UNIVERSITY OF CALIFORNIA, SAN DIEGO

Neuronal Development: Roles of Calcium Signaling

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Doctor of Philosophy

in

Neurosciences

by

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Chair

University of California, San Diego

2007
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ABSTRACT OF THE DISSERTATION

Neuronal Development: Roles of Calcium Signaling

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Within a neuron, calcium can depolarize the membrane to initiate or propagate an action potential and can serve as a second messenger to influence gene transcription or cytoskeletal arrangement. In these capacities, calcium plays diverse roles during neuronal development. Here I describe novel roles that calcium plays in neurotransmitter specification, neurite outgrowth, and sensory innervation.

Neurotransmitters are essential for interneuronal signaling, and the specification of appropriate transmitters in differentiating neurons has been related to intrinsic neuronal identity and to extrinsic signaling proteins. Altering the distinct patterns of Ca$^{2+}$ spike activity spontaneously generated by different classes of neurons.
embryonic spinal neurons in vivo changes the transmitter that neurons express without affecting the expression of markers of cell identity. Regulation seems to be homeostatic: suppression of activity leads to an increased number of neurons expressing excitatory transmitters and a decreased number of neurons expressing inhibitory transmitters; the reverse occurs when activity is enhanced. The imposition of specific spike frequencies in vitro does not affect labels of cell identity but again specifies the expression of transmitters that are inappropriate for the markers they express, during an early critical period. These results identify a new role of patterned activity in development of the central nervous system.

Axons and dendrites of developing neurons establish distributed innervation patterns enabling precise discrimination in sensory systems. I describe the role of the extracellular matrix molecule, laminin β2, interacting with the Cav2.2 calcium channel in establishing appropriate sensory innervation. In vivo, Cav2.2 is expressed on the growth cones of Xenopus laevis sensory neurites and laminin β2 is expressed in the skin. Culturing neurons on a laminin β2 substrate inhibits neurite outgrowth in a specific and calcium-dependent manner. Blocking signaling between laminin β2 and Cav2.2 leads to increased numbers of sensory terminals in vivo. These findings suggest that interactions between extracellular matrix molecules and calcium channels regulate connectivity in the developing nervous system.
I. Introduction
Calcium signaling in early development

Early neuronal development can be divided into three stages. During proliferation, neuronal precursors generate large numbers of neurons. Cells then migrate to appropriate locations to establish the scaffold of structure that will eventually underlie neural circuits. Finally, neurons differentiate, acquiring the morphology, transmitter expression, and connectivity that will allow them to perform their unique functions. Calcium signaling plays key roles in each of these phases of development.

During proliferation, calcium waves through radial glia in the ventricular zone promote cell division\(^1\). This calcium activity occurs in response to purinergic signaling\(^1,2\). In contrast, GABA- and glutamate-induced calcium activity within the ventricular zone decreases proliferation\(^3\), indicating that multiple sources of calcium signaling function together to regulate the rate of proliferation.

During neuronal migration, calcium signaling regulates the motility of cerebellar granule cells\(^4\). Increased calcium influx through N-type calcium channels or NMDA receptors leads to faster migration whereas blocking these channels slows migration\(^5,6\). Similarly, calcium influx following NMDA or GABA\(_A\) receptor activation promotes migration of hippocampal pyramidal cells\(^7\). In neural progenitors of the subventricular zone, GABA\(_A\) receptor activation disrupts internal calcium signaling and slows the rate of migration along the rostral migratory stream\(^8\).
During differentiation, neurons acquire their distinctive morphology, make connections, and establish a neurotransmitter phenotype that enables them to function in circuits. Axon pathfinding is an important part of this differentiation process and is regulated by calcium signaling within the growth cone\textsuperscript{9,10}. Spontaneous calcium waves in growth cones of \textit{Xenopus} spinal neurons slow neurite outgrowth \textit{in vitro} and \textit{in vivo}\textsuperscript{11,12}. Similarly, calcium signaling through the L-type calcium channel in growth cones of hamster cortical neurons slows neurite outgrowth\textsuperscript{13}. In contrast, nicotinic acid adenine dinucleotide phosphate increases release of calcium from internal stores within the growth cone and promotes neurite extension\textsuperscript{14}. Calcium signaling also mediates growth cone adhesion\textsuperscript{15} and turning. Different substrates evoke differing frequencies of filopodial transients in growth cones of \textit{Xenopus} spinal neurons and growth cones turn away from the side of the growth cone with greatest filopodial calcium activity\textsuperscript{16}. Turning in response to soluble molecules such as netrin and myelin associated glycoprotein also requires calcium\textsuperscript{17,18}. Whether a calcium signal stimulates or inhibits growth or acts as an attractant or a repellent may depend on the amplitude of the signal relative to an optimal set point\textsuperscript{19,20}, or the ratio of cAMP to cGMP\textsuperscript{21}. Calcium responses to guidance cues are mediated by a diverse repertoire of calcium channels within the growth cone that include TRP channels and voltage-gated channels\textsuperscript{21-25}. 
Calcium signaling and neurotransmitter phenotype

Neurons signal to each other through the use of over 50 different neurotransmitters. Appropriate specification of neurotransmitter phenotype is required for a neuron to communicate with its postsynaptic partner and for the function of neural circuits.

Extensive work demonstrates the role of intrinsic genetic programs in specifying neurotransmitter phenotype. Ectopic expression of the homeobox transcription factor MNR2 leads cells to express a motoneuron phenotype, including expression of choline acetyl transferase, the synthetic enzyme for acetylcholine. Similarly, the Unc-30 homeodomain protein regulates development of GABAergic neurons in *C. elegans* and the *islet* homeodomain protein is responsible for serotonergic and dopaminergic phenotypes in the *Drosophila* ventral nerve cord. While in these cases a single transcription factor is able to induce a particular neurotransmitter phenotype, transmitter specification can occur through the action of several transcription factors using a combinatorial code. For example, the homeodomain transcription factor Dbx2 specifies GABAergic fate, but GABAergic identity is then suppressed by Dbx1. Ectopic co-expression of Islet1 and Lhx3 induces a cholinergic phenotype. In the *Drosophila* ventral nerve cord, two groups of multiple transcription factors distinguish between FMRFamide and Npl1 peptidergic phenotypes.

Extrinsic factors within the environment surrounding a neuron also affect neurotransmitter phenotype. Rat sympathetic neurons grown alone in culture are
noradrenergic and have been demonstrated to synthesize, store, release, and uptake norepinephrine. However, when the sympathetic neurons are grown in the presence of cardiac myocytes, some of the neurons become cholinergic\textsuperscript{32}. Within the media conditioned by cardiac myocytes are multiple cytokines that affect expression of substance P, somatostatin, and vasoactive intestinal polypeptide-related peptides as well as acetylcholine\textsuperscript{33}. The cholinergic phenotype is also induced by factors in the sweat glands of the rat foot pad, and developing rat sympathetic neurons undergo a switch from noradrenergic to cholinergic phenotypes \textit{in vivo}\textsuperscript{34}. While the factor responsible for inducing this switch to cholinergic phenotype remains unidentified, it functions through the same receptor on sympathetic neurons that binds to cytokines that induce a cholinergic phenotype including leukemia inhibitory factor, ciliary neurotrophic factor, and cardiotrophin-1\textsuperscript{35}.

The effects of extrinsic factors on neurotransmitter phenotype may be mediated by calcium. Induction of the cholinergic phenotype in sympathetic neurons by cardiac myocytes is blocked by depolarizing the neurons\textsuperscript{36} and this effect is mediated by calcium and cyclic adenosine monophosphate\textsuperscript{37}. Embryonic \textit{Xenopus} spinal neurons exhibit spontaneous calcium spike activity, and the frequency of calcium spiking affects the GABAergic phenotype in culture\textsuperscript{11,38}. Increased frequency of spiking leads to greater numbers of GABA-immunoreactive neurons in culture as well as upregulation of GAD 67 transcripts. Chapter 2 describes experiments that demonstrate the role of patterned calcium activity in homeostatically regulating neurotransmitter expression \textit{in vivo}. Different classes of neurons exhibit distinct
patterns in frequency of calcium signaling. Increasing calcium spike activity leads to an increased expression of inhibitory transmitters and a decreased expression of excitatory transmitters. Suppressing activity has the opposite effect, leading to greater numbers of neurons that are glutamatergic and cholinergic and decreasing the number of GABAergic and glycineergic neurons. This developmental plasticity occurs during a critical period corresponding to the period of calcium spiking and electrophysiology demonstrates that neurotransmitter switches are functional.

**Screen for novel calcium signals**

Proliferation, migration, axon pathfinding, and neurotransmitter expression are all regulated by calcium signaling. These observations prompted me to ask what other neuronal phenotypes are regulated by early forms of calcium-dependent activity. In order to identify novel forms of calcium signaling that mediate embryonic development, I searched for calcium channels that may underlie early excitability. Large amounts of movement occur throughout embryonic development, including morphogenesis, cell migration, and axon outgrowth. This leads to the idea that movement may serve as a cue for developmental decisions. Mechanosensitive channels could serve to sense the movement of a cell or its environment and signal this information to the cell. For this reason, I focused my search on mechanosensitive calcium channels.

The appendix describes the channels selected for study. Chapter 3 briefly describes the expression patterns of each of these channels based on RT-PCR and *in
situ hybridization. Due to the expression pattern of Ca\textsubscript{v}2.2 in the developing neural tube, I focused my further investigations on this channel. Particularly, I have demonstrated a role for signaling through this channel upon binding to the extracellular matrix molecule laminin β2 in regulating neurite extension and sensory innervation.

**Role of laminin β2 in development**

Laminins are heterotrimeric glycoproteins composed of an α, β, and γ chain. Currently 5α, 3β, and 3γ chains have been identified\textsuperscript{39}. The laminin β2 chain is localized to the synaptic but not extrasynaptic regions of the basal laminae of the neuromuscular junctions\textsuperscript{40}, as well as the retina\textsuperscript{41}, and subplate and floorplate of the developing central nervous system\textsuperscript{42}. The laminin β2 chain is needed for proper synapse assembly\textsuperscript{43,44} and organizes presynaptic nerve terminals through direct interactions with Ca\textsubscript{v}2.1 and Ca\textsubscript{v}2.2\textsuperscript{45}. Furthermore, laminin β2 acts as a guidance signal for outgrowing motor axons\textsuperscript{46}. I have tested whether the laminin β2 interactions with Ca\textsubscript{v}2.2 are responsible for this laminin β2 guidance signal, or “stop signal”\textsuperscript{46}. A stop signal may function to indicate that neurites have reached their targets where they should form synapses or terminal endings\textsuperscript{47,48}. Alternatively, a stop signal may inhibit a neuron from overgrowing into non-target regions\textsuperscript{49}. As described in chapter 3, I have found that a laminin β2 – Ca\textsubscript{v}2.2 stop signal restricts neurite growth to maintain appropriate distribution of sensory innervation.
Development of sensory innervation

During neuronal development, axonal and dendritic processes must distribute appropriately within target tissues. In sensory systems, this distribution establishes cellular receptive fields that allow accurate perception of stimuli. In *Xenopus*, electron microscopy has revealed that Rohon-Beard sensory neurites initially run along the inner surface of the skin, continue for an undetermined length between the two layers of skin cells and form sensory varicosities in pits in the lateral or basal plasma membrane between superficial skin cells. Most of these varicosities occur in clusters in an en passant manner but some are terminal. The most heavily innervated skin cell type is the conical cell also termed intercalating non-ciliated cell. Electrophysiological studies of receptive fields of Rohon-Beard neurons estimate from the size of receptive fields and the numbers of Rohon-Beard neurons that the skin is covered between 1.5 and 2.5 times, although this may be an overestimate due to a recording bias for larger cells with larger receptive fields. In order for the animal to make correct judgments about the location of stimuli, sensory neurites must innervate correct target regions, in both *Xenopus* and other systems.

Neurite arbors efficiently cover sensory surfaces while maintaining discrete spatial resolution. In the mammalian retina, on-center and off-center ganglion cells thoroughly tile the retina surface with minimal overlap with dendritic fields of the same cell type. In the body wall of the leech, cone cells send out non-overlapping processes that serve as a scaffold for myocyte migration. In *Drosophila* larvae, class IV sensory neurons completely cover the body wall without overlapping each other.
In the larval *Xenopus* head, contact-mediated repulsion prevents trigeminal ganglia axons from crossing the midline\(^{56}\). In zebrafish trigeminal ganglia, the axons of 50% of neuron pairs repel each other but the remaining 50% cross each other with no response to the presence of the other neuron, suggesting that the distribution of dendrites may rely on other factors\(^{57}\). The leech cone cells, *Drosophila* type IV cells, *Xenopus* trigeminal neurons, and some zebrafish trigeminal and Rohon-Beard neurons maintain tiled distribution by contact mediated repulsion\(^{54,57,58}\). However, in other cell types sensory distribution occurs by separate or additional mechanisms\(^{55,57,59,60}\). Growth of sensory neurites is mediated by neurotrophic factors such as BDNF\(^{61}\) and NT-4\(^{62}\) and guidance molecules such as ephrins\(^{63}\). Branching and tiled patterning of sensory neurites relies on the NDR (nuclear Dbf2-related) kinase family in both like-repels-like tiling\(^{64}\) and non-contact mediated distribution\(^{59}\). Other mechanisms of maintaining dendritic distribution may include intrinsic limitation of neurite growth or instruction of neurite distribution by pre-existent tiled patterns of extracellular matrix molecules within target tissue\(^{65}\). Particularly, “stop signals” may direct termination or instruct neurites to avoid inappropriate regions.

Chapter 3 describes the role that a Cav2.2 – laminin β2 stop signal plays in neurite outgrowth and in establishing sensory innervation. Cav2.2 is expressed in the growth cone of developing spinal neurons and laminin β2 is expressed in a tiled pattern in the skin. *In vitro*, laminin β2 – Cav2.2 interactions specifically inhibit neurite outgrowth in a calcium – dependent manner. Blocking this calcium signal *in vivo* leads to an increase in sensory innervation of the skin.


II. Activity-dependent homeostatic specification of transmitter expression in embryonic neurons
Activity-dependent homeostatic specification of transmitter expression in embryonic neurons

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Neurotransmitters are essential for interneuronal signalling, and the specification of appropriate transmitters in differentiating neurons has been related to intrinsic neuronal identity and to extrinsic signalling proteins. Here we show that altering the distinct patterns of Ca\(^{2+}\) spike activity spontaneously generated by different classes of embryonic spinal neurons in vivo changes the transmitter that neurons express without affecting the expression of markers of cell identity. Regulation seems to be homeostatic: suppression of activity leads to an increased number of neurons expressing excitatory transmitters and a decreased number of neurons expressing inhibitory transmitters; the reverse occurs when activity is enhanced. The imposition of specific spike frequencies in vitro does not affect labels of cell identity but again specifies the expression of transmitters that are inappropriate for the markers they express, during an early critical period. The results identify a new role of patterned activity in development of the central nervous system.

The determination of neuronal phenotypes is a substantial developmental challenge, given the complexity of the nervous system. The classical low-molecular-mass, peptide, gaseous and growth-factor neurotransmitter number 50 or more. The appearance of a particular transmitter in a given class of neurons is a crucial step in differentiation because it enables the neurons to communicate with others with which they make synaptic connections. Expression of an incorrect transmitter could isolate neurons from their normal networks. The absence of synaptic signalling could also reduce trophic support from its postsynaptic partner, leading to neuronal death.

Several classes of mechanism regulate transmitter expression. Specification by intrinsic transcription factors has been demonstrated by the ectopic expression of MNDR2 or Lhx3/Id1 in the chick spinal cord, which drives inappropriate expression of the motor neuron transmitter acetycholine in interneurons. In mice that are mutant for the Drox1 transcription factor, neural progenitors from the Drox1 domain give rise to interneurons with an inappropriate GABergic phenotype. Gain-of-function and loss-of-function experiments with homeodomain transcription factors in Caenorhabditis elegans and Drosophila lead to the misexpression of GABA and a loss of synthesis of dopamine and serotonin. Cytokines and neurotrophic factors can also regulate transmitter expression and can drive the expression of acetylcholine instead of noradrenaline in rat sympathetic ganglion neurons, both in culture and in vivo. Additionally, the imposition of activity can regulate the choice of neurotransmitter in cultured neurons by means of Ca\(^{2+}\) influx and can differentially affect the regulation of transmitter expression by protein factors. The incidence of neurons expressing the transmitter GABA and its synthetic enzyme, glutamic acid decarboxylase (GAD), is upregulated in cultured embryonic spinal neurons by increasing the frequencies of Ca\(^{2+}\) spikes that mimic endogenous spontaneous activity.

Here we have examined the role of electrical activity and Ca\(^{2+}\) influx in the specification of neurotransmitter expression in the developing spinal cord of Xenopus laevis embryos. This is an attractive system to study because it contains only eight classes of neurons that collectively express four classical transmitters. Imaging neurons of four of these classes, we find they generate distinct patterns of Ca\(^{2+}\) spikes in vivo shortly after neural tube formation, starting before the synapse formation that enables network activity. We show that suppressing or enhancing this activity in the neural tube, either by misexpression of K\(^+\) or Na\(^+\) channels or with pharmacological antagonists and agonists, alters the expression of acetylcholine and glutamate without affecting markers of neuronal identity. In a homeostatic manner, spike suppression increases the incidence of excitatory transmitter expression and decreases the incidence of inhibitory transmitters. Conversely, enhancing spike production decreases the expression of excitatory transmitters and increases the expression of inhibitory transmitters. Cell cultures permit the imposition of specific spike frequencies, and we find that transmitter expression in vitro is dependent on Ca\(^{2+}\) spike frequency. We demonstrate transmitter release from neurons that is inappropriate for the markers they express, indicating that transmitter switches are functional. Finally we show that the effects of activity on transmitter specification are restricted to a critical period at this early stage of development.

Neuron-specific patterns of spike activity in vivo

To determine patterns of neuronal activity in the embryonic spinal cord, we imaged spontaneous Ca\(^{2+}\) spikes in dorsal sensory Rohon–Beard neurons (RB), dorsolateral interneurons (DLI), ventral motoneurons (MN) and ventral interneurons (VI) (Fig. 1a, c). We classified neurons on the dorsal surface of the neural tube as dorsomedial and dorsolateral neurons by their positions. Neurons located along the midline of the dorsal spinal cord are sensory RB neurons; whole-mount immunocytochemistry with antibodies against HNK-1—a membrane glycoprotein specifically expressed on RB cell bodies and processes—confirmed this identity (Fig. 1b).

We classified neurons on the ventral surface of the neural tube on the basis of the presence of coactivity. Ventral neurons that fired in concert were considered to be MNs, whereas neurons that did not spike together with others were designated VI. The clusters of MN identified by Ca\(^{2+}\) spike coactivity (Fig. 1c) and Supplementary Fig. 1 colocalized with neurons immunostained with limb-3 transcription factor in the ventral neural tube (Fig. 1d). All limb-3-immunoreactive neurons stained for choline acetyltransferase (ChAT), a generic MN marker, and vice versa (see below). Zebrafish VeL interneurons express limb-3 and are GABA-immunoreactive, but we observe no GABA immunoreactivity in limb-3 neurons. These
results indicate further that at the Xenopus developmental stages evaluated, lim-3 is expressed only in MNs. During a 10-h developmental period after closure of the neural tube, the incidence of spike activity increases for RB, DL1, and MN but decreases for VI. The frequency patterns of spikes are different for RB (low and constant), DL1 (monotonically increasing), MN (step from low to high) and VI (high throughout) (Fig. 1c, d). These observations indicated that neurons might be recognizable by signatures of activity as well as by their position and expression of molecular markers, and raised the possibility that activity drives neurotransmitter phenotype. This notion led to the hypothesis that perturbing these patterns of activity would alter the specification of neurotransmitters.

**Channel overexpression alters transmitter phenotype**

To examine the role of activity in neuronal differentiation, we suppressed Ca²⁺ spikes by overexpression of human inward rectifier K⁺ channels (hKir2.1) and later assessed the presence of neurotransmitters immunocytochemically (Fig. 2a). Imaging embryonic neural tubes loaded with the voltage-sensitive indicator bisoxonol revealed that unilateral expression of hKir2.1 causes the hyperpolarization of neurons only on the ipsilateral side. Imaging with fluo-4 demonstrated ipsilateral suppression of spontaneous spikes (Fig. 2b, c). Bilateral expression hyperpolarized neurons and silenced spikes on both sides of the neural tube. Glutamate immunoreactivity (Glu-IR) and glutamate vesicular transporter immunoreactivity (VGluT-IR), and choline acetyltransferase

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**Figure 1.** Ca²⁺ spike activity of four classes of neurons imaged in the embryonic spinal cord. **a.** Active Rohon–Beard neurons (RB, continuous circles) and dorsolateral interneurons (DL1, dashed circles) on the dorsal surface of a stage 23 neural tube; insets illustrate spike activity for cells indicated by arrows during a 1-h period. **b.** Fluorescence increase above baseline. **c.** Whole-mount immunoreactivity for HNK-1 identifies RBs on the dorsal surface of the same preparation. Dashed white lines indicate the margins of the neural tube. **d.** Coactive motor neurons (MN, continuous circles) and ventral interneurons (VI, dashed circles) on the ventral surface of a stage 24 neural tube; insets illustrate spike activity for cells indicated by arrows during a period of 1 h; scale as in a, d. **e.** Whole-mount immunoreactivity for lim-3 identifies MNs on the ventral surface of the same preparation. Profiles of nuclei are of different sizes as the result of the through-scan projection of a small stack of images and differences in nuclear orientation. **f.** Incidence of Ca²⁺ spiking percentage active cells for these neurons during three developmental periods. **g.** Frequency of Ca²⁺ spikes (spikes h⁻¹) for these neurons, excluding neurons that were silent during the imaging period. In e and f, n > 10 embryos were used for each period. Values are means ± s.e.m. and asterisks indicate significant difference from stages 20–22. Dotted columns, stages 20–22; hatched columns, stages 23–25; solid columns, stages 26–28. The neural tube is about 100 μm in diameter at all stages.

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**Figure 2.** Suppression of spike activity in vivo by overexpression of inward rectifier K⁺ channels increases the incidence of expression of glutamatergic and cholinergic phenotypes. **a.** Experimental design. hKir2.1 transcripts and fluorescent tracer were injected together into one or both blastomeres at the two-cell stage. Ca²⁺ imaging was performed on stage 22–26 neural-tube embryos (boxed region) and stage 40 larvae were sectioned for immunocytochemistry. **b.** Neural tube resulting from unilateral injection of transcripts plus tracer (left), loaded with bisoxonol (Blox); to image membrane potential (right), reveals that dorsolateral neurons containing transcripts are hyperpolarized (cells are pseudocoloured blue; n = 7 neural tubes). White dashed lines indicate margins of the neural tube. **c.** Neural tube resulting from unilateral injection of transcripts plus tracer (left), loaded with fluo-4 acetoxyethyl ester to image spikes (middle), reveals that spikes in dorsal neurons are suppressed on the side containing transcripts (active cells are circled). The incidence of spiking is reduced in both dorsal and ventral neurons marked with tracer (n = 15 dorsal and ventral neural tubes). Dotted columns, control; hatched columns, Kir2.1 unilateral; solid columns, Kir2.1 bilateral. **d.** Neural-tube sections from control embryos stained for glutamate (Glu) or the vesicular glutamate transporter (VGluT) in combination with HNK-1, and choline acetyltransferase (ChAT) in combination with lim-3. **e.** Embryos unilaterally silenced; f. bilaterally silenced and stained as in d. While dashed ovals indicate neural-tube diameters in both and subsequent figures. Numbers of immunoreactive neurons per 100 μm of neural tube are indicated beneath panels. For unilaterally silenced embryos these numbers are tabulated separately for each side of the neural tube. In e–f, values are means ± s.e.m. and asterisks indicate significantly different from control.
immunoreactivity (ChAT-IR), are normally observed only in RB and MN identified by HNK-1-IR and lim-3-IR, respectively (Fig. 2d). Strikingly, Glu-IR and VGluT-IR were present in HNK-1 cells expressing bKir2.1 either unilaterally or bilaterally (Fig. 2e, f). In contrast, ChAT-IR was present in lim-3 cells only after bilateral (and not unilateral) injections. Unilateral suppression might allow commissural axon projections from the contralateral side\(^{19}\) to prevent the expansion of the cholinergic phenotype. The numbers of HNK-1 and lim-3 cells were not affected by either unilateral or bilateral suppression of activity, and the cellular organization of the neural tube seemed normal (Supplementary Fig. 2). These results indicate that suppression of activity might lead to the spread of glutamatergic and cholinergic phenotypes.

To test whether increases in Ca\(^{2+}\) spike frequency suppress the incidence of Glu-IR and ChAT-IR, we overexpressed voltage-gated rat brain Na\(^+\) channels (rNa,2a0.3 and rNa,2a0.7). Both the incidence of spiking neurons and spike frequency were increased as a result (Fig. 3a–c). Glu-IR and VGluT-IR were decreased after overexpression of rNa,2a0.7 both unilaterally and bilaterally, and ChAT-IR was decreased in cells after bilateral increases in spike activity (Fig. 3d, e). Unilateral expression of Na\(^+\) channels caused a decrease in activity in contralateral, unlabelled V1 that might result from commissural axon projections. Consistent with this level of activity there was a significant increase in ChAT-IR in lim-3 cells on the contralateral side. In summary, suppression and enhancement of activity thus seem to exert opposing effects on expression of these excitatory transmitters.

**Pharmacological intervention alters transmitter phenotype**

To check the results of channel overexpression and to focus the manipulation of Ca\(^{2+}\) spikes on the period when this activity is manifested after closure of the neural tube, a pharmacological approach was adopted. We implanted agarose beads\(^{19}\) immediately before the time of tube closure, loaded either with Ca\(^{2+}\) spike blockers\(^{22}\) or with veratridine to activate Na\(^+\) channels and increase Ca\(^{2+}\) spike activity (Fig. 4a). The effectiveness of these agents was demonstrated by the suppression of spontaneous activity by the blockers and its enhancement by veratridine (Fig. 4b, c), with no effect of bovine serum albumin (BSA, control) when applied in the bath during imaging experiments. After implantation (Fig. 4d), Glu-IR and ChAT-IR were bilaterally increased when beads contained blockers, suppressed when beads contained veratridine (Fig. 4e, f), and unaffected when beads contained BSA. The

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**Figure 3** Enhancement of spike activity in vivo by overexpression of voltage-gated Na\(^+\) channels decreases the incidence of glutamatergic and cholinergic phenotypes. rNa,2a0.3 transcripts and fluorescent tracer were injected together into one or both blastomeres at the two-cell stage, followed by imaging and immunocytochemistry as in Fig. 2. a, Neural tube after unilateral injection of transcripts plus tracer (left) and fluo-4 imaging (right) shows that spike frequency is enhanced in dorsal neurons containing transcripts; spiking cells are circled. b, Spike incidence in dorsal and ventral neurons after unilateral or bilateral injection of transcripts and tracer. c, Spike frequency in RB, DL, MN and VI marked with tracer. In b and c, n ≥ 10 dorsal and ventral neural tubes were analysed. Dotted columns, control; striped columns, Na,2a unilateral, solid columns, Na,2a bilateral. d, e, Embryos with unilateral and bilateral enhancement of activity, stained for glutamate or VGluT in combination with HNK-1 for ChAT in combination with lim-3. For each condition, n ≥ 5. For unilaterally silenced embryos these numbers are tabulated separately for each side of the neural tube. In b-e, values are means ± s.e.m. and asterisks indicate significantly different from control.
difference between the effects of unilateral channel overexpression and unilateral bead implantation could be due to the diffusion of agents in the latter case. The results of these pharmacological perturbations confirm and extend the results of channel overexpression and demonstrate an inverse relationship between Ca\(^{2+}\) spike activity and the expression of excitatory transmitters.

**Activity regulates transmitter phenotype homeostatically**

To test the idea that transmitter specification is regulated homeostatically by Ca\(^{2+}\) spike activity, we investigated the expression of GABA and glycine. These transmitters are inhibitory for most of these embryonic Xenopus spinal neurons, although they are excitatory in other developing systems. Decreases in expression of Gly-IR and GABA-IR accompanied the increases in Glu-IR and ChAT-IR after bilateral suppression of activity (Fig. Sa, b). The increased incidence of excitatory transmitters seemed to lead to coexpression with inhibitory transmitters in some cases. In others, it was correlated with the disappearance of glycine and GABA, indicating the possible replacement of inhibitory by excitatory transmitters. In a reciprocal manner, the decrease in Glu-IR and ChAT-IR after bilateral enhancement of activity was accompanied by an increase in the incidence of GABA\(^{2-}\) and glycine\(^{2-}\) neurons relative to controls. Overexpression of Na\(_{a,2a}\beta\) led to the appearance of RB immunoreactive for HNK-1 and GABA or glycine, in the presence or absence of Glu-IR. Similarly, we observed MN immunoreactive for lim-3 and glycine or GABA, in the presence or absence of ChAT-IR (Fig. 5c). These findings imply homeostatic regulation of transmitter expression by activity, overriding other developmental cues. Downregulation of activity seems to stimulate the production of excitatory transmitters in inhibitory neurons and to suppress the expression of inhibitory transmitters in neurons that normally express them. Upregulation of activity leads to an increased incidence of neurons expressing inhibitory transmitters and to the expression of inhibitory transmitters in neurons normally expressing excitatory transmitters.

**Spike frequency regulates transmitter expression in vitro**

To determine more precisely the effect of different Ca\(^{2+}\) spike frequencies on neurotransmitter expression, we examined neurons in low-density dissociated cell cultures in the absence of synaptic connections. Suppression of spike activity during development in vitro increased the incidence of expression of Glu-IR and ChAT-IR, paralleling observations in vivo, without affecting the number of neurons expressing RB and MN markers of cell identity (HNK-1 and lim-3; Fig. 6a, b). We then imposed various Ca\(^{2+}\) spike frequencies on differentiating neurons and examined Glu-IR, VGluT-IR and ChAT-IR. The expression of HNK-1 and lim-3 was constant across the range of frequencies of imposed Ca\(^{2+}\) spikes, whereas the incidence of Glu\(^{2+}\)/VGluT\(^{2+}\) or ChAT\(^{2-}\) neurons varied inversely with frequency. Low frequencies enhanced the expression of these transmitters, and high frequencies suppressed their expression (Fig. 6c–e). The developmental progression of spike frequencies observed in the neural tube (Fig. 1f) was most effective in enhancement. These findings are consistent with results in vivo and provide further information about the spike activity patterns that are sufficient to regulate transmitter expression. However, transmitter expression also depends on factors related to cell identity over a wide range of the frequencies tested, because cells immunopositive for HNK-1 or lim-3 were preferentially immunoreactive for glutamate or ChAT. Activity overrides this dependence on intrinsic cell identity at the higher Ca\(^{2+}\) spike frequencies. The balance of excitatory and inhibitory neurotransmitters changed with the level of spike activity: low activity (0 mM Ca\(^{2+}\), spikes absent) stimulated increases in excitatory and decreases in inhibitory transmitters. In contrast, high activity (2 mM Ca\(^{2+}\), spikes present at 10 Hz) stimulated increases in inhibitory and decreases in excitatory transmitters (Fig. 6h). These results are consistent with the
increased incidence of expression of GABA and xGAD 67 with increasing spike frequency and indicate that activity-dependent regulation of transmitter expression might be cell autonomous.

**Demonstration of transmitter release**

Given the changes in incidence of Glu-IR and ChAT-IR when neurons are subjected to different patterns of spike activity, we then tested whether neurons are able to release these transmitters. When acetylcholine-receptor-expressing myoballs were brought into contact with neuronal growth cones, we recorded spontaneous synaptic currents (SSC) that were blocked by curare but not by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). We used blastomere

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**Figure 5** Suppression or enhancement of spike activity in vivo causes homeostatic superposition or replacement of one transmitter with another. A. Controls doubly stained for VGluT or ChAT (red, excitatory) plus glycinergic or GABAergic (purple, inhibitory) illustrate the normal distribution of immunoreactivity. B. Embryos in which spike activity was bilaterally suppressed by expression of HNK-1, stained as in A. Pink indicates coexpression of excitatory and inhibitory transmitters. C. Embryos in which spike activity was bilaterally enhanced by expression of Na,2ax3, stained for glutamate or ChAT and the respective marker of cell identity (HNK-1 or lim-3, green), plus glycine or GABA. Light blue denotes coexpression of marker and inhibitory transmitter, white shows coexpression of marker and both excitatory and inhibitory transmitters. Bottom, number of neurons immunoreactive for different transmitters per 100 μm of neural tube, means ± s.e.m.; n = 5 embryos for each condition. In B and C, hatched columns indicate number of neurons coexpressing both neurotransmitters. Asterisks indicate significantly different from control.
injection of rGluR2 messenger RNA to generate myoballs expressing glutamate receptors; when these myoballs were brought in contact with growth cones, we recorded SSCs that were blocked by CNQX but not by curare. The absence of cholinergic SSCs in this condition might have been due to downregulation of AChR by overexpression of GluR2. The percentages of neurons generating glutamatergic and cholinergic SSCs when cultured in medium containing 0 or 2 mM Ca\(^{2+}\) (Fig 7; \(n \geq 10\) ) were not distinguishable from the incidence of Glu-IR or ChAT-IR (Fig 6a, b), indicating that these altered phenotypes are functionally expressed. A parallel analysis of evoked synaptic currents elicited by the electrical stimulation of neurons yielded similar percentages (data not shown). The results can account for the high incidence of neuromuscular junctions in nerve–muscle cocultures\(^2\).

**Critical periods for regulation by Ca\(^{2+}\) spikes**

Regulatory roles of activity during neuronal development are often confined to critical periods\(^8,9\). To determine whether this is true of transmitter expression, we suppressed or imposed spikes for various times in vitro to identify the minimal stimulation required to achieve changes in incidence of transmitter expression. Five-hour periods of perturbation beginning near the time at which Ca\(^{2+}\) spikes are first expressed are sufficient to increase or decrease the incidence of Glu-IR or ChAT-IR to the same extent as that attained after 12 h. These seem to be critical periods, because the reversal of stimulation or deprivation after 5 h is ineffective in reversing the changes during the periods tested (Fig 8).

**Discussion**

Our results indicate that specific patterns of Ca\(^{2+}\) spike activity are necessary for normal expression of neurotransmitters in neurons in the embryonic spinal cord, because disrupting these patterns alters transmitter expression. Neuronal death or neurogenesis seems unlikely to contribute to these results because the numbers of RB and MN identified by HNK-1 and lim-3 do not change in vivo or in vitro. Ca\(^{2+}\) spike activity might also be sufficient to drive the expression of particular transmitters, because patterns of stimulation that more closely parallel the in vivo activity of RB or MN generate an increasing proportion of neurons expressing glutamate or ChAT in vitro. However, a large percentage of these neurons are immunoreactive for both glutamate and ChAT, and the mechanism by which neurons are led to express a single classical transmitter during normal development in vivo remains to be determined. Candidates include regulation by transcription factors, more subtle distinctions among patterns of spontaneous activity, the presence of synaptic activity, and signal transduction by means of protein factors. Natural changes in spike activity patterns could have a role in the restriction of the extensive early expression of GAD and

**Figure 6** Regulation of spike frequency in vivo drives novel expression of neurotransmitters.

- a. Examples of Glu\(^{+}\)HNK-1\(^{-}\) and Glu\(^{+}\)HNK-1\(^{-}\) phenotypes, and HNK-1\(^{-}\) (green) and Glu-IR in 2 mM Ca\(^{2+}\) (white) or after suppression of spike activity with 0 mM Ca\(^{2+}\) (solid gray). Culture medium with bis-(o-aminoephophenoxymethyl)-

- b. Examples of Chat\(^{+}\)Im-3\(^{-}\) and Chat\(^{+}\)Im-3\(^{-}\) phenotypes, and lim-3\(^{-}\) (green) and Chat-IR in 2 mM Ca\(^{2+}\) (white) or after suppression of spike activity as in a. Antibodies indicate significantly different from 0 mM Ca\(^{2+}\). c. Staining for Glu (circles), VGlUT (triangles) and HNK-1 (squares) as a function of frequency of imposed Ca\(^{2+}\) spikes. HNK-1\(^{-}\) neurons are Glu\(^{+}\)VGlUT\(^{+}\) at frequencies of 6 h \(^{-1}\) or lower. Bullets indicate the effect of stimulation with the RB pattern of spikes (Fig 8). Inset, examples of simulated spikes at 3 and 10 h \(^{-1}\).

- d. Staining of ChAT (circles) and lim-3 (squares) as a function of frequency of imposed Ca\(^{2+}\) spikes. lim-3\(^{-}\) neurons are Chat\(^{+}\) at frequencies of 15 h \(^{-1}\) or lower. Bullets indicate the effect of stimulation with the MN pattern of spikes (Fig 8). Inset, examples of simulated spikes at 6 and 25 h. e. Proportions of Glu\(^{+}\)Chat\(^{+}\) doubly stained neurons when cultured in 2 mM Ca\(^{2+}\) or 0 mM Ca\(^{2+}\) medium (indicated by 2 or 0, respectively). The percentage of neurons doubly labelled for glutamate and ChAT is consistent with predictions from a-d. f. Neurons doubly labelled for excitatory (red) and inhibitory (purple) transmitters; pink indicates coexpression. g. Incidence of neurons expressing excitatory and inhibitory transmitters when grown in 2 mM Ca\(^{2+}\) or 0 mM Ca\(^{2+}\) culture medium (indicated by 2 or 0, respectively). For a-e and g, values are means ± s.e.m. for at least 5 cultures containing \(n \geq 40\) neurons.
GABA<sub>2</sub>. The coexpresssion of several transmitters in single embryonic neurons after particular patterns of activity might provide the basis for transmitter coexpression in mature neurons<sup>23</sup>.

Shifts in transmitter expression in response to decreases or increases in spike activity seem to be directed towards the homeostasis of network excitability in the nervous system<sup>38</sup>. This process is conceptually similar to the homeostatic resetting of neuronal excitability and synaptic strengths that provides an important balance to hebbian plasticity<sup>39,40</sup> and to the homeostatic plasticity of excitatory networks that renders spontaneous output resistant to disruption of connectivity<sup>41,42</sup>. It was thus surprising to observe this process in neurons in dissociated cell culture in the absence of synaptic connections, in turn implying that the feedback loop might be intracellular. This regulatory programme indicates that abnormal electrical activity could regulate neurotransmitter expression. Although high frequencies of Ca<sup>2+</sup> spikes generated in paediatric epilepsy can be detrimental to brain development, seizure activity seems to regulate the expression of neurotransmitter homeostatically to suppress this activity. Mossy fibres normally generate excitatory glutamatergic postsynaptic potentials on hippocampal CA3 pyramidal cells, but generate inhibitory GABAergic postsynaptic potentials after kindled seizures<sup>43</sup>.

The effects of activity on transmitter specification probably operate through dynamic interplay with the molecular context provided by transcription factors, but the rules that govern this interaction are still unclear. For example, are the peptide, gaseous and growth-factor transmitters specified in the same way as the classical transmitters, or are these other categories of transmitters regulated differently? What is the range of transmitters that can be expressed in an activity-dependent manner? Can transmitters that are not normally expressed be synthesized in spinal neurons in response to particular patterns of activity, or are switches restricted to the population of classical transmitters normally present in the network? Additionally, it will be of interest to ascertain whether the time frames of action of transcription factors and activity are coextensive or the effects of one ultimately outweigh the other.

We propose a model in which the hierarchical expression of transcription factors defines fields of cells<sup>36–38</sup> that express constellations of ion channels. These channels produce patterns of activity that are modulated by protein factors. This modulated activity further engages transcription factors that stipulate transmitter expression. The transmitter that is specified then depends on the transcription factors expressed by the postmitotic neuron, the appropriate type of activity, interaction with signalling proteins, and further transcriptional regulation. This scheme enables the integration of genetic coding with signals that stimulate spike activity or the secretion of factors. A consequence of this proposal is that knockouts of neuronal class-specific transcription factors might not lead to transmitter switches unless they are involved in programming electrical activity<sup>39</sup>. Moreover, the robustness of transmitter phenotype in cells dissociated and grown in culture<sup>44</sup> implies the preservation of patterns of activity as observed in this study. Consistent with this model is the observation that misexpression of a lim-class homeobox gene in epidermal cells leads to ectopic expression of a putative voltage-dependent Na<sup>+</sup> channel gene normally expressed in motor neurons<sup>45</sup>, and neurotrophins modulate Ca<sup>2+</sup> and K<sup>+</sup> channels<sup>46</sup>. The signal transduction cascades by which low frequencies of spikes exert these effects are likely to engage RNA synthesis<sup>3,9</sup> by means of activity-dependent transcription factors<sup>39,40</sup>.

Our results lead to several predictions. On the one hand, activity-dependent changes in transmitter expression can be expected to affect axon guidance. Growth cones of developing Xenopus neurons release transmitter spontaneously<sup>47</sup> and turn in response to acetylcholine, glutamate and GABA<sub>2</sub><sup>48,49</sup>. In agreement with this view is the finding that the depletion of transmitters in vivo alters axon outgrowth<sup>40</sup>. On the other hand, neurons rerouted by novel neurotransmitter expression can be expected to make synaptic connections with novel postsynaptic partners. Embryonic Xenopus neurons express multiple classes of transmitter receptors<sup>46</sup>, providing key components necessary for the formation of functional connections. Alternatively, the expression of postsynaptic receptors might be regulated homeostatically in parallel with the regulation of transmitter expression. However, mismatches between presynaptically released transmitter and postsynaptically expressed receptors seem likely to arise. It will be of interest to determine whether the resulting neuronal networks promote behavioural homeostasis.

**Methods**

**Imaging**

Neuronal tubes dissected from three embryonic epochs<sup>47</sup> and dissociated cell cultures prepared from neural-plate-stage embryos<sup>50</sup> were loaded with 1 μM fluor-4-acetylmethyl azol or 1 μM bisfenol and imaged were acquired at 0.1 Hz for 1 h periods with a BioRad MRC1024 laser confocal system. Spikes were stimulated at different frequencies in vitro by culturing neurons in 250 μM Ca<sup>2+</sup>-free saline medium by using a volume reducer and continuously superfusing them with this medium at 2.5 ml min<sup>-1</sup>. The composition of saline was automatically switched for 15–20 s by computer-controlled solenoid valves (General Valve Corp.) to a solution containing 100 mM KCl and 2 mM Ca<sup>2+</sup>.

**Molecular biology and pharmacology**

SK12.1, eNav2a1d and eGlur2 were gifts from E. Marban, W. Catterall and S. Heinemann. The genes were subcloned into a Bluescript vector and complementary DNA was
Supplementary Figure 1 Coactively spiking clusters of MNs in the embryonic neural tube. a, active neurons on the ventral surface of a stage 24 neural tube (circled), imaged for a 1 hr period. Position and number of coactive cells corresponds to lim-3 staining (Fig. 1d). The neural tube is 100 µm in diameter. b, incidence of coactive clusters during early development. n>5 neural tubes. c, size of coactive neuron cluster. n=3 neural tubes.
Supplementary Figure 2 The cellular organization of the neural tube appears normal following bilateral suppression of Ca$^{2+}$ spike activity. Phase contrast images (left) and immunofluorescence images (right) of cryostat sections. a, from a control embryo. b, from an embryo following bilateral expression of Kir2.1 (indicated by tracer). Both stage 40 embryos were immunostained for VGluT.
III. Neurite outgrowth and *in vivo* sensory innervation mediated by a CaV2.2 – laminin β2 stop signal
Axons and dendrites of developing neurons establish distributed innervation patterns enabling precise discrimination in sensory systems. We describe the role of the extracellular matrix molecule, laminin β2, interacting with the CaV2.2 calcium channel in establishing appropriate sensory innervation. *In vivo*, CaV2.2 is expressed on the growth cones of *Xenopus laevis* sensory neurites and laminin β2 is expressed in the skin. Culturing neurons on a laminin β2 substrate inhibits neurite outgrowth in a specific and calcium-dependent manner. Blocking signaling between laminin β2 and CaV2.2 leads to increased numbers of sensory terminals *in vivo*. These findings suggest that interactions between extracellular matrix molecules and calcium channels regulate connectivity in the developing nervous system.

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 such as the mammalian retina\(^1\), the body wall of the leech and *Drosophila*\(^2,3\), and vertebrate trigeminal ganglia and sensory Rohon-Beard neurons\(^4,5\). In some cases, a tiled distribution is maintained by contact mediated repulsion\(^2,5-7\) whereas in other cases separate or additional mechanisms are involved\(^3,5,8,9\). These may include intrinsic limitation of neurite growth or instruction of neurite distribution by preexistent tiled patterns of extracellular matrix molecules within target tissue\(^10\). 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\textsuperscript{11,12}. Elevations in intracellular calcium upon growth cone contact with soluble guidance molecules\textsuperscript{13,14} or extracellular matrix molecules\textsuperscript{15,16} can lead to inhibition of growth or neurite avoidance. Calcium responses to guidance cues are mediated by a diverse repertoire of calcium channels within the growth cone that include TRP channels and voltage-gated channels\textsuperscript{17-21}. We describe the expression and role of Ca\textsubscript{v}2.2, a canonical voltage-gated calcium channel\textsuperscript{22}, in the growth cone of neurites innervating the skin. Ca\textsubscript{v}2.2 is notable among voltage-gated calcium channels in that it is also mechanosensitive\textsuperscript{23} and binds directly to the extracellular matrix molecule laminin β2\textsuperscript{24}.

Laminins are heterotrimeric glycoproteins composed of an α, β, and γ chain. Currently 5α, 3β, and 3γ chains have been identified in the mouse and human genomes\textsuperscript{25}. The laminin β2 chain is localized to the synaptic but not extrasynaptic regions of the basal laminae of the neuromuscular junctions\textsuperscript{26}, as well as the retina\textsuperscript{27} and subplate and floorplate of the developing central nervous system\textsuperscript{28}. The laminin β2 chain is needed for proper synapse assembly\textsuperscript{29,30} and organizes presynaptic nerve terminals through direct interactions with Ca\textsubscript{v}2.1 and Ca\textsubscript{v}2.2\textsuperscript{24}. Furthermore, at least \textit{in vitro}, laminin β2 acts as a “stop signal” for outgrowing motor axons\textsuperscript{31}.

We have tested whether the laminin β2 interactions with Ca\textsubscript{v}2.2 are responsible for the laminin β2 stop signal. We show here that interactions between laminin β2 and Ca\textsubscript{v}2.2 lead to inhibition of neurite outgrowth \textit{in vitro}. We describe a tiled expression pattern of laminin β2 in the developing skin and demonstrate that
blocking the signal between Ca$_{V}2.2$ and laminin $\beta2$ \textit{in vivo} leads to changes in sensory innervation of the skin.

**RESULTS**

**Expression and localization of Ca$_{V}2.2$**

Calcium signaling is important for many aspects of early neuronal development, including neuronal proliferation and migration, specification of neurotransmitter phenotype, and axon guidance$^{11,15,21,32-41}$. These observations prompted us to ask what other neuronal phenotypes are regulated by early forms of calcium-dependent activity. We therefore screened candidate genes encoding calcium-permeable channels and receptors that could contribute to early excitability due to their expression patterns at early stages of development. \textit{Xenopus} homologues of Ca$_{V}2.2$, TRPV1, TRPV4, and Osm-9 were detected by RT-PCR beginning at the time of neural plate formation (stage 13) until the time of synapse formation (stage 30; Supplementary Fig. 3-1, Fig. 3-1a). Ca$_{V}2.2$ mRNA continued to be detected until the swimming tadpole stage (stage 48; data not shown). \textit{In situ} hybridization with a 272 bp fragment of Ca$_{V}2.2$ revealed expression in the spinal cord and hindbrain beginning at the time of neural tube closure (Fig. 3-1b) leading us to pursue further investigation of this channel. \textit{In situ} hybridization with TRPV4 revealed expression around the blastopore and in the notochord, while TRPV1 and Osm-9 probes gave no signal distinguishable from control (data not shown).
To localize Ca\textsubscript{v}2.2 we immunostained whole mount preparations (Fig. 3-1c). Tailbud (stage 26) \textit{Xenopus} embryos were stained for the neuronal marker HNK-1, revealing longitudinal tracts running antero-posteriorly, commissural axons crossing the ventral midline, and sensory Rohon-Beard axons traversing the skin. Co-staining with an antibody to Ca\textsubscript{v}2.2 revealed expression in growth cones of extending commissural and Rohon-Beard sensory axons (Fig. 3-1d-f). Expression was also seen in the growth cones of the longitudinal tracts and growth cones initially emerging from the spinal cord (Supplementary Fig. 3-2). Similarly Ca\textsubscript{v}2.2 is expressed in over 90% of growth cones of spinal neurons grown \textit{in vitro}, specifically along the distal margin of the lamellipodia and in the filopodia (Fig. 3-1g,h). Expression of Ca\textsubscript{v}2.2 in the growth cone suggested that it may participate in developmental growth decisions such as initiation, outgrowth, guidance, and stopping.

\textbf{Expression and localization of laminin β2}

Peripheral processes of sensory neurons in \textit{Xenopus} and other organisms innervate the skin in a distributed, tiled fashion\textsuperscript{4,6,8}. In some cases, tiled patterns may be specified from external guidance cues, such as stop signals that limit neurite growth. Laminin β2 has been shown to act as a stop signal for neurites of developing motor neurons\textsuperscript{31}. Because laminin β2 interacts directly with Ca\textsubscript{v}2.2\textsuperscript{24}, we investigated its expression in \textit{Xenopus} skin.

\textit{Xenopus} skin is composed of two layers. The inner sensorial layer gives rise to precursor cells that intercalate into the superficial layer and differentiate into ciliated
cells and intercalating non-ciliated cells also termed conical cells. The superficial layer contains hexagonal mucus-secreting epidermal cells as well as the ciliated cells and intercalating non-ciliated cells (Fig. 3-2a)\textsuperscript{42,43}. Immunostaining 2-day old larvae (stage 35) with monoclonal antibodies to laminin β2 revealed association with specific cell types. Regions defined by the hexagonal cells express laminin β2, whereas regions around ciliated cells and intercalating non-ciliated (or conical) cells do not express laminin β2. Similar results were obtained with two monoclonal antibodies, C4 (Fig. 3-2b) and D79 (data not shown), which recognize distinct epitopes of laminin β2.

**Ca\textsubscript{v}2.2 interaction with laminin β2 in culture**

Because Ca\textsubscript{v}2.2 and laminin β2 bind directly to each other\textsuperscript{24} and are expressed where they may interact \textit{in vivo}, we sought to determine whether this interaction affects neurite outgrowth. The eleventh extracellular loop of Ca\textsubscript{v}2.2 binds a leucine arginine glutamate (LRE) motif on laminin β2\textsuperscript{24}. We grew \textit{Xenopus} neurons \textit{in vitro} on a substrate of laminin-111 that does not have a β2 chain, on laminin-111 plus a solublized 20 kDa C-terminal fragment of the laminin β2 chain containing the LRE, or on laminin-111 plus a point mutated (LRE→QRE) laminin β2 fragment. Neurons, identified by immuno-staining for neuron-specific β-tubulin, extended significantly fewer processes on substrates containing the LRE fragment (28 ± 3%) compared to QRE (42 ± 4%) or non-laminin β2 substrates (49 ± 2%; n ≥ 6 embryos per condition;
Fig. 3-3 a-c,f). These results demonstrate an effect of laminin β2 on neurite extension of *Xenopus* spinal neurons.

To test whether the inhibition of neurite outgrowth is specific to interactions between laminin β2 and CaV2.2, we incubated neuronal cultures grown on laminin-111 plus the laminin β2 LRE fragment with a peptide corresponding to the eleventh extracellular loop of rat CaV2.1. This peptide binds the LRE motif of laminin β2 and shares 91% homology with *Xenopus* CaV2.2. As a control, we incubated cultures with the 11th extracellular loop of rat CaV1.2 that shares only 61% homology with *Xenopus* CaV2.2 and does not bind to laminin β2 (Supplementary Fig. 3-3). The peptide corresponding to the 11th extracellular loop of CaV2.1 effectively rescued the inhibition by the laminin β2 LRE fragment with 50 ± 6% neurons extending processes (Fig. 3-3 d,f). The control peptide did not rescue the inhibition by laminin β2 LRE (31 ± 3%; Fig. 3-3e,f). Thus, the laminin β2 LRE inhibition is specific.

While the laminin β2 LRE fragment inhibits the number of neurons that initiate processes, it has no effect on the neurite length of those neurons that have extended a process (Supplementary Fig. 3-4; see also ref. 31). Laminin β2 LRE also has no effect on survival of neurons in culture, as indicated by the lack of significant differences in number of neurons per culture across conditions (data not shown).

To test whether laminin β2 inhibition of spinal neurite outgrowth is calcium-dependent, we incubated cultures grown on laminin β2 LRE in calcium-free medium or with 5 μM ω-conotoxin GV1A in standard 2 mM calcium medium. Both conditions relieve inhibition by laminin β2 LRE (45 ± 4%; 47 ± 5%, respectively, Fig. 3-4)
indicating that the stop signal relies on calcium influx through CaV2.2. Incubating cultures grown on laminin-111 alone in calcium-free medium does not affect the number of neurons extending processes (44 ± 6%), indicating a specific recovery of inhibition in the absence of calcium rather than a non-specific effect of growing cultures in calcium-free medium. In addition to demonstrating the calcium dependence of laminin β2 inhibition, these results reinforce the role of CaV2.2 in laminin β2 inhibition of neurite outgrowth, because ω-conotoxin GVIA blocks CaV2.2\textsuperscript{44}.

**CaV2.2 - laminin β2 interactions \textit{in vivo}**

To examine the role that CaV2.2 - laminin β2 interactions have in the skin \textit{in vivo}, we first blocked signaling through CaV2.2 by implanting an agarose bead releasing ω-conotoxin GVIA into the developing embryo from the time of neural tube formation until a late larval stage (stages 16 to 35) when sensory terminals have been formed. This method is effective in delivering drugs that block early neuronal activity\textsuperscript{38}. Using beads with fluorescently labeled ω-conotoxin, we found efficient diffusion at least 200 µm from the bead. Whole embryos were stained with HNK-1 to reveal sensory neurites traversing the skin and sensory nerve terminal clusters innervating the skin. Rohon-Beard sensory axons initially run along the inner surface of the skin, continue for an undetermined length between the two layers of skin cells and form sensory varicosities in pits in the lateral or basal plasma membrane between superficial skin cells\textsuperscript{45}. The majority of these varicosities occur in clusters in an en
passant manner but some are located at nerve endings. The most heavily innervated skin cell type is the intercalating non-ciliated cell (conical cell)\textsuperscript{42}, one of the two cell types not associated with laminin β2. Almost all nerve terminal clusters that we observe appear to form en passant as previously described by electron microscopy\textsuperscript{45}. Blocking signaling through \( \text{Ca}_V2.2 \) leads to a significant increase in numbers of sensory nerve terminal clusters in the skin (39 ± 8 clusters per 450 x 350 µm\(^2\) area) compared to embryos in which a vehicle bead (18 ± 6) or no bead (19 ± 3) was implanted (Fig. 3-5).

To ask whether the effect of \( \text{Ca}_V2.2 \) on sensory terminals was mediated by its interaction with laminin β2, we implanted beads that release the peptide corresponding to the eleventh extracellular loop of rat \( \text{Ca}_V2.1 \), which blocks this interaction. These beads resulted in significantly increased numbers of sensory nerve terminal clusters compared to embryos exposed to the control \( \text{Ca}_V1.2 \) loop peptide (67 ± 10 vs 36 ± 6, respectively; Fig. 3-6). Although both \( \omega \)-conotoxin and \( \text{Ca}_V2.1 \) loop-beads led to a doubling of the numbers of sensory nerve terminals in the skin, the absolute density varied in the two cases, possibly because the former were implanted during the summer and the latter during the winter; seasonal variation in amphibian development has been described previously\textsuperscript{46,47}.

**DISCUSSION**

\( \text{Ca}_V2.2 \) is expressed in the growth cones of sensory Rohon-Beard neurons as they grow under the skin, which expresses laminin β2. Either blocking \( \text{Ca}_V2.2 \) with \( \omega \)-
conotoxin GVIA or blocking Cav2.2-laminin β2 interactions with the Cav2.1 11th extracellular loop leads to generation of greater numbers of sensory terminals, potentially via more en passant varicosities, greater numbers of neurites, longer neurites, or less restricted branching. These results support a model in which neurite growth and subsequent innervation are normally restricted by a calcium-dependent stop signal generated by laminin β2 – Cav2.2 interactions (Fig. 3-7).

Specificity of laminin β2 – Cav2.2 inhibition of neurite outgrowth

Previous studies have demonstrated that laminin β2 binds directly to Cav2.2 via the 11th extracellular loop of Cav2.2 and the LRE motif of laminin β2. We present three lines of evidence indicating that these specific interactions mediate the inhibition of spinal neuron outgrowth. First, inhibition is observed when neurons are grown on a laminin β2 fragment containing the LRE motif but not one containing the point mutated QRE. Second, inhibition is rescued when cultures are incubated with the competing peptide corresponding to the eleventh extracellular loop of Cav2.2. Finally, ω-conotoxin GVIA, a specific inhibitor of Cav2.2, blocks laminin β2-mediated inhibition. These results indicate that laminin β2 acts through Cav2.2 to inhibit neurite outgrowth.

Calcium dependence of laminin β2 – Cav2.2 inhibition of neurite outgrowth

Inhibition of neurite outgrowth by laminin β2 is blocked by incubating cultures in calcium-free medium or ω-conotoxin GVIA, demonstrating calcium dependence.
The reliance of laminin β2 – Cav2.2 inhibition on calcium influx distinguishes this effect from the role that laminin-β2 plays in clustering presynaptic calcium channels and other active zone proteins in the presynaptic nerve terminal, a calcium-independent effect^{24}. 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 pre-synaptic calcium channel to which it binds. However, Cav2.1 heterologously expressed in HEK cells is not activated by presentation of the laminin β2 LRE fragment either in solution or as a fixed substrate (W. Catterall, personal communication)^{24}. 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 in order 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^{49}. Indeed, laminin β2 clusters Cav2.1 at the mammalian neuromuscular junction^{24}. Similar changes in distribution of Cav2.2 in 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^{23}. 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. It would be interesting to characterize the changes in calcium
signals that laminin β2 – CaV2.2 interactions elicit in growth cones and to determine the developmental consequences of these interactions in the central nervous system.

**Regulation of sensory innervation by laminin β2 – CaV2.2 interactions**

Laminin β2 functions in synapse formation at neuromuscular junctions, in glomerular filtration in the kidney, and in cell fate determination in the retina. Here we describe the expression of laminin β2 in the skin and its role regulating sensory innervation. This expression is consistent with evidence for expression of human laminin β2 in embryonic dermis and epidermis, and in human melanomas. Indeed, incorporating a mixture of laminins, including the laminin β2 chain, into skin grafts improves sensory perception achieved during regeneration.

Growth of sensory neurites is mediated by neurotrophic factors such as BDNF and NT-4 and guidance molecules such as ephrins. Branching and tiled patterning of sensory neurites relies on the NDR (nuclear Dbf2-related) kinase family in both like-repels-like tiling and non-contact mediated distribution. We have identified a role for calcium signaling elicited by an extracellular matrix molecule in sensory neurite innervation. The activation of NDR kinases is calcium-dependent, providing a potential mechanism by which calcium influx through CaV2.2 may lead to changes in sensory innervation.
Classes of stop signals

Laminin β2 has been described as a “stop signal” for motor neurite outgrowth\textsuperscript{31}. A stop signal may function to indicate that neurites have reached their targets where they are to form synapses or terminal endings\textsuperscript{63,64}. Alternatively, a stop signal may inhibit a neuron from growing into non-target regions\textsuperscript{65}. Our results are consistent with the latter function because inhibiting the signal through Ca\textsubscript{V}2.2 leads to increased numbers of sensory nerve terminals. Behaviorally, this may lead to hypersensitivity to light touch and/or mislocalization of sensory signals. Pierson syndrome, a rare human disorder caused by mutations to the LAMB2 gene that encodes laminin β2, has been characterized by kidney failure, blindness, muscle dystonia, microcephaly, and neurological disorders\textsuperscript{51,66}. It is likely that these infants would also have abnormalities in somatosensation.

Ziconotide, an inhibitor of Ca\textsubscript{V}2.2 derived from conus snail venom, has recently begun to be used for pain treatment, delivered intrathecally\textsuperscript{67}. Although the amount of this drug present in general circulation is quite low, with 56% of patients having less than 0.15 nM plasma levels, the IC\textsubscript{50} for its inhibition of Ca\textsubscript{V}2.2 heterologously expressed in \textit{Xenopus} oocytes is 0.45 nM\textsuperscript{68}. The role that we have demonstrated for Ca\textsubscript{V}2.2 in axon guidance and sensory innervation during development suggests that the use of Ziconotide in breast feeding or pregnant women is ill advised. Laminin β2 is also expressed centrally in the mammalian developing cortex in the subplate\textsuperscript{28}, a transient population of neurons that is crucial to
establishment of thalamocortical connections and maturation of inhibition. Ziconotide could disrupt normal innervation here as well.

**METHODS**

*Generation and staging of embryos*

Adult female *Xenopus laevis* were injected with human chorionic gonadotropin (Sigma) and oocytes were fertilized *in vitro*. Embryos were staged according to Nieuwkoop and Faber (1967).

*RT-PCR and in situ hybridization*

RNA was isolated from the dorsal region of stage 12-30 *X. laevis* embryos using RNAqueous-4-PCR (Ambion). cDNA was generated using random hexamer primers and superscript II enzyme (Gibco/Invitrogen). CaV2.2 primers were designed from *X. tropicalis* genomic sequence by aligning it with CaV2.2 sequence from other species to determine introns and exons. Sequence accession numbers and forward and reverse primers for PCR were as follows:

CaV2.2 (JGI TKS329692.x1)

5’GAATTCTTCTGACTGACAACGTTACTGCTGGCGTGC

5’GGATCCAGGGTACTCCGACATGACCTTATCTC

RT-PCR products were cloned into pBluescript and sequenced (UCSD Center for AIDS Research). Anti-sense probes for *in situ* hybridization were generated from the PCR product of CaV2.2 using the DIG RNA Labeling Kit (Boehringer-Mannheim).
Sense probes were used as controls. *In situ* hybridization was performed as described\textsuperscript{71} and modified\textsuperscript{72}.

**Culture**

Plastic culture dishes (Corning) with 200 µL volume reduction rings or 10 µL microwells (Nunc) were incubated with 5 µg/mL laminin (Sigma) alone or with 200 µg/mL 20 kD c-terminus of laminin-β2 LRE fragment solublized by linkage to maltose binding protein or a mutated version (LRE → QRE; Nishimune et al., 2004) in phosphate-buffered saline (PBS) for 2 hr. After rinsing with filtered PBS, dishes were blocked for 2 hr with 30 mg/mL heat inactivated, filtered BSA. Dishes were rinsed at least five times with PBS and 2 times with modified Ringer’s solution (MR, in mM: 100 NaCl, 2 KCl, 1 MgSO\textsubscript{4}, 2 CaCl\textsubscript{2}, 5 HEPES, pH adjusted to 7.4 with HCl). For loop peptide experiments, cultures were incubated with 20 µM peptide corresponding to rat Ca\textsubscript{V}2.1 or Ca\textsubscript{V}1.2 (Nishimune et al., 2004) for 1 hr prior to addition of cells. Stage 15-17 neural plates were dissected in MR containing 1mg/ml collagenase-B (Boehringer-Mannheim) and then dissociated in calcium-free medium for 1 hr. Dissociated cells were cultured in MR for 14-18 hr, fixed, and stained for β-tubulin as described below. For experiments with conotoxin, 5 µM ω-conotoxin GVIA (Sigma) were added 1-2 hr after plating. Tubulin-positive cells were scored for presence of a neurite longer than one cell body in diameter. There was no difference in percent of neurons extending processes between 200 µL and 10 µL cultures (Supplementary Fig. 3-5).
Immunocytochemistry

Whole or dissected *Xenopus* embryos were fixed with 4% paraformaldehyde (PFA), 0.1% glutaraldehyde in PBS for 30 min at 4°C. For open book dissections and HNK-1 whole embryo staining, embryos were incubated in 0.5% triton-X 100 PBS for 1 to 5 days prior to dissection or staining. Embryos were then dissected by making a ventral incision and removing the gut, notochord and myotomes to reveal the ventral surface of the neural tube and the internal surface of the skin. Cultured neurons were fixed in 4% PFA, 0.1% glutaraldehyde for 5 min. All preparations were blocked with 2% bovine serum albumin in 0.1% Triton-X 100 in PBS prior to staining overnight at 4°C at the following concentrations: rabbit anti-CaV2.2 (Alomone) 1:200; mouse IgG anti β-tubulin (Sigma) 1:500; mouse IgM anti-HNK-1 (Sigma) 1:100; mouse IgG anti-laminin β2 C450 1:500; mouse IgM anti-laminin β2 D79 1:10. HNK-1 whole embryo staining was incubated at 4°C for 1 to 5 days. Alexa-Fluor secondary antibodies (Molecular Probes) were used at 1:300 for 1-2 hr at room temperature. Dissected embryos were mounted in 80% glycerol and whole-mount HNK-1 stained embryos were dehydrated in methanol and mounted in 2:1 benzyl benzoate: benzyl alcohol for clearing. Other preparations were visualized in PBS. Images of dissected embryos were acquired on a Bio-Rad (Hercules CA) MRC-1024 confocal attachment on an Olympus BX-50WI microscope. Whole-mount embryos and cultured cells were imaged with a Zeiss Axiocam MRm camera on an Axioskop 2 microscope using a 20X Achromplan water immersion objective (whole mount and some cultures) or a Plan
10X objective (microwell cultures). Z-stacks of whole-mount HNK-1 stained embryos were deconvolved using the nearest neighbor algorithm on AxioVision.

Bead implants

100-200 mesh Affigel Blue beads (Bio-Rad) were soaked overnight in 10 µM ω-conotoxin GVIA (Sigma) in bead buffer (20 mM Na Citrate, 150 mM NaCl, 1 mM Mg(OAc)₂, 20% glycerol, 0.02% CHAPS) or 1 mg/mL loop peptide in PBS. Beads were implanted in stage 16-18 embryos. Embryos were fixed and stained at stages 34-36.

Analysis

Cultured neurons stained with tubulin were scored for presence of a neurite longer than the diameter of the cell body. Length was measured using AxioVision software. Statistical analyses were ANOVAs followed by Dunnett’s multiple comparison test against the laminin 111 control. In vivo, a sensory nerve terminal cluster was defined by the presence of 2 or more HNK-1 positive puncta no more than 3 diameters away from each other in the plane of the skin. Analyses were ANOVAs followed by Tukey’s multiple comparison test between all pairs of conditions. Descriptive statistics are reported as mean ± SEM.
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This chapter is a preprint of “Sann SB, Nishimune H, Sanes JR, Spitzer NC. Neurite outgrowth and in vivo sensory innervation mediated by a Ca\(_{\text{V}_{2.2}}\) – laminin \(\beta_2\) stop signal. (In preparation)”. Hiroshi Nishimune, Joshua Sanes, and Nicholas Spitzer have given permission to include this manuscript in this dissertation.
Figure 3-1. Cav2.2 is expressed in the developing *Xenopus* neural tube and is localized to the growth cone during axon outgrowth *in vivo* and *in vitro*. a) RT-PCR of a 272 base pair fragment of Cav2.2 using mRNA collected from dissections of dorsal halves of embryos from stages 12 to 30. b) *In situ* hybridization using the probe made from the same fragment reveals expression of Cav2.2 in the developing neural tube. Scale bar is 500 µm. c) Schematized dissection for immunostaining: a ventral incision was made to remove the myotomes and notochord and reveal the neural tube and skin. Staining with the neuronal marker, HNK-1 reveals longitudinal tracts running antero-posteriorly, axons of sensory Rohon-Beard neurons innervating the skin, and commissural interneurons crossing the ventral midline. d) Ventral view of a spinal cord and skin of a dissected embryo stained for HNK-1 (purple) and Cav2.2 (green). Boxes outline the Cav2.2 positive growth cones pictured in e and f. Scale bar is 50 µm. e) Sensory Rohon-Beard growth cone expressing Cav2.2 (green). Scale bar is 10 µm. f) Commissural interneuron expressing Cav2.2 (green). Scale bar is 5 µm. g) Cultured neuron stained for β-tubulin (purple) and Cav2.2 (green) expressing Cav2.2 in the growth cone. Scale bar is 50 µm. h) Growth cone boxed in g. Scale bar is 10 µm.
Figure 3-2. Laminin β2 is expressed in a tiled pattern in the skin. a) Schematic of Xenopus skin. b) Whole embryo staining with the C4 antibody to laminin β2 revealed expression coextensive with hexagonal epithelial (H) cells but not ciliated (C) or intercalating non-ciliated cells (INC) in stage 34 larva. Scale bar is 25 µm.
Figure 3-3. Laminin β2 specifically inhibits spinal neurite outgrowth. *Xenopus* neurons from stage 17 embryos were cultured on different substrates and analyzed at 14-18 hr *in vitro*: a) laminin (LM) with a β1 but not a β2 chain; b) a solublized 20 kDa LRE containing C-terminal fragment of laminin β2; c) a point-mutated (LRE→QRE) laminin β2 fragment. Neurons identified by immunostaining for neuron-specific β-tubulin, extended significantly fewer processes on substrates containing the LRE fragment compared to QRE or non-laminin β2 substrates. Scale bar is 50 μm. d) Incubating neurons cultured on laminin plus the β2 LRE fragment with a competing peptide corresponding to the 11th extracellular LRE-binding loop of rat CaV2.1 (91% homologous to *Xenopus* CaV2.2) rescued neurite extension. e) Incubation with a control peptide corresponding to the 11th extracellular loop of rat CaV1.2 (61% homologous to *Xenopus* CaV2.2) did not prevent inhibition by laminin β2 LRE. f) Laminin β2 LRE significantly reduces the number of neurons extending processes *in vitro*. This effect is rescued by incubation with the competing CaV2.1 loop peptide (**p < 0.01, *p < 0.05, comparison to LM control; N ≥ 300 neurons from ≥ 6 cultures).
Figure 3-4. Laminin β2 inhibition is calcium-dependent. Stage 16 dissociated neural tubes were cultured and neurons were identified by staining for β-tubulin. a,b) Neurite outgrowth on LM is inhibited by addition of the laminin β2 fragment (LRE; p < 0.05). Scale bar is 50 µm. c,d) Incubating neurons cultured on LM plus the β2 LRE fragment in 5 µM ω-conotoxin or in calcium-free medium prevents inhibition of neurite outgrowth indicating calcium dependence of LRE inhibition. e) Incubating neurons grown on LM alone in calcium-free medium does not lead to increases in neurite extension. f) Neurite extension on LRE is rescued by preventing calcium influx through Cav2.2 (N ≥ 250 neurons from ≥ 7 embryos).
Figure 3-5. Blocking signaling through Cav2.2 in vivo leads to increased innervation of the skin. a) Agarose beads soaked in 10 µM ω-ctx were implanted in stage 16 embryos. After one day, stage 34 embryos were fixed and stained with anti-HNK-1 to reveal sensory processes and sensory nerve terminals innervating the skin. A 450 µm x 350 µm area centered around the bead was imaged and analyzed (box). b-d) Deconvolved and projected z-series through the skin of embryos in which no bead (b), a control bead (c), or an ω-ctx bead (d) were implanted. Scale bar is 50 µm. e) Enlarged image of the boxed region in d showing three sensory nerve terminal clusters, circles. Scale bar is 10 µm. f) Embryos exposed to ω-ctx exhibited significantly greater numbers of sensory nerve terminal clusters compared to embryos implanted with a vehicle bead or no bead (*p < 0.05).
Figure 3-6. Blocking CaV2.2–laminin β2 interactions in vivo leads to increased innervation of the skin. a-c) Agarose beads soaked in 1mg/mL CaV2.1 or CaV1.2 11th extracellular loop peptide were implanted in stage 16 embryos. After one day, stage 34 embryos were fixed and stained with anti-HNK-1 to reveal sensory processes and sensory nerve terminals innervating the skin as in Fig. 5. Scale bar is 50 µm. d) Embryos exposed to CaV2.1 loop peptide (c) exhibited significantly greater numbers of sensory nerve terminals than embryos exposed to CaV1.2 loop peptide (b) (*p < 0.05; N ≥ 3 embryos for each condition).
Figure 3-7. Model of laminin β2 and CaV2.2 interactions in development of skin innervation. a) In control conditions, outgrowing sensory terminals are inhibited or slowed by laminin β2-rich regions of the skin coextensive with hexagonal cells (white). b) The number of sensory nerve terminals increases when signaling interactions between laminin β2 and CaV2.2 are blocked.
Supplementary Figure 3-1. A screen for calcium channels expressed during development identified expression of Osm9, TRPV1, and TRPV4 from the time of neural plate formation (stage 13) through the time of synapse formation (stage 30). RT-PCR identifies a 358 bp fragment of a sequence with homology to Osm9, a 454 bp fragment of TRPV1, and a 378 bp fragment of TRPV4. mRNA was collected from dissections of dorsal halves of embryos from stages 12 to 30.
Supplementary Figure 3-2. 
$\text{Ca}^2+\text{V}_{2.2}$ is expressed in the growth cones of ventral longitudinal tracts, commissural interneuron axons crossing the neural tube, and Rohon-Beard axons exiting the neural tube. 

b) scale bar 50 µm. c) dashed box in b; scale bar 20 µm. d) solid box in b; scale bar 5 µm.
Supplementary Figure 3-3. Alignment of the 11th extracellular loop of *Xenopus tropicalis* Ca\(\text{v}2.2\) with rat Ca\(\text{v}2.2\), Ca\(\text{v}2.1\), and Ca\(\text{v}1.2\). Bold amino acids are identical to *Xenopus* Ca\(\text{v}2.2\) and underlined amino acids are identical or have conserved changes. Compared to the 11th extracellular loop of *Xenopus* Ca\(\text{v}2.2\), rat Ca\(\text{v}2.2\) is 91% similar and 76% identical. Rat Ca\(\text{v}2.1\) is 91% similar and 73% identical. Rat Ca\(\text{v}1.2\) is 61% similar and 38% identical.

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Supplementary Figure 3-4. Growth on laminin β2 has no effect on neurite length. p > 0.45; N ≥ 70 neurons from ≥ 6 cultures.
Supplementary Figure 3-5. There is no difference in the effect of laminin β2 inhibition between neurons cultured in 200 μl culture dishes or 10 μl microwells. p > 0.75; N ≥ 300 neurons from ≥ 8 embryos.


IV. Summary and Perspectives
SUMMARY AND PERSPECTIVES

Role of calcium signaling in neurotransmitter specification

Summary

The pattern of calcium-dependent electrical activity regulates neurotransmitter expression in a homeostatic manner. *In vivo*, different classes of neurons exhibit different patterns of calcium spike activity. Suppressing calcium spiking during a critical period leads to an increase in the numbers of neurons expressing the excitatory transmitters glutamate and acetylcholine and a decrease in the numbers of neurons expressing the inhibitory transmitters glycine and GABA. Conversely, increasing spike activity leads to a decrease in the population of neurons expressing excitatory transmitters and an increase in the population of neurons expressing inhibitory transmitters. Imposing particular patterns of calcium activity *in vitro* demonstrates that expression of transmitters is dependent on the frequency of calcium spiking but independent of molecular markers of neuron identity. Transmitter switches are functional because neurons release transmitter that is atypical of the molecular markers of neuron identity.

Current and future directions

Current work aims to understand what generates calcium spike activity as well as the downstream mechanisms by which calcium signals interact with transcription factors to regulate neurotransmitter expression. Calcium spikes require T-type, N-type, and L-type currents through voltage-gated calcium channels\(^1\). These spikes may be triggered by neurotransmitters themselves, similar to other forms of embryonic
excitability. Indeed GABA and glutamate are expressed prior to onset of spike activity and blocking neurotransmission prevents activity (Root, Velazquez, Monsalve, Minakova, and Spitzer, in preparation). Calcium signaling has been shown to influence transcription through CREB, CaRF, DREAM, and NFAT pathways. Calcium spikes may work through these or other pathways to regulate transcription factors known to influence transmitter specification.

In order for a circuit to remain functional following a switch in presynaptic neurotransmitter expression, cells must pathfind to the appropriate targets and postsynaptic cells must express appropriate receptors. Early activity is needed for initial targeting of thalamocortical axons in cats. Activity regulates developmental expression of NMDA receptors in the mammalian visual system, kainate receptor expression at thalamocortical synapses, GABA_A receptor subunits in the rat superior colliculus, and acetylcholine receptor subunits at the mouse neuromuscular junction. In *Xenopus*, neurotransmitter receptor expression in striated muscle depends on neuronal spike activity. It would be of interest to further examine the mechanisms by which circuits establish or respecify correct pre- and post-synaptic neurotransmitter – receptor matches, both through axon targeting and expression of transmitter synthetic enzymes and receptors in the peripheral and central nervous system.
Role of calcium signaling in neurite outgrowth and sensory innervation

Summary

By interacting with the extracellular matrix molecule laminin β2, Cav2.2 mediates neurite inhibition and distribution of sensory innervation.

Cav2.2 was identified in a screen for calcium channels contributing to early excitability based on its mechano sensitivity and its known expression in the nervous system of other organisms. In *Xenopus laevis*, Cav2.2 is expressed embryonically in the growth cones of commissural axons, longitudinal axons, and sensory Rohon-Beard axons as they innervate the skin. Expression in the growth cones of sensory neurites is particularly intriguing because laminin β2 is expressed in the skin in a tiled manner.

Culturing *Xenopus* spinal neurons on a substrate of laminin β2 reduces the number of neurons that extend processes. This inhibition is rescued when the interaction between laminin β2 and Cav2.2 is disrupted by incubating cultures with a competing peptide corresponding to the binding loop of Cav2.2. Furthermore, this inhibition is rescued by incubating cultures in calcium–free medium or with the Cav2.2 inhibitor ω-conotoxin GVIA, demonstrating dependence on calcium influx through Cav2.2.

Blocking signaling through Cav2.2 in vivo leads to greater numbers of sensory nerve terminals in the skin. This may occur because there are greater numbers of en passant varicosities, greater numbers of neurites, longer neurites, or less restricted branching. These results support a model in which neurite growth and subsequent innervation are normally restricted by a calcium-dependent stop signal generated by
laminin β2 – CaV2.2 interactions. This stop signal may ensure appropriate distribution of sensory innervation that is needed for touch discrimination in the developing larvae\textsuperscript{15}.

**Future directions**

Laminin β2 has been described as a “stop signal” for motor neurite outgrowth\textsuperscript{16}. A stop signal may function to indicate that neurites have reached their targets where they are to form synapses or terminal endings\textsuperscript{17,18}. Alternatively, a stop signal may inhibit a neuron from growing into non-target regions\textsuperscript{19}. My results are consistent with the latter function because inhibiting the signal through CaV2.2 leads to increased numbers of sensory nerve terminals. Behaviorally, this may lead to hypersensitivity to light touch and/or mislocalization of sensory signals. To further address the nature of the stop signal from laminin β2, it would be informative to identify the cell types innervated by the sensory Rohon-Beard neurons and the changes in innervation that occur when signaling between laminin β2 and CaV2.2 is blocked. Other studies report that the primary cell type being innervated is the conical cell or intercalating non-ciliated cell\textsuperscript{20}, one of the two cell types not associated with laminin β2. Evidence that innervation spreads to regions expressing laminin β2 when the interaction between laminin β2 and CaV2.2 is blocked would further clarify the role of laminin β2 as a stop signal.

As noted above, laminin β2 inhibition of neurite outgrowth is blocked by incubating cultures in calcium-free medium or ω-conotoxin GVIA, demonstrating calcium dependence. The reliance of laminin β2 – CaV2.2 inhibition on calcium
influx distinguishes this effect from the role that laminin β2 plays in clustering presynaptic calcium channels and other active zone proteins in the presynaptic nerve terminal, a calcium-independent effect\textsuperscript{14}. It would be interesting to characterize the changes in calcium signals that laminin β2 – \( \text{Ca}_V 2.2 \) interactions elicit in growth cones both \textit{in vitro} and \textit{in vivo}.

Within the central nervous system, laminin β2 is expressed in the subplate of the developing rat cortex (E15) and the the floorplate of the developing spinal cord\textsuperscript{21}. The subplate is an early transient population of cells that sends the first pioneer axons from the cerebral cortex to the thalamus\textsuperscript{22} and is required for guidance of thalamic axons innervating the cortex\textsuperscript{23}. Lateral geniculate axons grow toward the cortex before cortical migration is complete and pause at the subplate at (E18) until cortical laminar structures are established (P4)\textsuperscript{24}. Absence of subplate neurons leads to overgrowth of the thalamocortical axons\textsuperscript{23}. Further study may reveal whether interactions between laminin β2 and \( \text{Ca}_V 2.2 \) are the subplate stop signal that prevents overgrowth while the cortex matures. Similarly, signals within the floorplate guide commissural axons while crossing the midline\textsuperscript{25}. \( \text{Ca}_V 2.2 \) – laminin β2 interactions may be part of the complex set of cues that guide axons here as well.

The LRE motif of laminin β2 is the binding site for \( \text{Ca}_V 2.2 \textsuperscript{14,26} \). \( \text{Ca}_V 2.2 \) may mediate axon guidance in response to other molecules with an LRE or a functionally equivalent motif (L,A, or I; R or K; E)\textsuperscript{26}. Indeed, netrin has an ARE motif and the oligodendrocyte myelin glycoprotein has an LKE motif. Both of these molecules have known roles in axon guidance\textsuperscript{27,28}. Further investigations may reveal a role for \( \text{Ca}_V 2.2 \)
in the inhibitory signals generated by these molecules and may uncover other molecules that interact with Ca\textsubscript{V}2.2.

I have described roles for calcium signaling in specifying neurotransmitter phenotype as well as regulating neurite outgrowth and sensory innervation. These are only two of many roles that calcium plays during neuronal development, acting both as an ion that contributes to voltage changes and as a second messenger\textsuperscript{29}. Certainly future investigations will demonstrate additional roles that calcium plays during development. A current challenge is understanding how these signals remain distinct or become integrated in order to effect appropriate changes.


Appendix

Selection of calcium channels for study
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Channels for study were selected based on four criteria: involvement in stretch-sensitive apparatus; calcium permeability; expression in the nervous system of the model systems in which they have been studied; and availability of homologous sequences in *Xenopus laevis* or *tropicalis*. *Xenopus* sequences were identified by blast searching genome databases of NCBI, Sanger Center, and the Joint Genome Institute as available in December 2002. Blast results were considered for study if their Expect value was < e-5. The following four channels met the above criteria.

**CaV2.2** is a member of the family of voltage-gated calcium channels. Calabrese et al.\(^1\) demonstrated that this high-voltage activated N-type calcium channel is also stretch sensitive. When CaV2.2 – transfected HEK cells were inflated by positive pressure through the pipette, the whole cell current elicited by depolarization increased, as did the frequency of single channel openings. These effects were not seen for current through T-type calcium channels (CaV3.1) transfected into the same cells\(^1\).

CaV2.2 is expressed in human central nervous system\(^2\), cerebellum and hippocampus of the rat brain\(^3\), and DRG of chick and rat\(^4,5\). Blocking this channel with ω-conotoxin slows cerebellar granule cell migration in slice culture\(^6,7\). RNAi reduction of CaV2.2 expression in *Drosophila* decreases synaptic growth at the neuromuscular junction\(^8\). CaV2.2 binds directly to the extracellular matrix molecule laminin β2 and clusters active zone proteins at the presynaptic terminal\(^9\).
Blast searches revealed 3 *X. tropicalis* genomic sequences and 1 *X. laevis* cDNA with homology to the human CaV2.2.

**Transient receptor potential cation channel (TRPV4)** *(also called OTRPC4, VR-OAC, VRL-2)* is an osmotically sensitive, non-selective cation channel. Whole cell and single channel recordings as well as calcium imaging show that cells heterologously expressing TRPV4 have increased calcium currents in hypotonic solutions\(^{10-12}\). By immunocytochemistry, TrpV4 is expressed in sympathetic and parasympathetic nerve endings in humans\(^{13}\); and by *in situ* hybridization, it is localized to in the subfornical organ; the vascular organ and median preoptic area of the lamina terminalis; the ependymal cells lining the choroid plexus of the lateral ventricles; scattered neurons in the cerebral cortex, thalamus, hippocampus, and cerebellum; hair cells of the inner ear; the trigeminal ganglia; large somata sensory neurons; and Merkel cells on the sinuses of the vibrissae of the snout in mouse\(^{10}\). The relative calcium to sodium permeability of TRPV4 is between 6 and 10\(^{14}\). Blast searching revealed 4 *X. laevis* cDNAs, 9 *X. tropicalis* cDNAs, and 1 *X. tropicalis* genomic sequence with homology.

**Osm9** is a non-selective cation channel identified in *Caenorhabditis elegans*. Loss of function Osm-9 mutants fail to avoid high osmotic strength stimuli or to respond to light nose touch\(^{15}\). Osm-9::GFP fusion genes are expressed in a subset of *C. elegans* sensory neurons. Osm-9 shares 26% amino acid identity with rat TRPV4, and expression of rat TRPV4 in *C. elegans* Osm-9 mutants recovers osmotic and nose touch responses (but not lost odorant responses)\(^{15}\). Blast searching revealed 9 *X.
laevis ESTs and 6 X. tropicalis ESTs. Of these, 4 in each group had been previously identified by Blast searches with TRPV4.

**TRPV1** is another member of the TRPV/Osm-9 family of non-selective cation channels. TRPV1 mutant mice exhibit a decreased voiding reflex during bladder filling, indicating a potential role for this channel in mechanosensation\(^\text{16}\). TRPV1 is expressed in cutaneous nociceptive neurons and visceral neurons\(^\text{17}\) as well as rat hippocampus, spinal cord, hindbrain, cerebellum, mesencephalon, cortex, olfactory bulb, and dorsal root ganglia\(^\text{18}\). Blast searching revealed 2 X. laevis ESTs in addition to those found by blasting with Osm9 or TRPV4.

Additional channels that were considered for further study but did not fully meet the criteria of a mechanosensitive, calcium permeable channel with known expression in the nervous system include NOMPC\(^\text{19}\), members of the EnaC/DEG/ASIC family\(^\text{20}\), and the bacterial MscL\(^\text{21}\). The L-type calcium channel Ca\textsubscript{V}1.2 is also mechanosensitive\(^\text{22}\); however, the *Xenopus* channel homologous to Ca\textsubscript{V}1.2 is expressed only in muscle during development\(^\text{23}\). The more recently described TRPA1 meets the above criteria and would be interesting for similar study\(^\text{24}\).


