadhyaya et al., 2004). Combining the strength of our single-cell targeting method with either molecular markers or neuron-type-specific promoters will achieve precise targeting, which may be expected to facilitate revealing the mechanisms that specify neurotransmitters in different classes of neurons.

Explore Intracellular Pathways Activated by Extracellular BDNF Responsible for Neurotransmitter Specification

Our data suggest that BDNF is a mediator integrating Ca^{2+} spike activity and neurotransmitter specification. However, the intracellular mechanisms downstream of BDNF signaling have not been determined. BDNF can induce a transient elevation in the intracellular calcium concentration by activation of TrkB signaling, predominantly as a result of calcium release from internal stores (Berninger et al., 1993; Kafitz et al., 1999; Lang et al., 2007). Calcium influx can trigger the expression of GABAergic markers such as GAD (Gu and Spitzer, 1995; Ohba et al., 2005; Patz et al., 2003; Spitzer et al., 1993; Watt et al., 2000; Yamada et al., 2002), and thus promote GABA expression and the appearance of GABAergic neurons. It would be interesting to examine the expression of GAD65/67 after application of BDNF *in vivo*. BDNF can also stimulate pathways regulated by MAP kinase, PI 3-kinase and phospholipase-C-gamma by activation of TrkB receptors (Huang and Reichardt, 2003; Segal and Greenberg, 1996). The MAP kinase pathway has been shown to regulate the phosphorylation of cJun (Pulverer et al., 1991; Whitmarsh and Davis, 2000). In *Xenopus*, Ca^{2+} spikes lead to phosphorylation of cJun that then acts as a repressor of the *tlx3* gene, which functions as a switch specifying glutamatergic over GABAergic phenotype (Marek et al., 2010). Therefore, the BDNF-activated TrkB signaling cascade may increase cJun phosphorylation and thus suppress *tlx3* transcription to generate increased numbers of GABAergic and decreased numbers of glutamatergic neurons in the embryonic *Xenopus* spinal cord. This mechanism could be further

probed by investigation of the expression of phosphorylated cJun and *tlx3* expression following BDNF application *in vivo*.

Explore other possible mediators of non-cell-autonomous activity-dependent neurotransmitter specification

Neurotrophins other than BDNF may play a role in non-cell-autonomous activity-dependent transmitter specification. Although NGF has generally been shown to act as a survival factor for neurons and has limited distribution in the CNS, like BDNF its expression and release are also regulated by neuronal activity (Blochl and Thoenen, 1995; Ernfors et al., 1991; Gall and Isackson, 1989; Zafra et al., 1990). Although NT-3 expression and release are often considered to be regulated by muscle cell activity and independent of neuronal activity (Poo, 2001; Thoenen, 1995), it may be regulated by neuronal activity in some cases (Boukhaddaoui et al., 2001). Furthermore, activation of Trk receptors by NGF and NT-3 is the principal pathway by which they initiate downstream signaling. The effects generated by NGF-TrkA or NT-3-TrkC signaling can be blocked by K252a as well. Thus NGF and NT-3 are interesting candidates as mediators for activity-dependent neurotransmitter specification.

Gap junctions could also mediate activity-dependent neurotransmitter respecification. Gap junctions between neurons serve two distinct functions (Spray, 1996). First, they are readily permeable to small ions, allowing current carried largely by potassium ions to pass directly between neurons and thus function as electrical synapses. This electrical coupling is dependent on ions moving down a potential

gradient. Second, gap junctions are also permeable to metabolites with molecular weights below ~1 kDa. This metabolic coupling is dependent on molecules diffusing down a concentration gradient. Therefore, the amino acid transmitters glycine (75 Da), GABA (103 Da), and glutamate (147 Da) should pass readily through neuronal gap junctions. The elevated levels of glycine in some types of cone bipolar cells arise by metabolic coupling through gap junctions with glycinergic amacrine cells, rather than by direct uptake or synthesis (Vaney et al., 1998). Gap junction channels span two plasma membranes and are formed by the alignment of two hemichannels, each consisting of an oligomer of structural subunit proteins, called connexins (Cxs). The blockade of spontaneous synaptic activity reduces the function of Cx43, the major Cx in astrocytes from embryonic stages, suggesting that neurons may control gap junctions in astrocytes through an activity-dependent mechanism (Rouach et al., 2000). Thus activity might regulate gap junction function to enable passage of neurotransmitters between different groups of neurons.

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APPENDIX

NEURITE OUTGROWTH MEDIATED BY A CAV2.2-LAMININ-BETA2 STOP SIGNAL

INTRODUCTION

During neuronal development, axonal and dendritic processes must distribute appropriately within target tissues. Neurite arbors efficiently innervate sensory surfaces while maintaining discrete spatial resolution in systems including the mammalian retina (Wassle et al., 1981), the body wall of the leech and *Drosophila* (Baker and Macagno, 2007; Grueber et al., 2003), and zebrafish and *Xenopus* skin (Kitson and Roberts, 1983; Sagasti et al., 2005). What is the mechanism responsible for establishment and maintenance of a tiled distribution of neurites? In many cases, contact-mediated repulsion is involved. Particularly, "stop signals" may direct termination or instruct neurites to avoid inappropriate regions.

Calcium signals play key roles in mediating growth cone extension, turning, collapse, and stopping (Spitzer, 2006). Calcium responses to guidance cues are mediated by a diverse repertoire of calcium channels within the growth cone that include transient receptor potential channels and voltage-gated channels (Lipscombe et al., 1988). We screened candidate genes encoding calcium-permeable channels that possibly contribute to early excitability and found Cav2.2, a canonical voltage-gated calcium channel, expressed in growth cones of extending commissural and Rohon-Beard sensory axons at early stages of *Xenopus* development. Cav2.2 not only plays a role in the growth cone of neurites innervating the skin (Nowycky et al., 1985), but is also mechanosensitive (Calabrese et al., 2002) and binds directly to the extracellular matrix molecule laminin β2 (Nishimune et al., 2004).

Laminins are heterotrimeric glycoproteins composed of α, β, and γ chains. The laminin β2 chain is needed for proper synapse assembly (Noakes et al., 1995) and

organizes presynaptic nerve terminals through direct interactions with Cav2.1 and Cav2.2 (Nishimune et al., 2004). Furthermore, at least *in vitro*, laminin β2 acts as a stop signal for outgrowing motor axons (Porter et al., 1995). In the present study, we found that laminin β2 is expressed in *Xenopus* skin. Therefore, we have determined whether laminin β2 interacts with Cav2.2 and generates a stop signal for neurites in *Xenopus*.

MATERIALS AND METHODS

Coating cell culture dishes

Plastic culture dishes (Corning, Corning, NY) were fitted on the bottom with 50 µl volume reduction rings cut from 0.5 ml microtubes (Eppendorf, Hauppauge, NY) and incubated with 200 µg/ml 20 kDa C terminus of laminin β2 leucine-arginine-glutamate (LRE) fragment solubilized by linkage to maltose binding protein or incubated with a mutated version (LRE→QRE (glutamine-arginine-glutamate)) (Nishimune et al., 2004) in 2 mM calcium saline for 2 hr. After rinsing with calcium saline, interleaved bands of native and denatured laminin β2 were generated by placing three electron-microscopic section multislot support grids (RB-90-Ni; Electron Microscopy Sciences, Hatfield, PA) in each dish and exposing dishes to UV illumination (12–15 cm from an 8 W bulb) for 2 hr (Porter et al., 1995). This procedure yields alternating 184-µm-wide bands of native and 90-µm-wide bands of denatured laminin; the boundary is clearly visualized with illumination at 442 nm. Dishes were then coated with 20 µg/ml laminin (Sigma-Aldrich, St. Louis, MO) before addition of cells. Stage 15–17 neural plates were dissected in calcium saline containing 1 mg/ml collagenase-B (Boehringer Mannheim) and then dissociated in calcium-free medium for 1 hr. Neurons were plated rapidly across both native and denatured stripes.

Imaging.

Neurons plated across interleaved bands of native and denatured LRE or QRE were loaded with 5 µM of the Fluo-4 AM calcium indicator (Invitrogen, Carlsbad,

CA)/0.01% Pluronic F-127 detergent (Invitrogen, Carlsbad, CA) in 2 mM calcium saline for 45 min. Neurons were gently washed in calcium saline for 5 min prior to imaging to minimize background fluorescence and imaged as growth cones approached the boundary between denatured and native laminin β2 fragments from the denatured side. Images were captured at 2 Hz on a Leica SP5 confocal microscope (Nussloch, Germany) and analyzed with Image J. Comparison with the grid at the end of acquisition with the grid taken at the beginning of acquisition confirmed that the dish had not moved during the time that the movie was shot.

Analysis.

Cultured neurons were scored into cross-border and stop-at-border categories respectively. $n > 80$ growth cones were calculated for each group. Statistical analyses were performed using STATA 8.0. Significance was assessed with Fisher's exact test. Values are considered different when $p < 0.05$.

RESULTS

To determine whether neurites initially grown on a control substrate stall or stop when their growth cones encounter laminin β2, we generated 184-μm-wide stripes of native and 90-μm-wide stripes of UV-denatured laminin β2 LRE or QRE (Figure appendix 1) (Porter et al., 1995), plated neurons on them, and analyzed the behavior of growth cones migrating from denatured onto native laminin β2. We imaged intracellular calcium with Fluo-4 AM to assess calcium dynamics as growth cones palpate the border. Growth cones stalled at the LRE border generated calcium transients 10–70% above baseline fluorescence that were 1 s to 2 min in duration at half maximal amplitude and correlated with the contact of the border by one or more filopodia (Figure appendix 2. A) ($n = 9$ of 9 growth cones at the border). These transients were observed at a significantly lower frequency at the QRE border (Figure appendix 2. B) ($n = 2$ of 6 growth cones at the border; $p < 0.05$, Fisher's exact test). The calcium transients associated with LRE contact are distinct from the other classes of calcium transients described for these growth cones (Conklin et al., 2005; Gomez et al., 2001; Gu and Spitzer, 1995). Fifty-seven percent of growth cones stopped or turned back at the LRE border, whereas only 9% of growth cones stopped or turned at the QRE border (Figure appendix 2. C, D) ($p < 0.0001$; $n = 80$ growth cones; Fisher's exact test).

DISCUSSION

Growing neurons on stripes of native and denatured laminin β2 LRE demonstrates that neurites already growing on a denatured laminin β2 substrate are inhibited by initial contact with an active laminin β2 substrate. This initial contact appears to be signaled through the growth cone by a calcium transient.

Laminin β2 has been described as a stop signal for motor neurite outgrowth (Porter et al., 1995). A stop signal may function to indicate that neurites have reached their targets where they are to form synapses or terminal endings (Becker et al., 2000; Dimitropoulou and Bixby, 2005; Poskanzer et al., 2003). Alternatively, it may inhibit a neuron from growing into non-target regions. Behaviorally, this may lead to hypersensitivity to light touch and/or mislocalization of sensory signals.

The calcium dependence of laminin β2-Cav2.2 neurite inhibition raises the intriguing possibility that laminin β2 may activate Cav2.2 or Cav2.1, the other presynaptic calcium channel to which it binds. However, channel properties of Cav2.1 heterologously expressed in human embryonic kidney (HEK) cells are not changed by presentation of the laminin β2 LRE fragment (Nishimune et al., 2004). There are several mechanisms by which laminin β2 may produce a change in the calcium signal through Cav2.2 that would affect neurite outgrowth but that would be undetected in recordings from HEK cells. First, Cav2.2 and Cav2.1 may require an additional molecule not present in HEK cells to be activated by laminin β2. Second, laminin β2 may serve to cluster Cav2.2 to a microdomain in growth cones at which it can effectively transduce a calcium signal. Indeed, laminin β2 clusters Cav2.2 at the

mammalian neuromuscular junction (Nishimune et al., 2004). Similar changes in distribution of Cav2.1 HEK cells might not lead to changes in the current recorded. Third, the involvement of Cav2.2 in generating a stop signal may rely on the mechanosensitivity of this channel (Calabrese et al., 2002). The interaction of the moving growth cone expressing Cav2.2 channels with the static LRE motif of laminin β2 may constitute an adequate stimulus for channel activation that is absent from Cav2.1 expressed in non-motile HEK cells. Indeed, calcium influx through other mechanosensitive channels within the growth cone inhibits neurite outgrowth (Jacques-Fricke et al., 2006).

We propose a model in which neurite growth is normally restricted by a stop signal generated by laminin β2-Cav2.2, which is most likely calcium-dependent.

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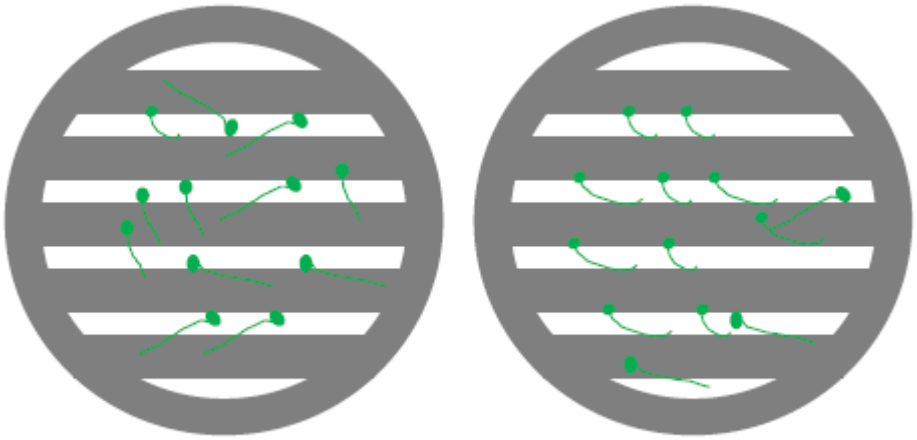

Figure appendix 1 Neurons plated on stripes of native and stripes of denatured laminin β2 QRE or LRE. Neurons are shown in green. Laminin β2 is underneath the entire grid area. The grey stripe area is protected from UV exposure by 184 μm-wide grid stripe and generates native laminin β2. The white stripe area is exposed to UV light and laminin β2 is denatured.

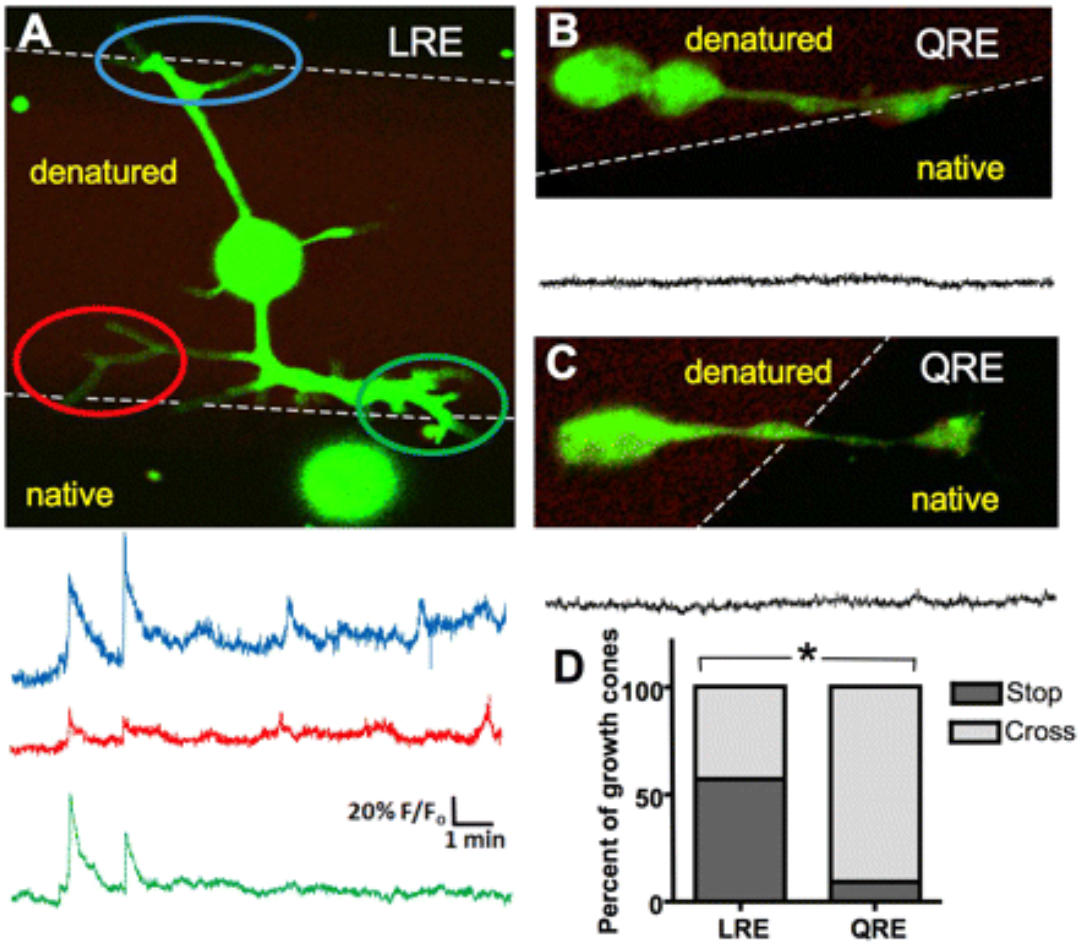

Figure appendix 2. Neurons at a border of laminin β2 generate calcium transients and stall. **A**, Neurons were cultured on stripes of native and UV-denatured laminin β2 LRE fragment and internal calcium concentrations were imaged using Fluo-4. Images were captured at 2 Hz. Fluo-4 is in green, denatured laminin β2 is in red, and native laminin β2 is in black. Three images were averaged to enhance visualization of the neuron. Traces show the calcium activity in each of three growth cones as they encounter the border. Axis scales apply throughout the figure. **B**, **C**, Less calcium transient activity is seen when a neuron approaches (**B**) or has crossed onto (**C**) activated point mutated laminin β2 (LRE→QRE). **D**, Significantly more neurons stop or turn back at an LRE border than a QRE border (*$p < 0.001$).

The appendix is part of the content that has been published in the Journal of Neuronscience, 2008; 28(10):2366-2374. Neurite Outgrowth and in vivo Sensory Innervation Mediated by a CaV2.2-laminin β-2 Stop Signal. Sann SB, Xu L, Nishimune H, Sanes JR, Spitzer NC. I am a co-author of this paper. I thank Sharon Sann for having me involved in a large part of her thesis project.