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Molecular mechanisms integrating genetic and calcium activity-dependent neurotransmitter specification

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in

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By

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LIST OF ABBREVIATIONS

ANPE	anterior neural plate element
AP1	activator protein 1
BDNF	brain-derived neurotrophic factor
Bg	β-globin basal promoter
bHLH	basic helix-loop-helix
BLAST	basic local alignment search tool
Bmp	bone morphogenetic protein
bp	basepair
Ca	calcium
CamKIV	calcium/calmodulin-dependent protein kinase IV.
cAMP	cyclic adenosine monophosphate
CAR	cardiac actin promoter
CBP	CREB-binding Protein
ChAT	choline acetyltransferase
ChR2	channelrhodopsin
CRE	cAMP response elements
CREB	cAMP response element binding
Dbx1	developing brain homeobox 1
eCFP	enhanced cyan fluorescent protein
ECR	evolutionary conservation of genomes
eGFP	enhanced green fluorescent protein
EMSA	electrophoretic mobility shift assay
EST	expressed sequence tag
FRET	Förster resonance energy transfer
GABA	γ-aminobutyric acid
Gad67	glutamic acid decarboxylase 67
GOF	gain of function
hr	hour
IR	immunoreactive
JGI	Joint Genome Institute
Kb	kilobase
K_{ir}	human Kir2.1
lbx1	ladybird homeobox 1
lhx8	LIM-homeobox gene 8
LSE	lateral stripe element
MCS	multiple cloning site
MN	motor neuron
Morf4L2	mortality factor 4 like 2
MOs	morpholinos
Na_v	$rNa_v2a\alpha\beta$
nBgal	nuclear-localized β-galactosidase
NCAM	neural cell adhesion molecule

NFAT	nuclear factor of activated T-cells
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NFY	nuclear factor Y
Ngn1/2	neurogenin1/2
NMDA	N-methyl-D-aspartate
NT	neurotransmitter
p-cJun	phosphorylated cJun
PFA	paraformaldehyde
PG	petrosal ganglion
Phox2a/2b	paired-like homeobox 2a/2b
PKC	protein kinase c
ptf1a	pancreas specific transcription factor, 1a
RB	Rohon Beard
RFP	red fluorescent protein
RT-PCR	reverse transcriptase PCR
RyR	ryanodine receptor
Shh	sonic hedgehog
STAT	signal transducers and activators of transcription
TF	transcription factor
tlx1	T-cell leukemia homeobox 1
tlx3	T-cell leukemia homeobox 3
UAS	upstream activation sequence
VaChT	vesicular acetylcholine transporter
VGluT1	vesicular glutamate transporter1

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ABSTRACT OF THE DISSERTATION

Molecular mechanisms integrating genetic and calcium activity-dependent neurotransmitter specification

by

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Excitability regulates many aspects of neuronal differentiation in addition to forming the basis for rapid signaling in the mature nervous system. Several key features that determine the functional activity of neurons are modulated by calcium signaling during development, including neurotransmitter specification. The focus of the dissertation is on understanding the signaling cascades that mediate calcium spike-dependent specification of neurotransmitter phenotype in embryonic spinal neurons.

I describe the identification and characterization of novel pathways that act in the specification of activity-dependent neurotransmitter phenotype by targeted screening of molecules known to regulate the expression of excitatory and inhibitory neurotransmitters. In addition, I describe a gain-of-function screen to identify novel molecules that were previously unknown to play a role in the activity-dependent specification of neurotransmitter phenotype. Finally, I describe the generation and development of novel transgenic *Xenopus* lines in which specific subsets of neurons are fluorescently labeled. These lines serve as the basis for further genetic screening and can be used to characterize the activity-dependent specification of neurotransmitters as well as numerous other processes in neuronal development.

I. Introduction

The brain is composed of a greater diversity of cells than any other human organ, with cells in the nervous system collectively expressing close to 80% of predicted genes in the human genome (Lein et al., 2007). Each neuron expresses only a subset of these genes, and this distinct and highly regulated expression profile leads to differentiation of a particular cell type. Neuronal differentiation is due to both genetic factors and environmental cues throughout development. Differential responsiveness to these external cues is essential for appropriate neuronal signaling in a given environment. Waves of transcriptional cascades in response to unique environmental signals act in a combinatorial manner to determine neuronal identity and fate. Many of these transcription factors are expressed only transitorily during development, acting in a temporally specific manner to affect neuronal characteristics that persist into adulthood. One of the fundamental questions in neuroscience is therefore to develop an understanding of the mechanisms by which this extensive cellular diversity is established during development.

Alterations in gene expression are responsible for the plasticity of neurons that underlies learning and memory, in addition to numerous neuronal diseases. The integration of signaling that influences neuronal development is not yet fully understood although, in recent years, many studies have contributed to our understanding of the processes that regulate context-dependent neuronal differentiation in embryonic development. While

combinatorial transcription factor expression defines a basic framework of the developing nervous system, additional control mechanisms contribute to the generation of cell diversity, including calcium signaling activity.

Activity-regulated genes in nervous system development

Interestingly, there are a number of activity-dependent transcription factors that have been shown to control many differing aspects of neuronal development, including the balance between excitation and inhibition, synapse formation and elimination, and synaptic plasticity (reviewed in Greer & Greenberg, 2008). In each of these processes, activity-dependent transcription factors regulate the expression of distinct subsets of genes in only partially overlapping pathways, suggesting precise decoding of temporal, spatial, and stimulus-specific cues.

Calcium transients in neuronal development

Spontaneous transient elevations of intracellular calcium have been observed in many systems at early stages of neuronal development. Calcium transients at frequencies of 1-10 hr^{-1} are generated in murine neural crest cells and in differentiating sensory neurons in culture during a limited neurogenic period (Carey & Matsumoto, 1999a; Carey & Matsumoto, 1999b; Carey & Matsumoto, 2000). Spontaneously active neural crest cells produce clones of cells containing neurons, while inactive cells do not. Blocking these calcium

transients suppresses neuronal differentiation, measured by the expression of Hu RNA-binding protein, voltage-dependent calcium channels and caffeine-releasable calcium stores. Spontaneous low frequency (20 hr^{-1}) calcium transients observed in cranial motor neurons in the embryonic mouse hindbrain have been suggested to play a role in circuit assembly (Gust et al., 2003). In postnatal rat Purkinje neurons spontaneous cytoplasmic calcium oscillations (mean 2.6 min^{-1}) generated by L-type calcium channels and RyR-activated calcium stores are communicated to the nucleus and correlate closely with patterns of spontaneous and evoked electrical activity recorded in the neurons (Liljelund et al., 2000).

At earlier stages of development, low frequency (7 hr^{-1}) calcium transients are a prominent feature of mouse egg fertilization (Halet et al., 2003). Calcium transients are also observed in amphibian blastomeres during blastula and gastrula stages, prior to morphological differentiation of the nervous system. These calcium transients (3 hr^{-1}) are generated by L-type calcium channels during a 5 hr period of development and can be triggered by the neural inducer, noggin. They are strongly implicated in neural induction, and regulate expression of fos-related protein, the left-right asymmetry specification factor zic3, the DNA replication inhibitor geminin, and the cell adhesion molecule NCAM (Moreau et al., 1994; Leclerc et al., 1997, 1999, 2000). Body axis specification, cell motility, cell differentiation, heart and

kidney development are all regulated in varying ways by calcium signaling (reviewed in Webb & Miller, 2003).

Calcium signaling in neurotransmitter specification

Neuronal phenotype is specified in response to modulation of neuronal activity. Elevations of intraneuronal calcium levels can lead to altered expression and/or function of numerous transcription factors, and can lead to changes in neuronal fate as well. Some of these calcium-induced transcription factors have been well characterized for their role in influencing particular transcriptional cascades in response to waves of neuronal calcium activity, including CREB, CBP, and CamKIV. In many cases, phosphorylation of these transcription factors and their downstream targets mediates their activity at a given promoter (West et al., 2001; West et al., 2002).

Excitatory and inhibitory neurotransmitter specification has also been shown to be regulated by both transcriptional cascades and neuronal activity. Chronic NMDA receptor blockade in cultured hypothalamic neurons causes an increase in the number of cholinergic neurons. This phenotypic switch is mediated in a calcium-dependent manner and involves the CaMKII/IV and PKC signaling cascades (Belousov et al., 2001; Belousov et al., 2002). Furthermore, the induction of dopaminergic phenotype by depolarization of petrosal ganglion (PG) sensory neurons occurs only in the presence of Phox2a/2b and the developmental expression of Phox2a/2b corresponds to

the period when the neurons are capable of expressing tyrosine hydroxylase in response to depolarization (Brosenitsch & Katz, 2002). This observation suggests that the cellular context, in this case the expression of Phox2a/2b, can make a cell competent to respond to activity cues. The results imply that different cells, even when exposed to the same activity cues, may respond differently, and that an understanding of the interplay between activity, transcriptional cascades, and cellular context is essential for decoding these processes.

Calcium activity and neuronal phenotype specification in Xenopus

Calcium spikes, which involve developmentally transient calcium-dependent action potentials and calcium-induced calcium release, regulate several aspects of development of *Xenopus* spinal neurons (Gu & Spitzer, 1995; Watt et al., 2000; Borodinsky et al., 2004). Spikes are ~10 seconds in duration, raise intracellular Ca concentration to ~1 μM (Gu et al., 1994), and occur at frequencies of 1-20 hr^{-1} (Borodinsky et al., 2004). Interestingly, calcium spike frequency appears to homeostatically regulate specification of neurotransmitters during a 5 hr critical period *in vitro* (Borodinsky et al., 2004). When calcium spikes are suppressed using molecular or pharmacological approaches, a higher incidence of neurons expresses the excitatory neurotransmitters glutamate and acetylcholine. In contrast, when calcium spiking is increased, more neurons express the inhibitory neurotransmitters

GABA and glycine. Regulation involves transcription: suppression of RNA synthesis prevents calcium-dependent changes in incidence of the GABAergic phenotype (Spitzer et al., 1993) and specific frequencies of spontaneous calcium transients upregulate GAD67 transcripts in embryonic spinal neurons (Watt et al., 2000).

The signaling pathways downstream of calcium spiking that mediate neurotransmitter specification are unknown. Signal transduction cascades have been described that are capable of deciphering calcium influx delivered in pulses at 1-100 Hz in the mature nervous system, but these mechanisms are unlikely to be responsive to calcium influx at the frequency of spikes observed in the embryonic spinal cord (West et al., 2002). The low frequency with which these spikes are produced raises the question of the mechanism by which they exert their effects.

Molecules involved in neurotransmitter specification

The majority of work on neuronal differentiation, including neurotransmitter specification, has focused on transcription factor cascades and downstream gene regulation. This work has identified several general aspects of neuronal development. Cell position, defined largely by gradients of members of the Shh and Bmp signaling pathways, defines the dorsoventral axis in the spinal cord. Neuronal precursors express a combinatorial code of transcription factors that determine cell identity. As precursors exit the cell

cycle this combinatorial code initiates a cascade of transcription factors that determine the expression of effector genes and specify the various programs underlying a neuron's functional identity, including axon trajectory and neurotransmitter specification (Thor et al., 1999; Jessell, 2000).

Strong evidence exists for the involvement of transcription factors in neurotransmitter choice, with specific neuronal lineages specified by a unique combination of transcription factor networks. For example, the coordinated activity of homeodomain and bHLH (basic helix–loop–helix) transcription factors regulates the differentiation of neural progenitors into specific neural subtypes.

Neuronal phenotype is determined by expression of Ngn1/2 and Mash1, which act to select glutamatergic versus GABAergic cell fates in developing forebrain and spinal cord spinal neurons (Fode et al., 2000; Schuurmans et al., 2004; Mizuguchi et al., 2006). The ectopic expression of the homeobox gene MNR2 in neural cells can cause motor neuron differentiation, including the expression of acetylcholine (Tanabe et al., 1998). Dbx1 knockout mice have an increase in GABA-positive interneurons (Pierani et al., 2001). Gain and loss-of-function experiments have shown that transcription factors play a role in the expression of other classical neurotransmitters, glutamate and glycine (Mo et al., 2004). More recently, the central role played by transcription factors in neurotransmitter specification

was demonstrated by the ability of Tlx3, Tlx1, Ptf1a and Lbx1 to act as binary switches in determining glutamatergic and GABAergic phenotypes (Cheng et al., 2004; Cheng et al., 2005, Glasgow et al., 2005; Hoshino et al., 2005). A gain or loss of any one of these genes is sufficient to change neurotransmitter expression.

While both calcium activity and particular families of transcription factors have been shown to mediate neuronal fate specification during early development, the mechanism by which the two interact to influence differentiation has remained unclear. The coding that specifies commitment to one cell fate over another has yet to be determined. In this thesis, I describe recent experiments that delineate the pathways that integrate calcium spike activity and intrinsic, genetically determined, neurotransmitter specification. In addition, I describe the design and execution of genetic screens for novel genes that were previously unknown to mediate activity-dependent specification of neuronal phenotype in the developing embryonic nervous system.

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II. Tlx3 mediates calcium dependent neurotransmitter specification through phosphorylation of cJun

ABSTRACT

Neuronal differentiation is accomplished through cascades of intrinsic genetic factors initiated in neuronal progenitors by external gradients of morphogens. Activity was thought to be important only late in development, but recent evidence indicates that activity also regulates early neuronal differentiation. Activity in post-mitotic neurons prior to synapse formation can regulate phenotypic specification, including neurotransmitter choice, but the mechanisms are not clear. Here we identify a mechanism that links endogenous calcium spike activity with an intrinsic genetic pathway to specify neurotransmitter choice in the embryonic nervous system. Early activity modulates transcription of the GABAergic/glutamatergic selection gene *tlx3* and requires a variant cAMP response element (CRE) in its promoter. The cJun transcription factor binds to this CRE site, modulates transcription, and regulates neurotransmitter phenotype through its transactivation domain. Calcium signals through cJun N-terminal phosphorylation, thus integrating activity-dependent and intrinsic neurotransmitter specification. This mechanism provides a basis for early activity to regulate genetic pathways at critical decision points, switching the phenotype of developing neurons.

INTRODUCTION

Excitability regulates many aspects of neuronal development in addition to forming the basis for rapid signaling in the mature nervous system (Goodman & Shatz, 1993; Spitzer, 2006). Key features determining neuronal structure and function are regulated by calcium (Ca) signaling, including cell proliferation and migration, axon guidance, synapse refinement, cell survival and neurotransmitter (NT) specification (Borodinsky et al., 2004; Buffelli et al., 2003; Hanson & Landmesser, 2004; Hanson & Landmesser, 2006; Komuro et al., 1996; Weissman et al., 2004; Yano et al., 1998). Much work has been done to elucidate the way in which synaptic activity regulates these processes through neuronal gene expression (Flavell & Greenberg, 2008; Lin et al., 2008), but less is known about modulation of neuronal development by earlier forms of activity prior to synapse formation. Here we focus on understanding the signaling cascade that mediates Ca spike-dependent specification of neurotransmitter phenotype in embryonic *Xenopus* spinal neurons.

Specification of neurotransmitters is a crucial aspect of development because it determines the polarity of synapses and the function of microcircuits. Many studies of neurotransmitter specification have focused on transcription factor cascades and downstream gene regulation. Gradients of members of the Shh and Bmp signaling pathways define the dorsoventral axis in the spinal cord and the expression of a combinatorial code of transcription

factors that determine cell identity in neuronal progenitors (Liem et al., 1995; Yamada et al., 1993). As neuronal precursors exit the cell cycle this combinatorial code initiates a cascade of transcription factors that determine the expression of effector genes and launch the various programs underlying a neuron's functional identity, including axon trajectory, neurotransmitter specification and neurotransmitter receptor expression (Baumgardt et al., 2007; Jessell, 2001; Ma, 2006; Thor et al., 1999). Compelling evidence identifies roles for transcription factors in neurotransmitter choice. Ectopic expression of the homeobox gene MNR2 in neural cells can cause motor neuron differentiation, including the expression of acetylcholine (Tanabe et al., 1998). Dbx1 knockout mice exhibit an increase in GABA-positive interneurons (Pierani et al., 2001). Gain- and loss-of-function experiments in mice have shown that transcription factors play a role in the expression of other classical neurotransmitters, glutamate and glycine (Mizuguchi et al., 2006; Mo et al., 2004; Pillai et al., 2007). The central role played by transcription factors in neurotransmitter specification was demonstrated by the ability of Tlx3 and Lbx1 to act as switches in determining glutamatergic and GABAergic phenotypes in chick and mouse (Cheng et al., 2004; Cheng et al., 2005).

Activity also regulates neurotransmitter specification, including acetylcholine in mouse hypothalamic neurons (Liu et al., 2008) and dopamine in mouse petrosal ganglion neurons (Brosenitsch et al., 2002). In *Xenopus*, Ca spike activity regulates dopaminergic specification in the ventral

suprachiasmatic nucleus and four classical neurotransmitters, GABA, glutamate, glycine, and acetylcholine, in the spinal cord, via mechanisms that require transcription (Borodinsky et al., 2004; Dulcis & Spitzer, 2008; Gu & Spitzer, 1995; Watt et al., 2000). Prior to synapse formation, the frequency of Ca spikes, which involve developmentally transient Ca-dependent action potentials and Ca-induced Ca release, homeostatically regulates specification of neurotransmitters in the spinal cord during a brief critical period (Borodinsky et al. 2004; Root et al., 2008). When Ca spikes are suppressed using molecular or pharmacological approaches, fewer neurons express the excitatory neurotransmitters glutamate and acetylcholine. In contrast, when Ca spiking is increased, more neurons express the inhibitory neurotransmitters GABA and glycine.

Here we provide evidence for a molecular mechanism by which Ca spikes during the critical period cause a change in the neuron's intrinsic transcriptional cascade specifying GABA and glutamate. The end result is the mature neurotransmitter phenotype of the neuron, determined by both genetic factors and activity. This interplay of intrinsic cellular context and Ca signaling is likely to allow assembly of circuits with functions appropriate to an environment of changing activity.

RESULTS and DISCUSSION

Activity and *tlx3* interact to specify neurotransmitter fate

To discover possible integration points between early activity and genetic pathways regulating neuronal differentiation we sought to identify genes with activity-dependent expression by *in situ* hybridization for transcription factors involved in neurotransmitter specification. *Xenopus tropicalis* (*X. tropicalis*) embryos (Supplementary Fig. 1) (Chang & Spitzer, 2009) were injected in one cell at the two-cell stage with mRNA encoding myc-tagged K_{ir} to suppress Ca spike activity unilaterally (Borodinsky et al., 2004). Overexpression of this inward rectifier potassium channel hyperpolarizes neurons and suppresses generation of Ca spikes. Ca spike suppression caused an increase in both the area and intensity of expression of the t-cell leukaemia homeobox-3 (*tlx3*) gene compared to the unmanipulated side of the embryo (Supplementary Fig. 2). *tlx3* expression begins in post-mitotic lateral spinal neurons at the neural tube stage. These neurons migrate dorsally and medially, strongly suggesting they are glutamatergic Rohon-Beard cells. *tlx3* expression continues throughout the critical period for neurotransmitter specification when spontaneous Ca spike activity is present (Supplementary Fig. 3) (Patterson & Krieg, 1999). To manipulate its expression we generated splice- and translation-blocking morpholinos (MOs; Supplementary Fig. 4) and an overexpression construct. Knockdown of *tlx3* function with MOs caused an increase in the number of GABA-immunoreactive (-IR) neurons and a decrease in the number of vesicular glutamate transporter (VGluT1)-IR neurons in the spinal cord, demonstrating that *tlx3* functions as a switch

specifying the glutamatergic over the GABAergic phenotype as in mouse and chick (Cheng et al., 2004; Cheng et al., 2005). Overexpression of *tlx3* caused the opposite effect on neurotransmitter specification. In addition, the overexpression construct lacks the target sequence for the translation-blocking MO and reversed the MO phenotype when they were co-expressed (Supplementary Fig. 5).

To determine whether *tlx3* is an integration point for activity and a genetic pathway specifying neurotransmitter expression, we performed simultaneous manipulations of Ca activity and *tlx3* gene function. Overexpressing mRNA encoding K_{ir} to suppress Ca activity produced a decrease in the number of GABA-IR neurons and an increase in the number of VGluT1-IR neurons. MO knockdown of *tlx3* generated the opposite phenotype. Simultaneous suppression of Ca activity and knockdown of *tlx3* gene function phenocopied the result obtained by *tlx3* knockdown (Fig. 1a,b). Overexpressing mRNA encoding Na_v to increase sodium channel expression and enhance Ca activity (Borodinsky et al., 2004) caused an increase in the incidence of GABA-IR neurons and a decrease in the incidence of VGluT1-IR neurons, while overexpressing *tlx3* produced the opposite phenotype. Simultaneous enhancement of Ca activity and overexpression of *tlx3* phenocopied overexpression (Fig. 1c,d). These results demonstrate that *tlx3* is required for activity-suppression-dependent respecification of GABA and glutamate and that overexpression occludes the switch caused by increased Ca spike

activity. Furthermore, these data indicate that Ca activity regulates neurotransmitter specification upstream of a crucial genetic choice point.

A variant cAMP response element mediates activity-dependent *tlx3* transcription

The interaction between activity and *tlx3* in specifying neurotransmitter phenotype makes the regulation of this gene attractive for analysis of the integration of genetic factors and early activity-dependent processes controlling neuronal differentiation. Comparing human and mouse genomic sequences with more distantly related vertebrates has proven successful for identifying important cis-regulatory elements (Nakano et al., 2005; Ovcharenko et al., 2004). We adopted this approach to identify elements that may be important for imparting activity dependence. Comparative genomics revealed that only a single region of the gene is conserved among *X. tropicalis*, mouse, and human for ~15 kb upstream and ~40 kb downstream of the gene (see Methods). 384 bp of the promoter region upstream of the start ATG is 65% conserved including a 152 bp subregion that is 81% conserved (Fig. 2a). This 384 bp region drives eGFP expression in the dorsal spinal cord and sensory ganglia, similar to the endogenous expression pattern, demonstrating that it is sufficient to recapitulate native expression of *tlx3* (Supplementary Fig. 6a). In addition, expression is observed in dorsal muscle, indicating that the 384 bp region is likely missing an additional negative regulatory element that normally

represses expression in this tissue. Because this small region is highly conserved among frog, mouse, and human, we surmised that it may contain an activity-responsive element.

To test this hypothesis, we generated a wildtype reporter construct to express the firefly luciferase gene under the control of the conserved 384 bp of the upstream promoter region. This reporter was co-injected with a normalization vector (see Methods) and mRNA encoding K_{ir} or Na_v to suppress or enhance Ca-activity, respectively. Suppressing Ca-activity increased expression of the luciferase reporter while enhancing Ca-activity decreased reporter expression (Fig. 2c). The relatively smaller change observed when enhancing activity may indicate the presence of additional activity-dependent regulatory elements not contained in our reporter. No changes in reporter expression were observed when assays were performed on larvae from which the neural tube had been dissected (Supplementary Fig. 6b), indicating that expression of luciferase in other embryonic tissue is not activity-dependent and that the activity-dependent changes observed are occurring in the neural tube. In addition, no changes in reporter expression were observed when luciferase assays were performed on embryos prior to the period of Ca activity responsible for regulating neurotransmitter specification, demonstrating that activity present during earlier periods of development does not influence reporter expression (Supplementary Fig. 6c).

These data demonstrate that transcriptional regulation of the genetic pathway by Ca spike activity could determine neurotransmitter specification and suggest that there may be activity-responsive elements within this promoter. Analysis of the 384 bp promoter region for conserved transcription factor binding sites identified a variant form of the cAMP response element (CRE; TGATGTCA), an activating protein-1 (AP1) site, a signal transducer and activator of transcription (STAT) site, in addition to two nuclear factor Y (NFY) loci previously shown to be important for basal transcription of *tlx3* (Fig. 2b) (Borghini et al., 2006). The high conservation of this region between *X. tropicalis*, mouse, and human suggests the importance of these sites for regulation of *tlx3* transcription.

CRE and AP1 have both been implicated in activity-dependent transcriptional regulation (Flavell & Greenberg, 2008; Sugiyama et al., 2007). To determine the requirement of these sites for *tlx3* regulation by Ca activity, we performed additional luciferase assays with reporters containing either a mutated AP1 binding site ($AP1_{mut}$ reporter; Fig. 2d) or a mutated CRE (CRE_{mut} reporter; Fig. 2e). Mutating the AP1 site had no effect, while mutating the CRE abolished the activity-dependence of reporter expression. These data indicate that the CRE is required for activity-dependent regulation of *tlx3* and suggest that this site could integrate genetic and activity-dependent specification of glutamatergic and GABAergic phenotypes.

cJun regulates the intrinsic neurotransmitter specification pathway

We tested the ability of candidate transcription factors to bind the CRE sequence in the *tlx3* promoter using electrophoretic mobility shift assays (EMSAs). Previous work indicates that cJun and ATF2 preferentially bind the variant CRE sequence TGATGTCA (Heckert et al., 1996). Incubating probe containing the CRE and flanking sequence from the *tlx3* promoter with recombinant cJun or ATF2 caused a shift in migration of the probe that was blocked with excess unlabeled probe, but not by excess unlabeled probe containing a mutant CRE or a non-specific probe (Fig. 3a,b). Luciferase assays demonstrated that constitutively overexpressing a dominant negative mutant of cJun lacking the transactivation domain ($cJun_{TAM}$) (Peng et al., 2002), but not a similar dominant-negative $ATF2_{TAM}$, caused a CRE-dependent increase in the reporter (Fig. 3c). We confirmed the presence of cJun in the embryonic spinal cord by RT-PCR (Fig. 3d) and immunocytochemistry (Fig. 3e). These data suggest that cJun can act through the variant CRE site to regulate transcription of *tlx3*.

To avoid early developmental defects caused by constitutive manipulation of cJun with morpholinos, we generated hormone-inducible overexpression constructs for wildtype cJun and the dominant negative mutant $cJun_{TAM}$. These overexpressed fusion proteins remain inactive until injected embryos are incubated with dexamethasone (Picard, 2000). Inducing

overexpressed wildtype cJun decreased wildtype reporter levels in the luciferase assay (Fig. 4a), while induction of $cJun_{TAM}$ increased wildtype luciferase reporter expression (Fig. 4b). The effects were abolished when the CRE site was mutated in the luciferase reporter (Fig. 4a,b). These experiments show that cJun can control *tlx3* transcription via the CRE binding site.

We next directly assessed the role of cJun in neurotransmitter specification by overexpressing wildtype or dominant negative $cJun_{TAM}$ and immunostaining for GABA-IR and VGluT1-IR. Inducing overexpressed cJun increased the number of neurons expressing GABA and decreased the number of neurons expressing VGluT1 (Fig. 4c,d), similar to the results obtained for *tlx3* knockdown. In contrast, inducing $cJun_{TAM}$ generated the opposite phenotype (Fig. 4c,d), similar to *tlx3* overexpression. Thus, both Ca activity and cJun regulate intrinsic neurotransmitter specification by modulating *tlx3* transcription via the CRE site located in its promoter.

Phosphorylation of the cJun transactivation domain integrates activity-dependent and intrinsic neurotransmitter specification

To find out if cJun integrates genetic and activity-dependent neurotransmitter specification pathways we determined whether Ca spike activity regulates cJun function. The extent of cJun expression was unaffected by manipulation of Ca activity via unilateral injection of mRNA encoding K_{ir} or

Na_v (Fig. 5a). Phosphorylation of S63, S73, T91 and T93 is the primary basis of regulating the transactivation domain of cJun (Raivich, 2008). Because cJun regulation of *tlx3* was altered by deletion of the transactivation domain, we ascertained whether Ca activity regulates phosphorylation of amino acids in this region. Immunocytochemistry using phosphospecific antibodies showed that phosphorylation of S73 and T91 was decreased in response to Ca spike suppression and increased in response to Ca spike enhancement (Fig. 5b,c).

Mutations converting all four residues to alanines ($cJun_{mut}$) reproduced the CRE-dependent increase of *tlx3* reporter expression observed for the cJun transactivation domain mutant in luciferase assays (Fig. 6a). Overexpressing this phosphorylation mutant caused a reduction in GABA-IR and an increase in VGlut1-IR (Fig. 6b,c). These experiments demonstrate the functional role of these phosphorylation sites and identify cJun phosphorylation as the Ca spike entry point in the genetic pathway.

Our results reveal a mechanism by which early activity and genetic factors interact to drive differentiation. The data support a model in which Ca spike activity prior to synapse formation modulates the genetic pathway for specification of neurotransmitter phenotype (Fig. 7a). It is currently unclear whether Ca spike activity functions cell-autonomously. Ca spikes could cause phenotypic changes within the spiking cell or they could initiate a signaling cascade to neighboring cells via diffusible factors. The downstream signaling

cascade initiated by Ca activity, however, is cell-autonomous and involves phosphorylation of cJun that regulates *tlx3* transcription through a CRE site in its promoter (Fig. 7b). cJun appears to function as a repressor of *tlx3* transcription. Although cJun is most often described as an activator, it has been demonstrated to function as a repressor, operating by recruiting co-repressors to the promoter or sequestering other activation factors (Ghosh et al., 2005). The conservation of the variant CRE site in *Xenopus*, mouse and human *tlx3* suggests that activity-dependent regulation of *tlx3* is present in mammals, as well as in *X. tropicalis*. There may exist other species-specific sites that impart additional activity-dependence that we did not find in our cross-species analysis.

Many other transcription factors are involved in neurotransmitter specification, but their relation to activity-dependent processes is unclear. However, several examples of activity-dependent neurotransmitter regulation involve transcription: suppression of RNA synthesis prevents Ca-dependent changes in incidence of the GABAergic phenotype and specific frequencies of spontaneous Ca transients upregulate glutamic acid decarboxylase 67 (GAD67) transcripts in embryonic spinal neurons (Watt et al., 2000). Changes in Ca spike activity change the number of neurons expressing tyrosine hydroxylase transcripts in the postembryonic brain (Dulcis & Spitzer, 2008). Synaptic activation leads to appearance of GAD67 and vesicular GABA transporter transcripts in adult glutamatergic granule cells and $GABA_A$

receptor-mediated responses to granule cell stimulation (Gomez-Lira et al., 2005). These results suggest that the interplay between activity and genetic pathways in neurotransmitter specification will be extensive. Indeed, the difference between the changes in VGluT1-IR and GABA-IR that we observe when manipulating activity is likely caused by the involvement of other transcription factors, which may or may not be differentially regulated by activity. Future work will determine whether the mechanisms underlying activity-dependent specification of other neurotransmitters share common elements.

This integration of activity and genetic pathways may have important implications for development of the nervous system. The Ca activity-dependent switch from excitatory glutamatergic to inhibitory GABAergic phenotype through regulation of a binary selection gene could reverse the polarity of a developing neuronal circuit. Our findings raise the intriguing possibility that activity-dependent regulation occurs at other fundamental choice points during development. Activity-responsive genes may identify developmental switches where extrinsic factors impinge on intrinsic pathways.

Activity plays an important role in many aspects of neuronal development. The functions and mechanisms of synaptic activity have been extensively studied. For example, this form of activity regulates the number and strength of synapses formed (Flavell et al., 2006; Lin et al., 2008) and cell

survival (Hardingham et al., 2002). Less is known about the function of earlier forms of activity, although it has been identified throughout the nervous system in many organisms. Early activity regulates proliferation of neuronal precursors and neuronal migration as well as neuronal differentiation (Spitzer, 2006). Ca transients occur at many times and in many places during development, but the mechanisms by which their patterns regulate differentiation have not been fully elucidated. It is unclear how Ca transients generated at frequencies of 1-20 hr^{-1} initiate the signaling cascade regulating neurotransmitter phenotype. Coding of transcription by the frequency of Ca transients has been reported in T lymphocytes (Dolmetsch et al. 1998) and in basophilic leukemia cells (Li et al., 1998). Stimulation of CaM kinase II, NFAT and OCT/OAP transcription factor activity depends on relatively high frequencies of Ca transients (De Koninck & Schulman, 1998; Dolmetsch et al. 1998; Li et al., 1998). In contrast, activation of the NF-kB transcription factor by Ca oscillations occurs at low frequencies (Dolmetsch et al. 1998) that are within the range of those generated by developing *Xenopus* spinal neurons. It will be interesting to assess the role of cJun and CRE in other organisms and to identify signaling components linking Ca spikes to changes in cJun activity. N-terminal Jun kinases regulate cJun activity via phosphorylation of S63/S73 and T91/T93, making them attractive candidates.

METHODS

Neuronal culture and calcium imaging. *X. tropicalis* neuronal cultures and *in vitro* and *in vivo* calcium imaging were performed as previously described for *X. laevis* (Borodinsky et al., 2004; Chang & Spitzer, 2009).

Constructs and plasmids. hKir2.1 (K_{ir}) and rNa$_v$2aαβ (Na_v) were gifts from E. Marban and W. Catterall. pGL3 and pRL were gifts of C. Murre. *nβgal* was a gift from R. Harland. K_{ir} was myc tagged. The *tlx3* promoter was cloned into the pGL3 vector. The full-length *tlx3* cDNA clone was amplified from a stage 25 cDNA library (tlx3$_{FL}$-forward: 5'-ATG GAT CAG CCA ACA AGT GC; tlx3$_{FL}$-reverse: 5'-TTA CAC TAA TGA GGT ACA TGG G) and cloned into pCS2-MT. The *tlx3-eGFP* chimera was made by fusing 15bp of 3' UTR immediately upstream of the start codon and the first 4 amino acids of *tlx3* to *eGFP* in pCS2. Full-length or truncated cJun (lacking the first 123 amino acids corresponding to the transactivation domain) was amplified from genomic *X. tropicalis* DNA and cloned into pCS2-MT-GR.

***In situ* hybridization.** Antisense and sense RNA probes to *tlx3* were constructed by subcloning the coding region of *tlx3* into pCR2.1 (Invitrogen) and digoxigenin-labeled RNA probes were generated (T3 megascript kit, Ambion). *In situ* hybridization was performed as described (Sive et al., 2000) and followed by immunostaining (Sive et al., 2000) with a 9e10 myc antibody (Abcam ab32) to identify the injected side of the embryo. Images were

captured using AxioVision (Zeiss) and a Zeiss Discovery V12 stereomicroscope and analyzed using ImageJ. Background mean intensity was determined for a region of the embryo with no *in situ* signal and the threshold for positive signal set to two times the standard deviation above this value. Data for the injected side were normalized to the uninjected side.

Morpholino oligonucleotides. MO experiments were carried out with 25-mer MOs (GeneTools) directed against the splice junction between the second exon and intron of the *tlx3* gene ($MO_{tlx3\text{-}SB}$: AGT CGG AGC CAC TTT ATC TCA CCT C) or against the translation start site ($MO_{tlx3\text{-}TB}$: TGG CTG ATC CAT CCT CTG GCA ATA G). 10 ng of either *tlx3* MO were injected into *X. tropicalis* embryos at the one cell stage. $MO_{tlx3\text{-}SB}$ was fluorescein-tagged and $MO_{tlx3\text{-}TB}$ was coinjected with rhodamine-dextran where specified.

mRNA microinjection. mRNA was generated (mMessage mMachine kit, Ambion), and capped RNA (50-250 pg) was injected as described [9]. All injections were done at the one-cell stage except where indicated.

Immunocytochemistry. Embryos were fixed and sectioned; sections were immunostained by incubation in a blocking solution of 2% fish gelatin for 1 h at 22°C, followed by overnight incubation with primary antibodies to GABA (Chemicon AB175), VGluT1 (Sigma V0389), cJun (Santa Cruz Biotechnology sc45), p-cJun (S73) (Santa Cruz Biotechnology sc-7981) or p-cJun (T91) (Abcam ab28853) at 4°C. When embryos were manipulated by injecting K_{ir}

into one cell of two cell embryos, the injected side was determined by costaining with the 9e10 myc antibody. Similar experiments were performed by co-injecting *Na_v* and *nβgal* followed by costaining with a βgal antibody (Abcam ab9361) to determine the manipulated side. Sections were incubated for 2 h with fluorescently tagged secondary antibodies at 22°C. Immunoreactivity was examined on a Zeiss Axioscope with a 40x objective. Slides were blinded and randomized, and neurotransmitter phenotype was quantified by counting the number of immunoreactive cell bodies in the spinal cord in 10 consecutive 10 µm sections from a 100 µm length of the neural tube starting immediately posterior to the hindbrain. All stained cells were counted in each section, independent of cell size. Slides were costained with DAPI to visualize nuclei and staining was compared in consecutive sections to ensure that each cell was counted only once.

Genomic analysis. The ECR Browser (Ovcharenko et al., 2004) was used with default settings (100 bp, 70% identity) to identify regions of the *tlx3* gene conserved from *X. tropicalis* to mouse to human. Additional conserved intronic sequences of the *ranbp17* gene are located ~ 15 kb upstream of *tlx3* and an additional 100 bp conserved region of unknown function lies ~40 kb downstream of *tlx3*. There are no other conserved sequences for 150 kb downstream. Scans using less stringent parameters (down to 25 bp and 50% identity) revealed no additional elements.

Luciferase assays. *X. tropicalis* embryos were injected at the one-cell stage with pRL normalization vector and promoter driven pGL3 luciferase DNA at a ratio of 1:50. Embryos were pooled in groups of 10 and lysed at stage 28 (Dual Luciferase Reporter Assay kit, Promega). Luminescence was measured on a TD-20/20 luminometer (Turner Designs) as the ratio of pGL3 luciferase activity to pRL luciferase activity. Data were normalized to the reporter-alone condition for each experiment.

EMSAs. EMSAs were performed using the buffer system and protocol described in the Promega Gel Shift Assay System. Reactions were supplemented with 0.01 mg/ml poly(dI-dC)•poly(dI-dC) and 5 mM DTT. 0.6 µg of recombinant cJun (Promega E3061) or 1.1 µg of ATF2 (Santa Cruz Biotechnology sc-4007) were incubated with or without competitors at room temperature for 10 minutes, followed by incubation with biotinylated probe for 20 minutes at room temperature. probe: 5'- TGT TAA AAT GAT GTC ACC TTG GAA; $comp_{mut}$: 5'-TGT TAA AAG TCC TCG CCC TTG GAA; $comp_{NS}$: 5'-GAT CGA ACT GAC CGC CCG CGG CCC GT. EMSAs were run on 4% native PAGE gels as described in the Promega protocol and developed (Lightshift Chemiluminescent EMSA Kit, Pierce).

RT-PCR. Total RNA was isolated from embryos at the indicated stages (RNAqueous 4-PCR kit, Ambion) and cDNA was synthesized (Superscript III first strand synthesis kit, Invitrogen). tlx3-forward: GCG ATT CGT GAA GGA

AAG AT; tlx3-reverse: TCA CAG ATC TGC ACC CTT GA. cJun-forward: TCC TCC ACT GTC CCC TAT TG; cJun-reverse: GTT TGA GCT GGG CTA CTT GC.

Statistics. *In situs* were prepared from a single clutch of embryos and immunocytochemistry data were collected from three or more clutches. Values are presented as mean±SEM and significance was assessed with Student's *t*-test; * indicates $P<0.05$ with respect to control. Luciferase data are the mean±SEM from at least three clutches. Significance was assessed with the one-sample *t*-test; * indicates $P<0.05$ with respect to reporter alone.

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FIGURES

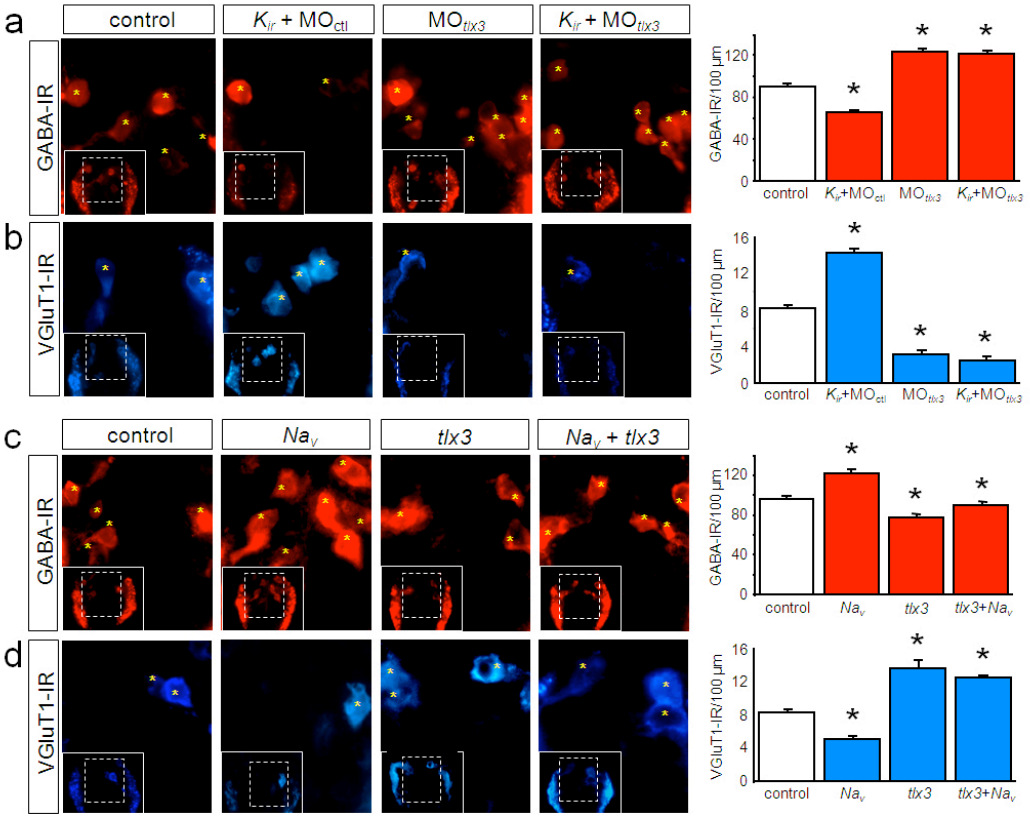


Figure 2.1 | **Activity-dependent specification of GABA and glutamate in embryonic spinal neurons requires *tlx3***. GABA and vesicular glutamate transporter immunoreactivities (GABA-IR, VGluT1-IR) are shown at left and quantified per 100 µm of spinal cord at right. Panels show central regions of the spinal cord (dashed outlines in insets) enlarged to make cell bodies clear (asterisks). Insets show the entire spinal cord, including cell bodies and lateral axon tracts. **a,** GABA specification is suppressed by overexpression of potassium channels (K_{ir}) together with a control morpholino (MO_{ctl}) and enhanced by reducing expression of *tlx3* by splice-blocking morpholino injection (MO_{tlx3}). Combining K_{ir} overexpression and MO_{tlx3} injection (K_{ir} + MO_{tlx3}) yields the MO_{tlx3} phenotype. **b,** glutamate specification is enhanced by K_{ir} overexpression and reduced by MO_{tlx3} injection. Combining K_{ir} overexpression and MO_{tlx3} injection produces the MO_{tlx3} phenotype. **c,** GABA specification is enhanced by overexpression of sodium channels (Na_v) and suppressed by overexpression of *tlx3*. Combining the two perturbations generates the *tlx*3 overexpression phenotype. **d,** glutamate specification is suppressed by overexpression of sodium channels and enhanced by overexpression of *tlx3*. Combining the two perturbations yields the *tlx*3 overexpression phenotype. Scale bar is 100 µm for insets and 25 µm for magnified panels. a-d, data are mean±SEM; n≥7 embryos; *, P<0.05.

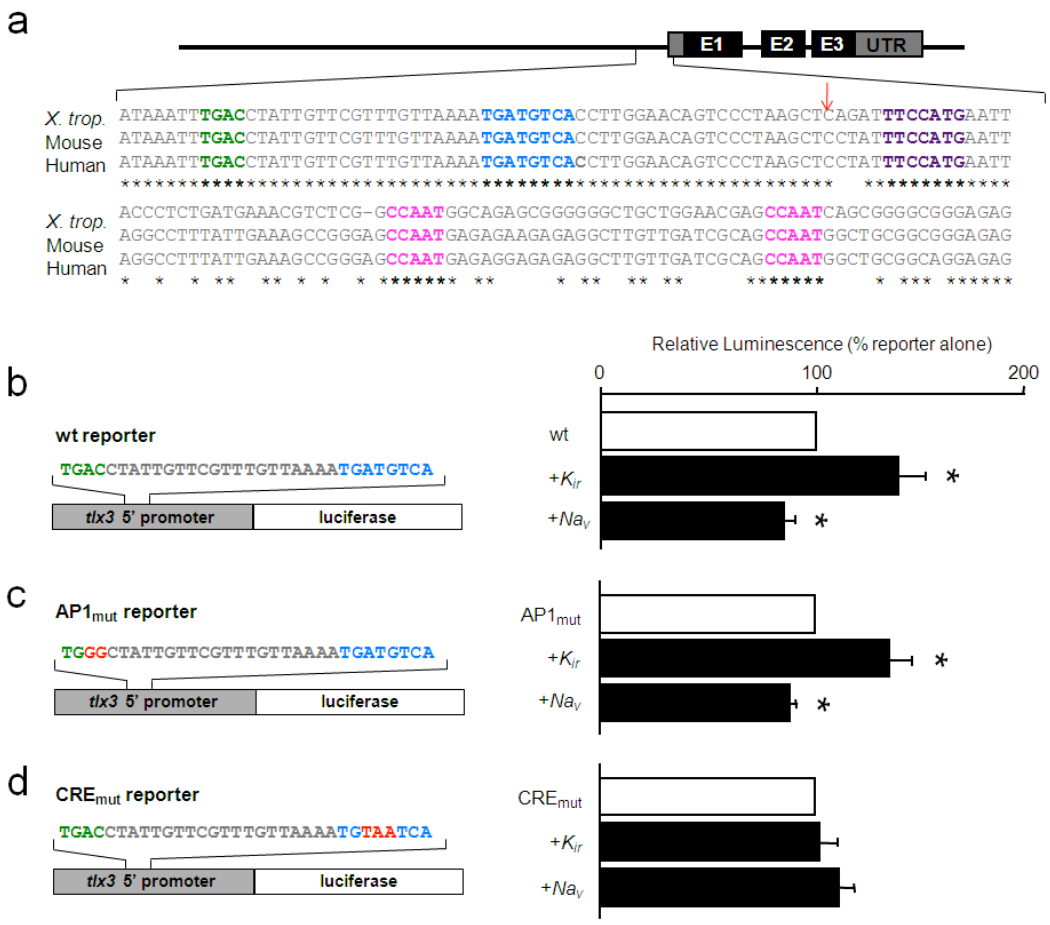


Figure 2.2 | **A variant CRE binding site is required for activity-dependent transcriptional regulation**. **a,** schematic of the *tlx3* gene showing the promoter, exons and UTRs, with alignment of 152 bp of the promoter region containing the AP1 (green), CRE (blue), STAT (purple), and NFY (magenta) elements showing conserved sequence (asterisks) and the major transcriptional start site identified for the human gene (red arrow). **b,** a control construct in which 384 bp of the *tlx3* promoter drives firefly luciferase reports an increase in relative luminescence (RL) when coinjected with K_{ir} and a decrease in RL when coinjected with Na_V. **c,** mutating the AP1 site has no effect on luciferase expression. **d,** mutating the CRE site eliminates the activity-dependence of luciferase expression. b-d, data are mean±SEM; n≥3 clutches; *, P<0.05.

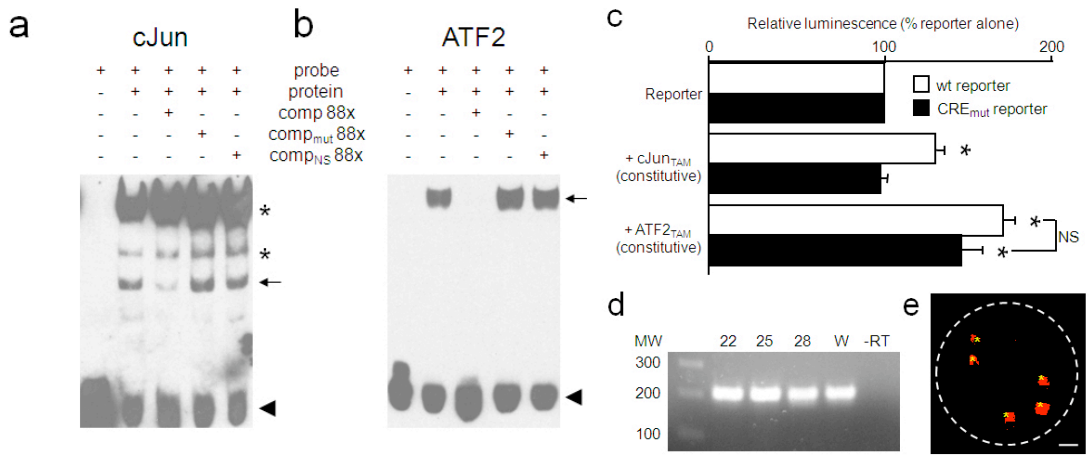


Figure 2.3 | **cJun interacts with the CRE site. a,b,** electrophoretic mobility shift assays show a shift in CRE probe migration in response to cJun (**a)** and ATF2 (**b**) that is eliminated by competition with excess unlabeled probe (comp), but not an unlabeled probe with a mutant CRE ($comp_{mut}$) or a non-specific probe ($comp_{NS}$). Specific bands are indicated by arrows and non-specific bands by asterisks; free probe is indicated by arrowheads. **c,** constitutively overexpressing a dominant negative cJun or ATF2 construct lacking the transactivation domain ($cJun_{TAM}$, $ATF2_{TAM}$) increases the activity of the wildtype *tlx3* luciferase reporter, but only the increase caused by $cJun_{TAM}$ requires an intact CRE. **d,** RT-PCR performed on isolated spinal cords shows *cJun* transcripts are expressed at stages 22, 25, and 28. W: stage 28 whole embryos as positive control, -RT: same as W, without reverse transcriptase in the reaction. **e,** cJun-IR in the spinal cord of a stage 25 embryo. Scale bar is 25 μm. c**,** data are mean±SEM for n≥3 clutches; *, P<0.05; NS, not significant.

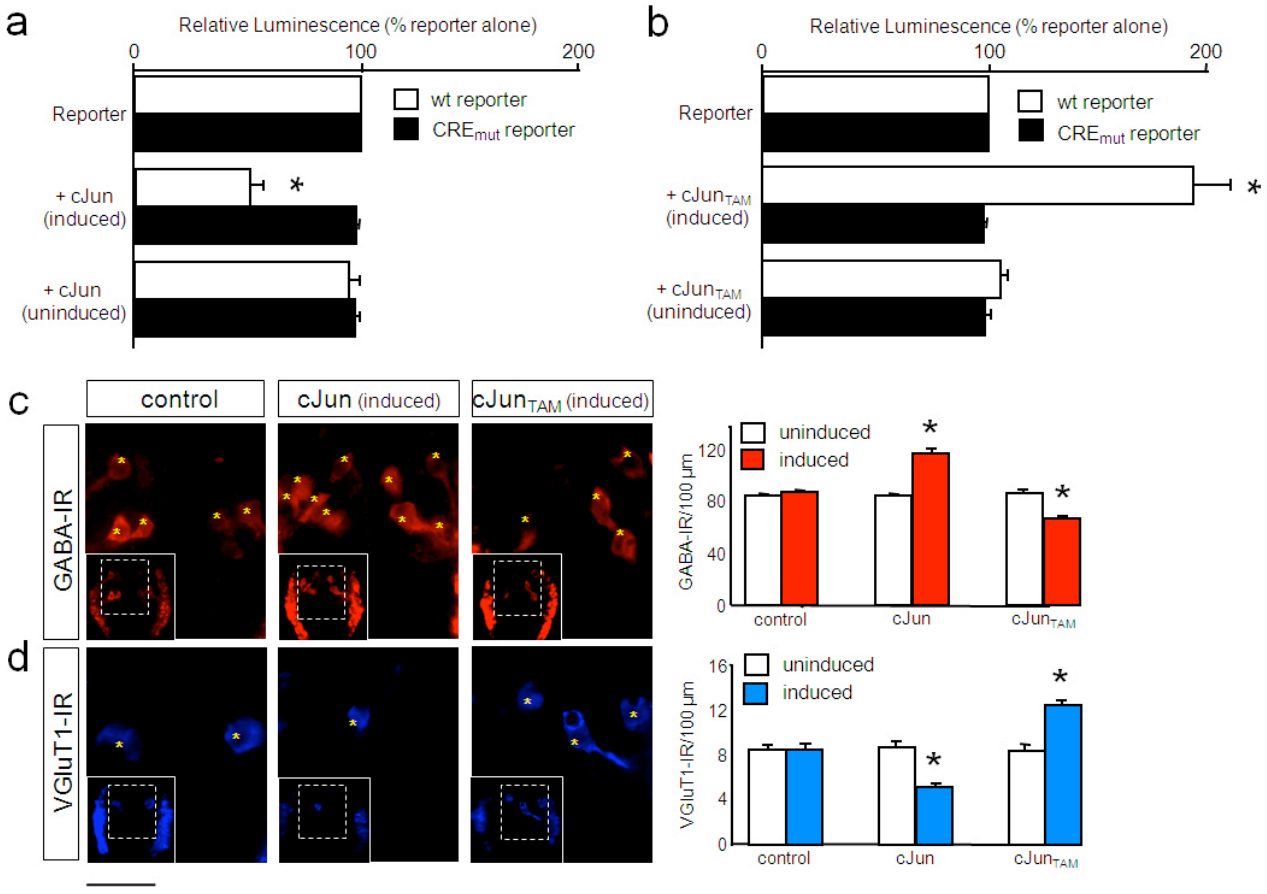


Figure 2.4 | cJun regulates transcription and specification of GABA and glutamate. a,b, inducing overexpressed wildtype cJun at stage 24 causes a reduction in activity of the wildtype *tlx3* luciferase reporter while inducing the dominant negative cJun produces the opposite result. Mutating the CRE site abolishes these effects. **c,d,** inducing overexpressed wildtype cJun at stage 24 causes an increase in the number of GABA-IR neurons and a decrease in the number of VGlutT1-IR neurons, while inducing the overexpressed dominant negative cJun results in a decreased incidence of GABA-IR neurons and an increase in the number of VGluT1-IR neurons. Overexpression of either construct without induction has no effect. Immunostaining for GABA-IR and VGluT1-IR formatted as in Figure 1. Scale bar is 100 µm for insets and 25 µm for magnified panels. Data are mean±SEM; a,b, n≥3 clutches; c,d, n≥15 embryos; *, P<0.05.

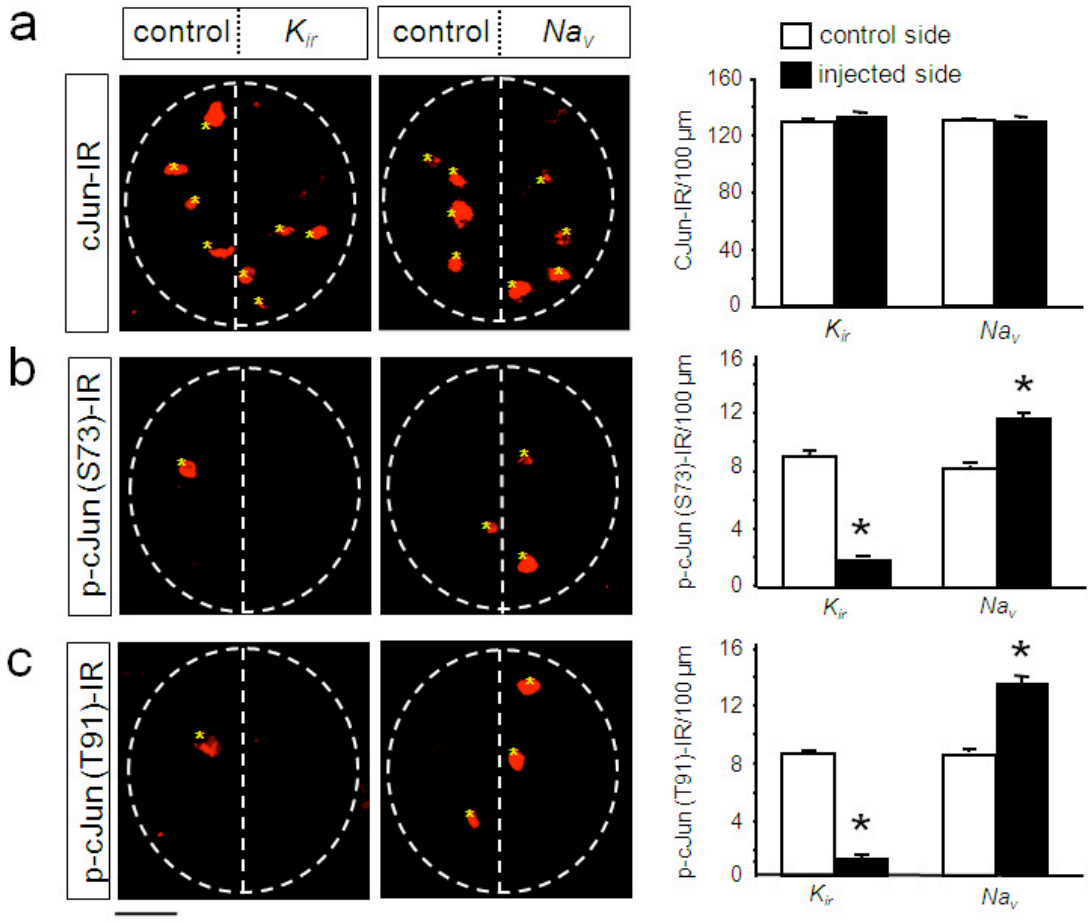


Figure 2.5 | **Ca spike activity regulates phosphorylation of cJun. a,** the number of cells expressing cJun is not changed by Ca activity manipulations. **b,c,** Ca spike suppression causes a decrease in the number of cells expressing cJun phosphorylated at residues S73 [p-cJun (S73)-IR) and T91 [p-cJun (T91)-IR], while Ca spike enhancement causes an increase in the number of cells expressing S73 and T91 phosphorylated cJun. a-c, K_{ir} or Na_v was injected into one cell of two-cell embryos to suppress or enhance Ca spike activity, respectively. Scale bar is 25 µm. Data are mean±SEM; n≥15 embryos; *, P<0.05.

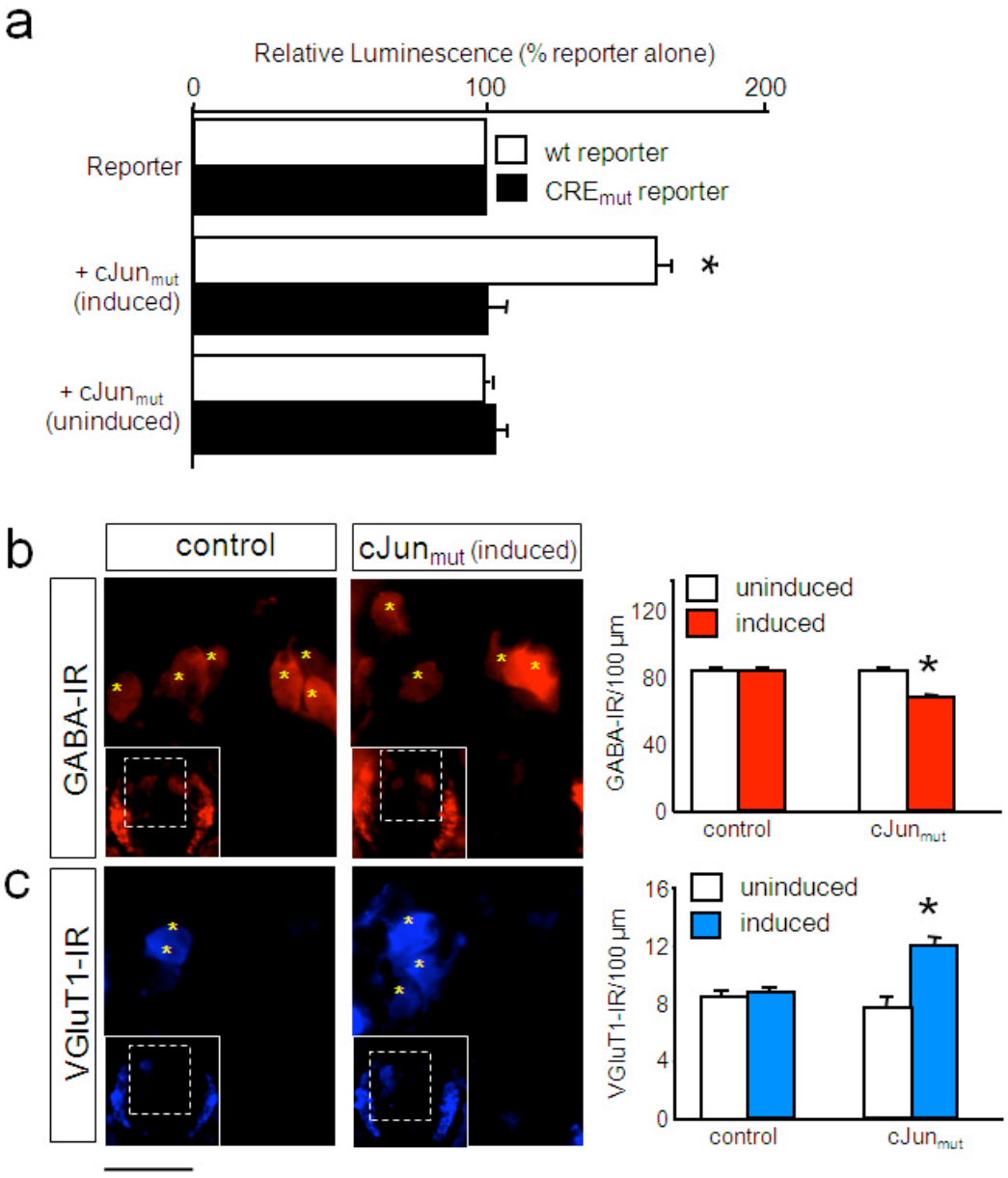


Figure 2.6 | cJun signaling integrates genetic and activity-dependent neurotransmitter specification. a, inducing an overexpressed phosphorylation mutant of cJun (cJun$_{mut}$; S63A, S73A, T91A and T93A) at stage 24 causes an increase in the activity of the wildtype *tlx3* luciferase reporter. Mutating the CRE site abolishes this effect. **b,c,** inducing the overexpressed phosphorylation mutant of cJun at stage 24 causes a decrease in the number of GABA-IR neurons and an increase in the number of VGlutT1-IR neurons. Overexpression of either construct without induction has no effect. Immunostaining for GABA-IR and VGluT1-IR formatted as in Figure 1. Scale bar is 100 µm for insets and 25 µm for magnified panels. Data are mean±SEM; a, n≥3 clutches; b,c, n≥15 embryos; *, P<0.05.

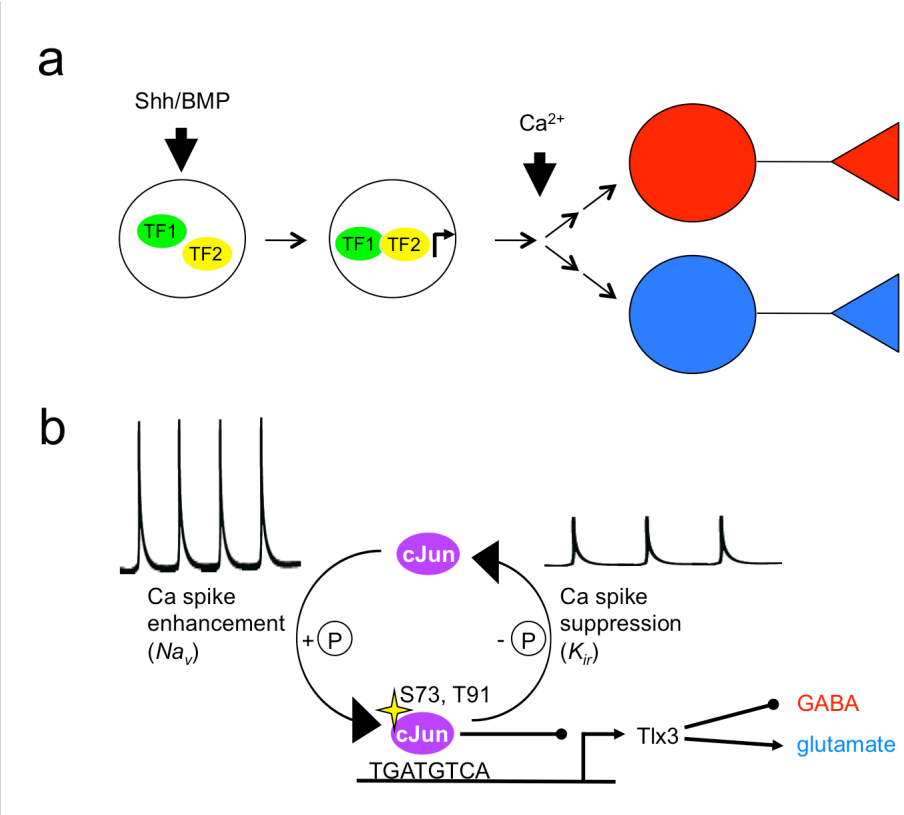
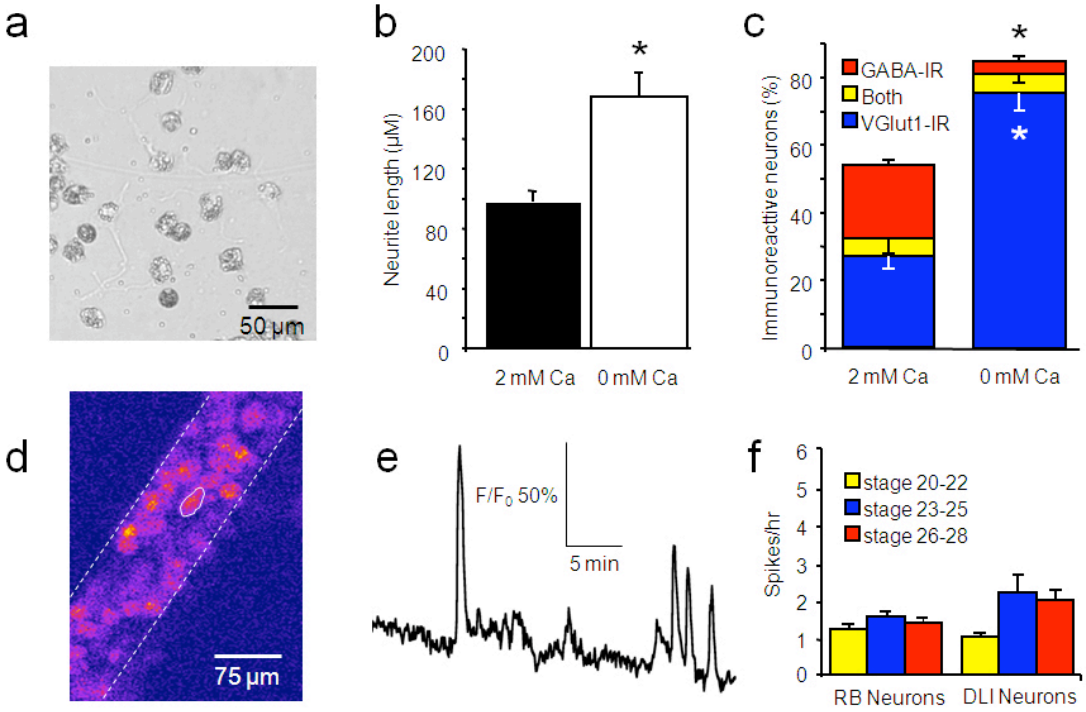
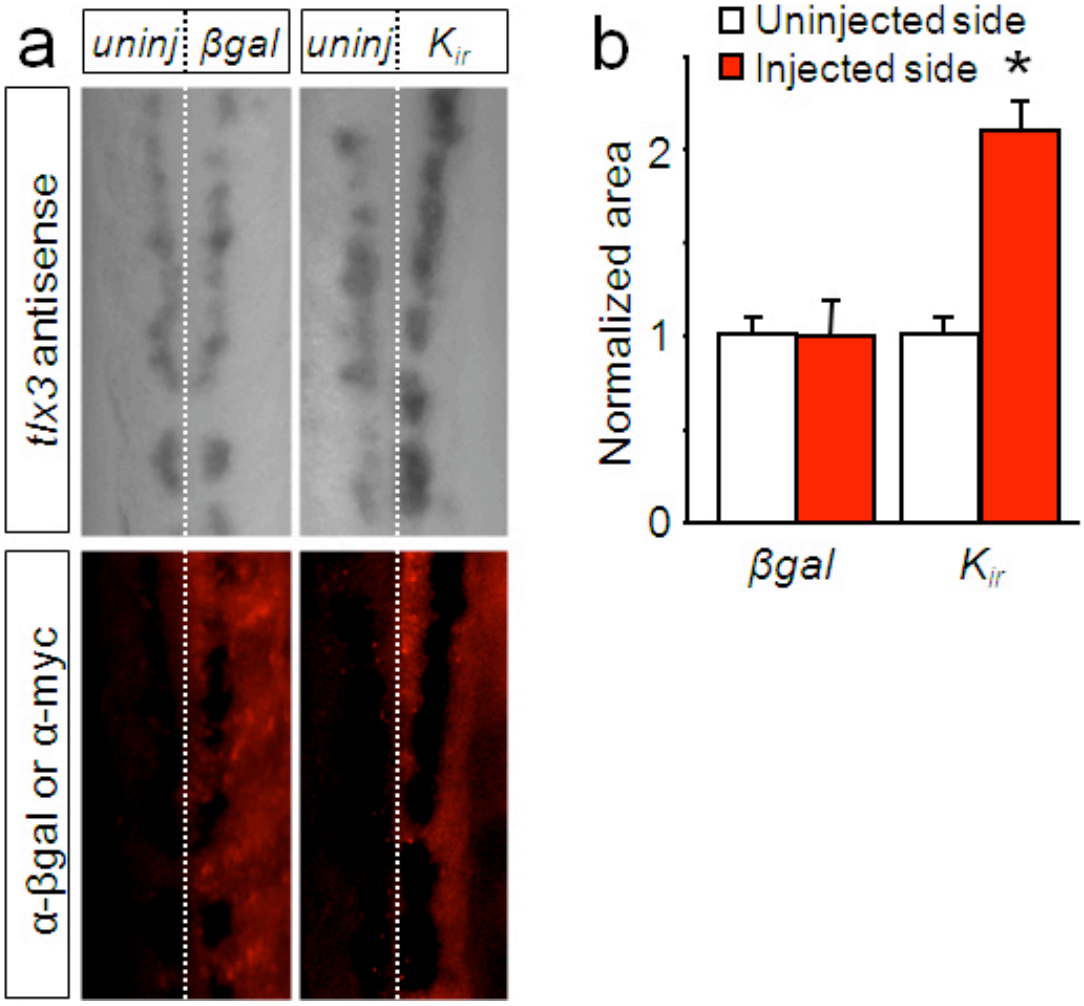


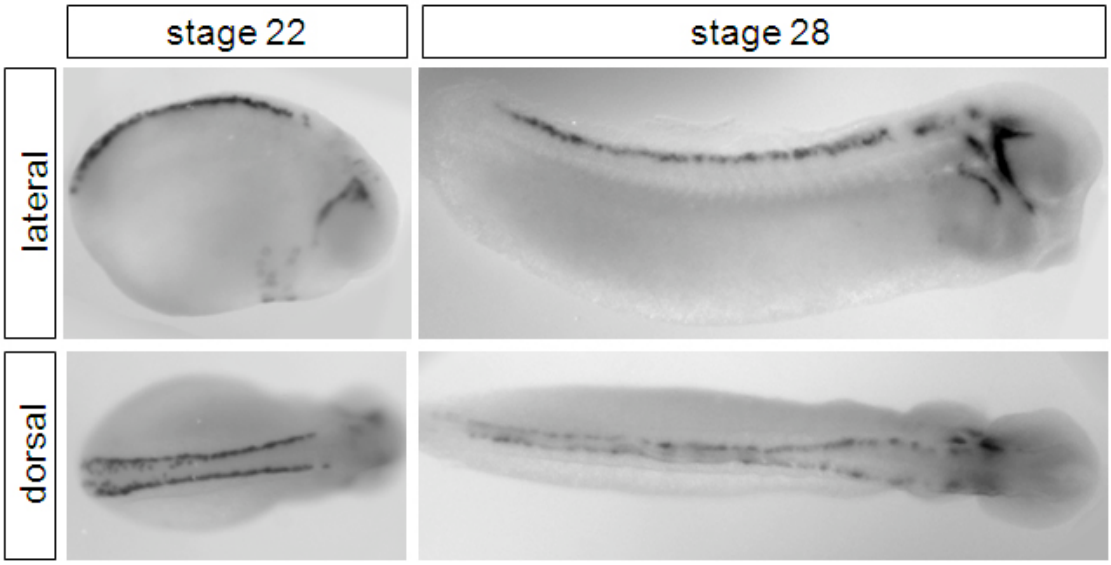
Figure 2.7 | **Model of integration of activity-dependent and intrinsic specification of neurotransmitters by cJun. a,** external morphogens cause the expression of a combinatorial code of transcription factors (TF1, TF2) in neuronal progenitors. Neurons express a cascade of transcription factors that lead to the ultimate neurotransmitter fate. Spontaneous Ca activity impinges on this genetic cascade at a crucial binary switch to regulate neurotransmitter specification. **b,** Ca spike enhancement (Na_v) and suppression (K_{ir}) modulate phosphorylation of cJun S73 and T91. The N-terminal phosphorylation state of cJun regulates transcription of *tlx3* through the CRE in the *tlx3* promoter. Tlx3 selects the glutamatergic fate over the GABAergic fate.



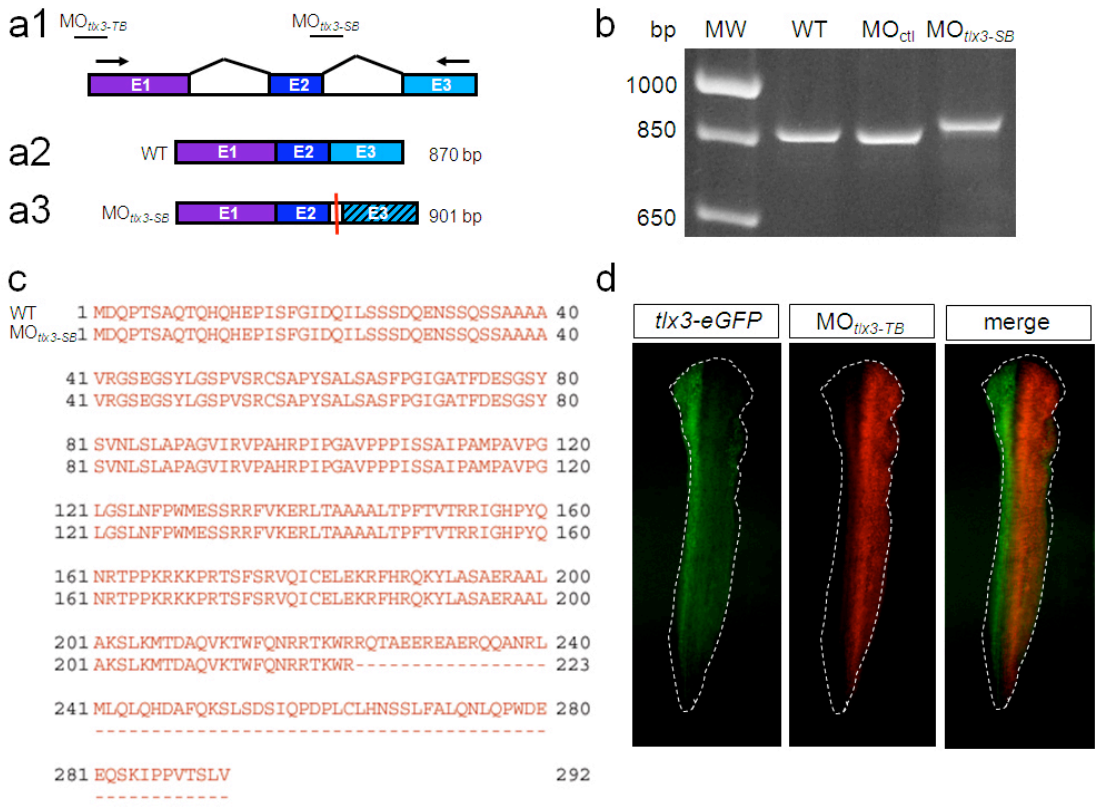
Sup. Figure 2.1 | *X. tropicalis* spinal cord neurons exhibit Ca-dependent development similar to that of *X. laevis* neurons. Previous work demonstrating the role of Ca activity in neurotransmitter specification was performed in *X. laevis*, a tetraploid anuran. *X. tropicalis*, a related species, was chosen for the present study because it is a diploid organism more amenable to genetic studies and its sequenced genome facilitates comparative genomic analyses. **a,** neuronal cultures derived from stage 15 embryos grown in 0 mM Ca culture medium. Scale bar is 50 µm. **b,** neurite length is increased in 0 mM Ca compared to 2 mM Ca, similar to cultures from *X. laevis* [29]. **c,** neurotransmitter phenotype is homeostatically regulated by Ca activity. For neurons grown in 0 mM Ca, 76±5% are immunoreactive for VGlut1, while only 4±2% of neurons are immunoreactive for GABA. In 2 mM Ca 27±3% of neurons are immunoreactive for VGlut1 and 22±4% of neurons are immunoreactive for GABA, similar to results for *X. laevis* neurons [9]. b,c, data are mean±SEM; n≥8 cultures; *, P< 0.01. **d,** a frame from a Ca imaging experiment showing a spiking Rohon-Beard neuron (circled and identified by position; dashed white lines indicate neural tube margins). Scale bar is 75 µm. **e,** the resulting trace from the RB neuron in **d** illustrating Ca-spike activity in a *X. tropicalis* embryo recorded at 0.2 Hz for 1 h after loading with Fluo-4 acetoxymethyl ester. **f,** *X. tropicalis* Rohon-Beard neurons (RB Neurons) and dorsolateral interneurons (DLI Neurons) exhibit Ca-spike frequencies similar to those in *X. laevis* from stage 20 to 28 [9]. Data are mean±SEM; n≥8; *, P<0.05.



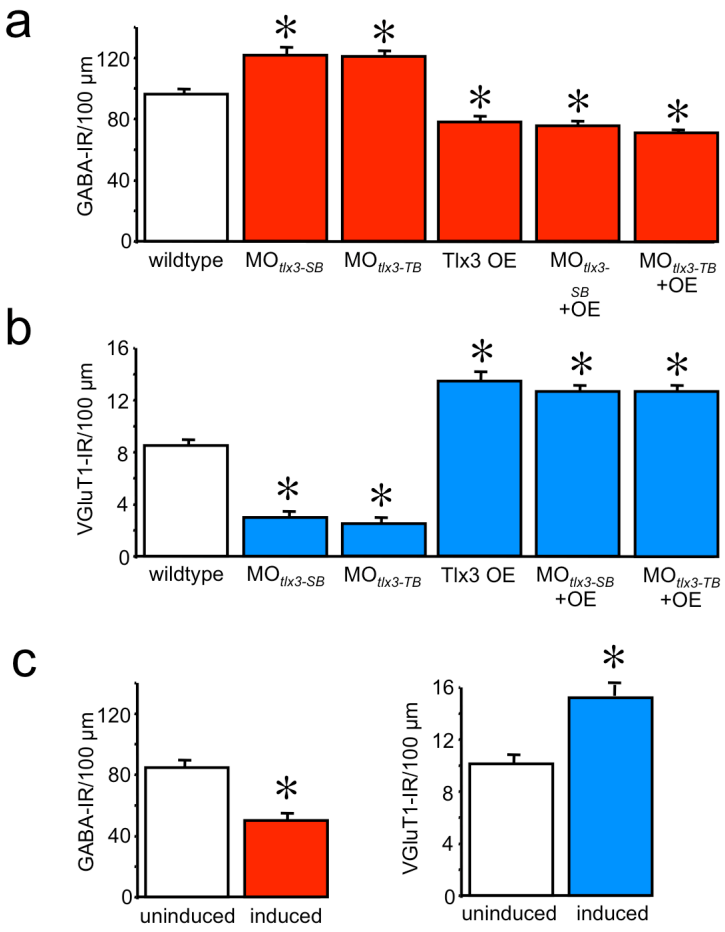
Sup. Figure 2.2 | **Activity regulates *tlx3* gene expression.** One cell of two-cell stage embryos was injected with mRNA encoding nβgal (*βgal)* or myc-tagged K_{ir}; at stage 28 larvae were processed for *in situ* hybridization for *tlx3* and immunohistochemistry for βgal or the myc tag. **a,** dorsal views of spinal cord immediately caudal to the hindbrain of whole mount embryos show no change of *in situ* signal (black) following injection of *βgal* (red) compared to the uninjected side (uninj). *K_{ir}* injection, identified by the presence of myc-immunoreactivity (red), caused an increase in *tlx3 in situ* signal compared to the uninjected side. Rostral is at top; caudal is at bottom. Scale bar is 50 μm. **b,** quantification of the area of *in situ* hybridization signal. Data are mean±SEM; n≥8 embryos; P<0.05.



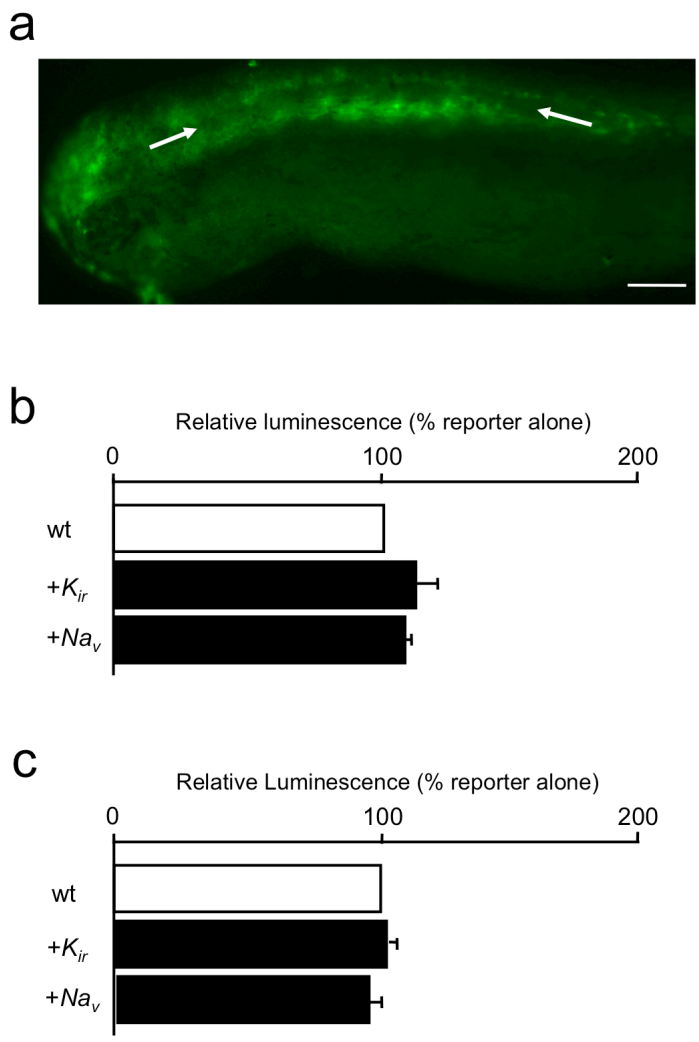
Sup. Figure 2.3 | *tlx3* is expressed in the dorsal spinal cord of *X. tropicalis*. Dorsal and lateral views of stage 22 and stage 28 *X. tropicalis* embryos processed for *tlx3 in situ* hybridization. Scale bars are 250 µm. Rostral is at right; caudal is at left.



Sup. Figure 2.4 | Translation and splice-blocking morpholinos knock down *tlx3* function. a1, schematic showing the location of the *tlx3* translation-blocking morpholino ($MO_{tlx3\text{-}TB}$), the splice-blocking morpholino ($MO_{tlx3\text{-}SB}$), and the PCR primers (arrows) used in **b**. **a2,** schematic of the 870 bp PCR product expected for the wildtype transcript. **a3,** schematic of the 901 bp PCR product expected for the mis-spliced transcript resulting from $MO_{tlx3\text{-}SB}$ that causes inclusion of 31 bp of the second intron (white box). This insertion puts exon three out of frame and introduces an early stop codon (red line). **b,** gel showing PCR products resulting from RT-PCR of RNA harvested from stage 25 wildtype (WT) embryos, and embryos injected with a control morpholino (MO_{ctl}) or $MO_{tlx3\text{-}SB}$. **c,** the PCR products shown in (**b**) were excised and sequenced. The translated sequence for the $MO_{tlx3\text{-}SB}$ product is truncated at amino acid 223. **d,** a one-cell embryo was injected with RNA encoding a chimeric *tlx3-eGFP* reporter with an intact target sequence for $MO_{tlx3\text{-}TB}$ followed by coinjection of $MO_{tlx3\text{-}TB}$ and rhodamine-dextran in one cell at the two-cell stage. *tlx3-eGFP* expression (green) is substantially reduced on the side of the embryo injected with $MO_{tlx3\text{-}TB}$ (red). Image is representative of results obtained from 20 embryos. Scale bar is 250 µm. Rostral is at top; caudal is at bottom.



Sup. Figure 2.5 | *tlx3* overexpression rescues morpholino knockdown of *tlx3* and is effective when induced at stage 24. Co-injecting *tlx3* RNA lacking the $MO_{tlx3\text{-}TB}$ target sequence rescues the increase in GABA-IR (**a**) and the decrease in VGluT1-IR (**b**) caused by knockdown of *tlx3* with either $MO_{tlx3\text{-}TB}$ or $MO_{tlx3\text{-}SB}$. **c**, inducing a *tlx3* overexpression construct with a hormone domain fused to the C-terminus at stage 24 caused a decrease in GABA-IR and an increase in VGluT1-IR similar to that obtained when constitutively overexpressing *tlx3*. Data are mean±SEM; n≥8 embryos *, P<0.05.



Sup. Figure 2.6 | Regulation of reporter expression by a 384 bp *tlx3* promoter. a, the promoter drives eGFP expression in the dorsal spinal cord (above arrows) and dorsal muscle (below arrows). Stage 28 larva. Scale bar is 250 µm. **b, c,** luciferase experiments reveal no activity dependence of reporter expression when the neural tube has been dissected from stage 28 larvae prior to assays (**b**) or when the assays are performed at stage 18, prior to the period of Ca activity that regulates neurotransmitter specification (**c**). Data are mean±SEM; n=3 clutches.

III. Identification of novel molecules that mediate calcium activity-dependent neurotransmitter specification

ABSTRACT

Calcium spikes generated by developmentally transient calcium-dependent action potentials regulate the expression of neurotransmitters. Although we have characterized some of the functional aspects of calcium spikes that lead to regulation of transmitter specification, we still have little understanding of the molecules involved. To discover genes involved in calcium-dependent transmitter specification, we performed an in-situ screen and a gain-of-function genetic screen sensitized to identify molecules in this process. Each of these screens resulted in the identification of several candidate molecules, whose role in neurotransmitter specification was further characterized. One candidate gene, Ptf1a, acts to specify a GABAergic phenotype in an activity-dependent manner in the embryonic spinal cord.

INTRODUCTION

Although we have characterized some of the functional aspects of calcium spikes that lead to regulation of neurotransmitter specification, the molecules involved remain largely unknown. For instance, we do not understand how the initial excitability of neurons is established by processes, such as the regulation of ion channel expression and trafficking to the plasma membrane. We also do not know the identity of the molecule(s) immediately responsive to changes in calcium concentration or downstream of these effectors, such as calcium-dependent kinases, phosphatases and transcription factors. Therefore, in an attempt to identify proteins involved in these aspects of calcium-dependent neuronal cell fate determination, we conducted two genetic screens for molecules involved in these processes.

Design of gain-of-function Screen

We performed a targeted gain-of-function (GOF) expression screen in which we injected pools of mRNAs from a commercially available cDNA library of unique and full-length clones derived from *Xenopus tropicalis* gastrula and neurula stages (Chen et al., 2005; Voigt et al., 2005). We have previously shown that injecting mRNA for hKir2.1 channels suppresses calcium spiking (Borodinsky et al., 2004). By injecting these channels along with mRNA pools in the GOF screen, we sensitized the screen for molecules specifically involved in the process of activity-dependent neurotransmitter specification.

A set of unique and full-length cDNAs was generated from gastrula and neurula stages, and was recently used to screen for genes involved in neurogenesis (Voigt et al., 2005) and mesoderm formation (Chen et al., 2005). Calcium spiking occurs during a ten-hour period starting at stage 20 (the time of closure of the neural tube). The molecules responsible for regulating this period of calcium spiking and the signaling pathways that decode calcium spiking are therefore expected to be initially expressed during the gastrula and neurula stages. For this reason, and because neurotransmitter specification is an early neuronal phenotype, this set of mRNAs is highly likely to contain transcripts of genes in the pathways for calcium spike-dependent neurotransmitter specification. The advantage of this condensed set of cDNAs is that small pools of mRNAs generated from these cDNAs can be injected into fertilized eggs to perform the expression screen. This increases the amount of each mRNA that can be injected, thereby increasing the probability of observing the effect of each mRNA. In addition, since efforts have been made to enrich this set for unique cDNAs there is less redundancy in positive hits identified in the screen (Voigt et al., 2005) (Figure 3.1).

We performed a BLAST search against the Conserved Domain Database on 1156 full-length clones from this *Xenopus tropicalis* cDNA library overlapping the period of calcium spiking, and selected clones containing known transcription factor domains. We also searched the *X. tropicalis* Full-length Database for EST clusters of transcription factors known to be involved

in transmitter specification, and included these clones when they could be verified as full-length. This resulted in 74 transcription factors, which we then divided into pools of 8 for injection into *Xenopus* embryos.

We have previously shown that injecting hKir2.1 to suppress calcium spike activity increases the number of glutamatergic spinal cord neurons and decreases the number of GABAergic neurons. To sensitize the screen for molecules involved in calcium-dependent regulation of transmitter specification, we co-injected hKir2.1 mRNA with pools of mRNA encoding the transcription factors identified above into embryos at the one cell stage. hKir2.1 alone was injected as a positive control. Surviving embryos were fixed at stage 41, sectioned, and immunostained with an antibody to GABA. The number of GABA-immunoreactive (-IR) neurons per 100 μm of spinal cord was assayed (Figure 3.2). Compared to uninjected control embryos, embryos injected with hKir2.1 alone displayed a decrease in GABA immunoreactivity. We identified pools that altered this pattern (Figure 3.3).

Pools that induced 40% penetrance of a phenotype were considered positive. Individual candidates from positive pools were then injected using the same paradigm outlined above to identify single mRNAs that are possibly involved in calcium activity-dependent neurotransmitter specification.

Design of in-situ Screen

In addition to the GOF screen, we performed a targeted in-situ screen of candidate genes that have been demonstrated to regulate neurotransmitter phenotype specification in the embryonic spinal cord. A literature search identified several genes that have been shown to play a role in specifying excitatory versus inhibitory neurotransmitter phenotype. We assessed these genes for their role in activity-dependent neurotransmitter specification in the *Xenopus* spinal cord.

Several transcription factors have been shown to act as binary switches in the selection of an excitatory versus inhibitory cell fate in early post mitotic neurons. Lbx1 acts to establish a basal GABAergic phenotype in the developing embryonic spinal cord (Müller et al., 2002). Tlx1 and Tlx3 play a role in the differentiation of glutamatergic phenotype, antagonizing the inhibitory phenotype established by Lbx1. Expression of Ptf1a drives a GABAergic phenotype at least partly through suppression of the tlx genes (Cheng et al., 2004; Cheng et al., 2005, Glasgow et al., 2005; Hoshino et al., 2005). It is not clear if or how activity interacts with these pathways in the establishment of neurotransmitter phenotype. To assay whether activity acts upstream of these genes to regulate their expression, we performed an in-situ screen with probes to these genes.

We designed and generated in-situ probes for *lbx1, ptf1a, tlx1,* and *tlx3*, and performed in-situs on stage 28 embryos that had been manipulated by unilateral calcium spike suppression via injection of hKir2.1 mRNA in one of two cells at the two cell stage. If expression of the transcript was altered (either increased or decreased) on the manipulated side compared to the control side of the embryo, the candidate gene was further characterized for its role in neurotransmitter specification (Figure 3.4).

RESULTS AND DISCUSSION

GABA expression changes in pools and individual clones for GOF screen

Embryos expressing three of the pools died prior to stage 41. Pool 2 completely occluded the decrease in GABA-IR neurons caused by hKir2.1-mediated calcium spike suppression, indicating that at least one of the clones in this pool may act downstream of calcium activity in determining neurotransmitter specification. We further investigated this pool by testing RNA for each clone separately to identify the specific clone(s) responsible for this activity. In addition, two pools (pools 2 and 4) partially occluded the effect of injecting hKir2.1, but did not return GABA-IR counts to wildtype levels (Figure 3.5a).

We further analyzed two of the pools that occluded neurotransmitter respecification caused by calcium spike suppression (pools 2 and 4). Pool 2 completely occluded while pool 4 partially occluded the reduction in GABA-IR neurons caused by hKir2.1 injection in the primary screen. We prepared mRNA for each individual clone and coinjected each with hKir2.1 mRNA into 1-cell embryos. Embryos were raised to stage 41, and then sectioned and stained for GABA-IR (Figure 3.5b).

Two clones from pool 2 (clones 2D and 2H) and one clone from pool 4 (clone 4A) occluded the hKir2.1-mediated reduction in GABA-IR neurons. Clone 2D had the greatest effect, resulting in an increase in GABA-IR neurons to 118% of wildtype levels (Figure 3.5b).

Candidate molecules from the GOF Screen

Clone 2D (TGas066p02) contains the full-length cDNA of mortality factor 4 like 2 (morf4l2), a member of the MRG family of transcription factors. Although little is known about Morfl2, the related family member MRG15 has been hypothesized to be involved in activity-dependent synaptic plasticity (Matsuoka et al., 2002) and this family of transcription factors is known to coordinate the action of other transcription factors (Bowman et al., 2006).

In contrast to clone 2D, clones 2H and 4A contain well-characterized genes. Clone 2H (TTpA022n08) contains the full-length cDNA of the *Xenopus*

tropicalis homolog of twist1, a basic helix-loop-helix transcription factor. Although involved in cell fate determination, there is no current evidence to suggest endogenous twist1 expression in the spinal cord.

Clone 4A (TTpA009c08) contains the full-length cDNA of LIM homeobox 8 (Lhx8), a transcription factor known to be involved in GABA specification (Bachy et al., 2006; Manabe et al., 2005). Lhx8 has been shown to repress the GABAergic phenotype in forebrain development. This is opposite to the effect we observe in the spinal cord when activity is suppressed. Furthermore, the evidence suggests that lhx8, like twist1, is not expressed in the spinal cord. Although it is possible that lhx8 is expressed at very low levels in the spinal cord, making it difficult to detect, and that it has the opposite effect on GABA specification in the spinal cord than described for it in the brain, we believe it is more likely that misexpression of lhx8 interferes with the function of other related LIM homeodomain transcription factors known to be expressed in the spinal cord. For instance, lhx1 is known to be involved in the maintenance of inhibitory neurotransmitters in the spinal cord (Pillai et al., 2007).

In further screening we identified additional candidate genes, two of which are expressed in the spinal cord (tetraspanin 7 (Tspan-7) and brain acid soluble protein 1 (Basp1)). The tetraspanin family is made up of transmembrane proteins that are involved in cell motility. Tspan-1 is also

expressed in the *Xenopus* spinal cord and is required for primary neurogenesis. Basp-1 is a growth-associated protein and regulates neurite outgrowth in rat hippocampal neurons.

Candidate molecules from the in situ screen

In situ hybridization on unilaterally calcium spike-suppressed embryos revealed calcium activity-dependence of both Tlx3 and Ptf1a transcription in the embryonic spinal cord. At stages 22 and 28 suppression of calcium spiking by hKir2.1 injection leads to a decrease in expression of *ptf1a* and an increase in expression of *tlx3* transcript compared to control (Sup figure 2.2). We further characterized the role these candidate genes play in the pathways regulating the activity-dependence of neurotransmitter specification (see below and Chapter 2).

Testing candidates' effect on neurotransmitter specification

We performed both knockdown and overexpression experiments to alter the expression levels of two of the candidate genes (see Chapter 2 for experiments involving Tlx3), and assayed changes in neurotransmitter expression. To knock down protein expression we designed morpholinos (MOs) to the start site or splice junctions of candidate genes. For overexpression studies, the coding regions of candidate genes were cloned into the pCS2 expression vector and mRNA was transcribed in vitro. MOs or

mRNAs were injected into one cell embryos. Embryos were then raised to stage 41 and fixed and stained with antibodies to GABA.

Identification of Ptf1a as a calcium activity-dependent regulator of neurotransmitter phenotype

We asked whether manipulations in Ptf1a expression lead to changes in GABA expression in the *X. tropicalis* spinal cord. We designed a morpholino to the translation start region of *ptf1a*, which prevents translation of the protein. In addition, we designed an overexpression construct which encoded the full length *ptf1a* transcript fused to a myc tag. We injected the Ptf1a morpholino or overexpression construct into embryos at the single cell stage and then fixed and immunostained them for GABA expression at stage 41.

Overexpression of Ptf1a led to a significant increase in GABA expression, while knockdown of Ptf1a expression led to a significant decrease in GABA expression in the spinal cord (Figure 3.6). Previous knockdown experiments in murine systems demonstrated that progenitor cells require Ptf1a for the generation of GABAergic Purkinje cells and interneurons in the cerebellum and spinal cord (Glasgow et al., 2005, Pascual et al., 2007). Likewise, overexpression of Ptf1a in the dorsal telencephalon produces ectopic GABAergic neurons whose migratory pattern and morphology resemble GABAergic neurons of the cerebral cortex (Hoshino et al., 2005).

The results of our knockdown and overexpression studies similarly indicate that Ptf1a is an important regulator of GABAergic differentiation in the embryonic spinal cord.

Our in-situs indicated that *pft1a* expression is increased by decreases in calcium spiking, suggesting that calcium activity acts in at least a partially overlapping pathway upstream of Ptf1a in regulating neurotransmitter expression. To assay whether activity also acts downstream of Ptf1a in regulating neurotransmitter phenotype, we performed experiments in which we simultaneously increased calcium activity by Na_V overexpression, and knocked down Ptf1a expression by morpholino injection. Morpholino injection alone caused an increase in GABA expression, while Na_V injection caused a decrease in GABA expression. When activity and Ptf1a expression were simultaneously manipulated, expression of GABA was increased, suggesting that calcium activity acts upstream of Ptf1a in regulating neurotransmitter phenotype (Figure 3.6).

Recently, Ptf1a has been shown to mediate glutamate specification in the hindbrain, suggesting that it functions in a region-specific manner to regulate neurotransmitter phenotype (Yamada et al., 2007). It will be interesting to further examine the role Pft1a plays in activity-dependent specification of other neurotransmitters in the spinal cord, and the extent to

which it interacts with additional identified candidate molecules that potentially act in this pathway.

METHODS

Array and plasmid prep. A set of unique full-length commercially available clones (Geneservice LTD, UK) was arrayed into 96 well plates. Each plate was divided into pools (eight clones per pool), plasmid preps were performed using a Qiagen miniprep kit and mRNA was transcribed *in vitro* (Ambion).

Constructs and plasmids. hKir2.1 and rNav2aαβ (NaV) were gifts from E. Marban and W. Catterall. The full-length *ptf1a* cDNA clone was amplified from a stage 25 cDNA library and cloned into a pCS2-MT vector.

Embryo injection. Embryos were injected with mRNA in one cell according to standard methods. 5 ng of mRNA from each pool of eight clones was injected along with 250 pg of myc-tagged hKir2.1 mRNA. For each pool, twenty embryos were injected with hKir2.1 alone and twenty were injected with the pool and hKir2.1. Injected embryos were raised at 25°C until stage 41.

Immunocytochemistry. Embryos were fixed by 30 minute incubation in 4% PFA at 4°C and sectioned; sections were immunostained by incubation in a blocking solution of 2% fish gelatin for 1 hr at 22°C, followed by overnight incubation with primary antibody to GABA (Chemicon AB175) at 4°C. When embryos were manipulated by injecting hKir2.1 into one cell of two cell

embryos, the injected side was determined by costaining with the 9e10 myc antibody. Similar experiments were performed by co-injecting *Na$_V$* and *n*β*gal* followed by costaining with a βgal antibody (Abcam ab9361) to determine the manipulated side. Sections were incubated for 2 hr with fluorescently tagged secondary antibodies at 22°C. Immunoreactivity was examined on a Zeiss Axioscope with a 40x objective. Neurotransmitter phenotype was quantified by counting the number of immunoreactive cell bodies in 10 consecutive 10 μm sections from a 100 μm region of the neural tube starting immediately posterior to the hindbrain.

Scoring of embryos. Slides were blinded and randomized and examined by fluorescence microscopy for changes in NT reporter expression. We identified pools that caused either an increase or decrease in the pattern of NT reporter fluorescence compared to uninjected embryos or embryos injected with channel mRNA alone. A pool was identified as positive if more than 40% of the injected embryos showed a phenotype. These positive pools were then divided into single clones, which were individually injected to identify the positive clone(s) in the pool.

Morpholino oligonucleotides. MO experiments were carried out with a 21-mer MO (GeneTools) directed against the translation start site of the *ptf1a*

gene (MO*ptf1a-TB*: GAA CTG CTC CAG TAC CGT TTC CAT G). 5 ng of the *ptf1a* MO were injected at the one cell stage.

***In situ* hybridization.** Antisense and sense RNA probes to myc-tagged *tlx1, tlx3, lbx1, and ptf1a* were constructed by subcloning the coding region of each gene into pCR2.1 (Invitrogen) and digoxigenin-labeled RNA probes were generated (T3 megascript kit, Ambion). In situ hybridization was performed as described (Sive et al., 2000) and followed by immunostaining with a 9e10 myc antibody (Abcam ab32) to identify the injected side of the embryo. Images were captured using AxioVision (Zeiss) and a Zeiss Discovery V12 stereomicroscope and analyzed using ImageJ. Background mean intensity was determined for a region of the embryo with no in-situ signal and the threshold for positive signal set to two times the standard deviation above this value. Data for the injected side were normalized to the uninjected side.

mRNA microinjection. mRNA was generated (mMessage mMachine kit, Ambion), and capped RNA (50-250 pg) was injected as described (Borodinsky et al., 2004). All injections were done at the one cell stage except where indicated.

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FIGURES

Stage/Library	Clusters in library	Clusters in full-length Geneservice clone set	Additional IMAGE clones
Gastrula libraries (6)	6962	4336	805
Neurula libraries (3)	5525	3638	713
Tailbud libraries (2)	5490	3593	731
Emb7 library	1922	1156	400
Stages 10-30 library	1454	985	223

Figure 3.1 | Basis of the GOF screen

The collection of unique and full-length cDNAs is based on thirteen libraries of clones expressed before and during the period of calcium spiking. A total of 8412 non-redundant clusters were identified from these libraries.

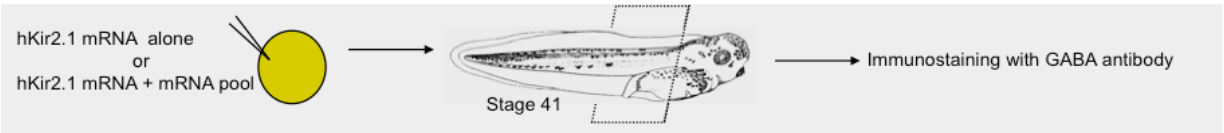

Figure 3.2 | Design of GOF screen

Clones were injected in pools of 8 into *Xenopus tropicalis* embryos at the one-cell stage, along with mRNA encoding hKir2.1. Surviving embryos were fixed at stage 41, sectioned, and immunostained with an antibody to GABA. Injection of hKir2.1 mRNA causes a decrease in GABA expression and clones of interest were identified by their ability to occlude this effect.

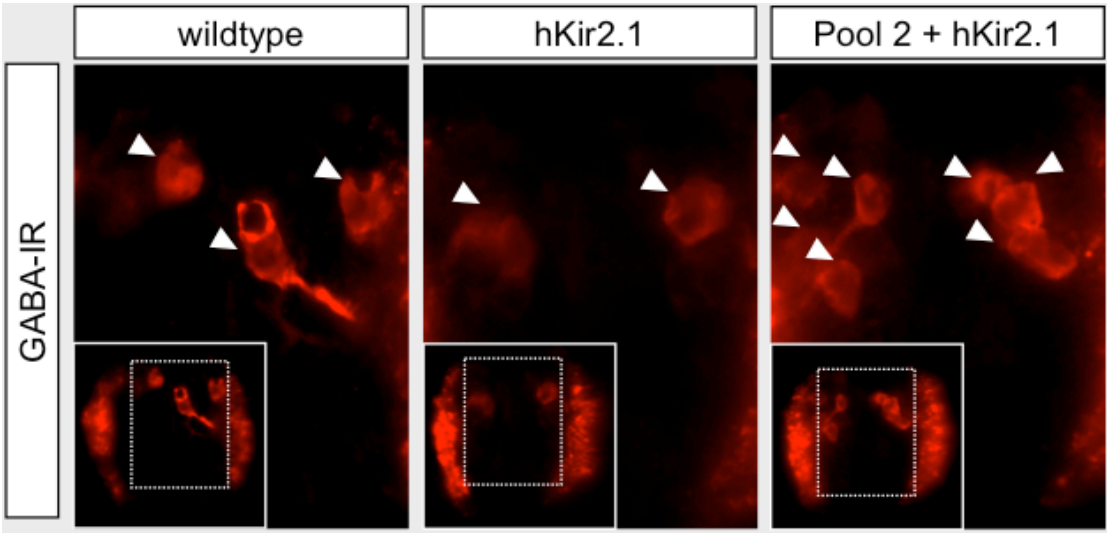

Figure 3.3 | Identification of activity-dependent genes that regulate transmitter expression

Transverse spinal cord sections of stage 41 embryos were stained with antibodies to GABA. Panels show central regions of the spinal cord (dashed outlines in insets) enlarged to make cell bodies clear (arrowheads). Insets show the entire spinal cord, including cell bodies and lateral axon tracts. Injection with hKir2.1 mRNA suppresses Ca activity and reduces the number of GABA-immunoreactive neurons. Co-injection of RNA from pool 2 with hKir2.1 mRNA occludes this effect.

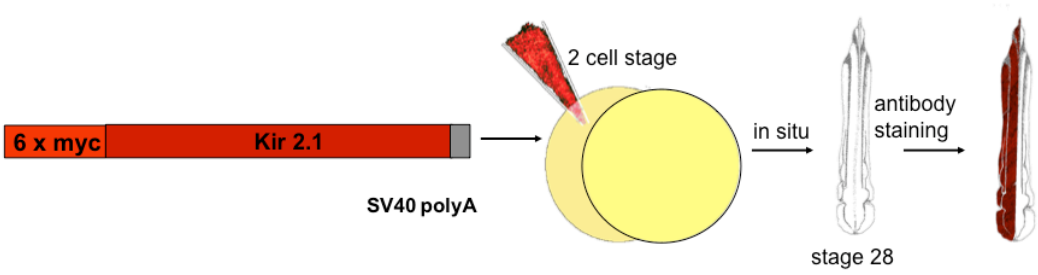

Figure 3.4 | Design of in situ screen

Myc-tagged hKir2.1 mRNA was injected into one cell of two-cell stage *X. tropicalis* embryos to unilaterally suppress calcium spike activity. The embryos were then raised to stage 28, and in situs were performed for candidate gene expression. Embryos were then immunostained for myc expression to determine which side had been manipulated.

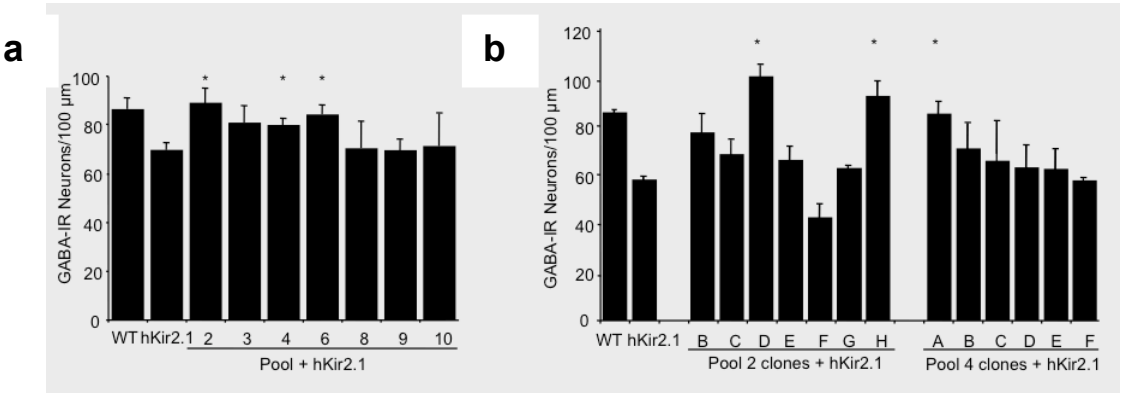

Figure 3.5 | Results of GOF screen: pools and individual clones

a, The number of GABA-IR neurons/100 µm is shown for wildtype (WT) embryos, embryos injected with hKir2.1 mRNA, and for embryos co-injected with hKir2.1 mRNA and mRNA for each pool. Significance ($p<0.05$) compared to hKir2.1 mRNA alone is indicated by asterisks. **b,** individual clones from pools 2 and 4 were injected with hKir2.1 mRNA, and compared to hKir2.1 mRNA alone. Clones 2D, 2H, and 4A significantly increased GABA expression.

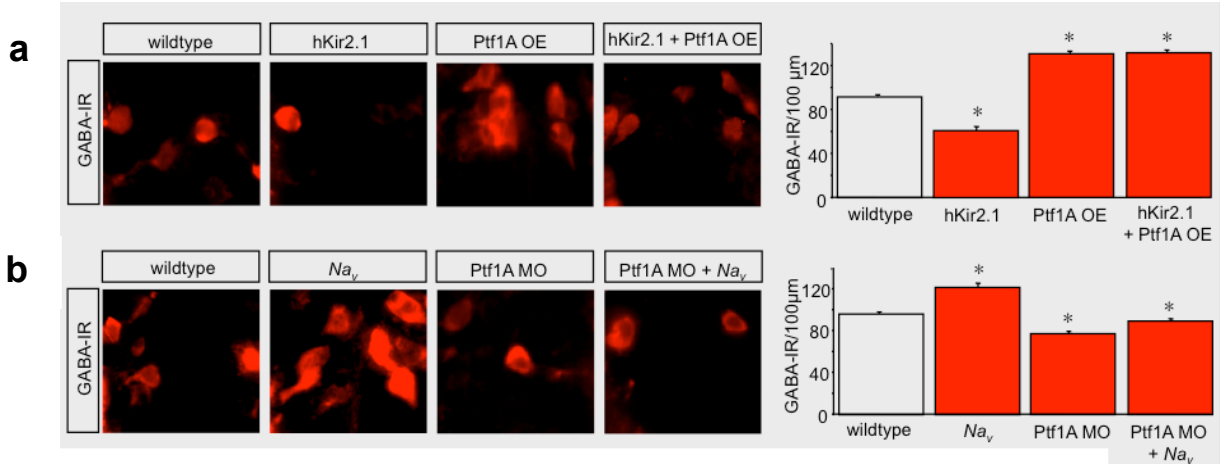

Figure 3.6 | Identification of ptf1a as an activity-dependent gene that regulates transmitter expression

a, mRNA encoding candidate gene Ptf1a was injected into embryos at the one-cell stage alone, or with hKir2.1 mRNA. Embryos were fixed at stage 41, sectioned, and immunostained with an antibody to GABA. Representative images depict GABA-IR in transverse sections of stage 41 embryos. The number of GABA-IR neurons/100 µm is shown for wildtype embryos, embryos injected with hKir2.1 mRNA, embryos overexpressing ptf1a mRNA alone (OE), and for embryos co-injected with hKir2.1 mRNA and ptf1a mRNA. Overexpression of ptf1a causes an increase in GABA-IR, when injected either alone, or with hKir2.1 mRNA. **b,** Knockdown of Ptf1a by morpholino (MO) injection causes a decrease in GABA-IR. Injection of mRNA encoding sodium channels (Na_V) alone increases calcium-spike activity and leads to an increase in GABA-IR. Coinjection of the Ptf1a MO and Na_V mRNA leads to a decrease in GABA-IR, indicating that Ptf1a acts downstream of activity to specify transmitter phenotype.

IV. Generation of transgenic *Xenopus* expressing fluorescent markers of neuronal phenotype

ABSTRACT

The generation of *X. tropicalis* transgenic reporter lines allows novel embryological manipulations and experiments studying neural development. We have generated transgenic reporter lines, based on the expression patterns of molecules involved in neurotransmitter biosynthesis, which express fluorescent reporters of neurotransmitter phenotype. We used the expression of GAD67 as a marker for GABAergic neurons (Watt et al., 2000), ChAT as a marker for cholinergic neurons (Naciff et al., 1999), Hb9 as a marker for motor neurons (Tanabe et al., 1998) and neurogenin as a marker for Rohon-Beard sensory neurons (Blader et al., 2003). In addition, through the use of a cameleon construct and a light-gated ion channel, we developed transgenic lines for observing and imposing calcium spike activity respectively during neuronal development. These lines are useful in facilitating the rapid screening of mutations involved in neurotransmitter fate determination, observing real time changes neurotransmitter expression, and as tools for monitoring and manipulating calcium spiking during embryonic development.

INTRODUCTION

Xenopus embryos are extremely useful for studying mechanisms of development because they are large and develop externally, which greatly facilitates experimental manipulations. Until recently, however, genetic analysis and manipulations have been difficult to perform using *Xenopus* because the most commonly studied species, *X. laevis*, is tetraploid with a generation time of several years. Unlike *X. laevis*, *X. tropicalis* is diploid and has a shorter generation time of 3-6 months. With the characterization and development of *X. tropicalis* as a model for studying development and the sequencing of its genome near completion, genetic experiments are now feasible in *Xenopus*, combining the power of genetics with the ease of manipulating embryos during neuronal development.

The generation of transgenic *X. tropicalis* lines has been optimized by several labs, and many reporter lines now exist in which subsets of neurons are labeled by expression of a fluorescent marker driven by a cell-type specific promoter (Hirsch et al., 2002). These lines have been used for gain-of-function screens (described in Chapter III) in early embryonic development, greatly increasing the speed and efficiency of mutant screening by eliminating the need to perform immunohistochemistry on manipulated embryos (Chen et al., 2005; Voigt et al., 2005) (Figure 4.1).

Previous studies in our lab investigating calcium-dependent neurotransmitter fate determination have relied on static end-point experiments using antibody staining to assay neurotransmitter fate (Borodinsky et al., 2004; Gu & Spitzer, 1995; Watt et al., 2000). We therefore generated transgenic reporter lines based on the expression patterns of molecules involved in neurotransmitter biosynthesis. These transgenic reporters enable us to determine neurotransmitter phenotypes rapidly in the living embryo and whether these phenotypes are altered by changes in calcium spike activity and genetic manipulations without the need for immunohistochemistry (Figure 4.1).

Transgenic reporter lines are also useful for monitoring temporal changes in protein expression in a living animal. By expressing a destabilized version of GFP, it is possible to monitor the real time changes in protein expression throughout embryonic development. In addition, it is possible to perform live imaging of transgenic animals in order to observe changes in axon outgrowth in response to specific manipulations and cues.

Transgenic *Xenopus* embryos are useful for deciphering the precise patterns of calcium activity that are responsible for neurotransmitter specification. By genetically expressing calcium sensors in specific subsets of neurons, we can observe the calcium spike patterns that are generated during neuronal development. Similarly, by genetically expressing a light-gated ion

channel, it is possible to impose specific patterns of activity on the developing embryo to manipulate transmitter phenotype.

Thus *X. tropicalis* transgenic lines are useful in facilitating the rapid screening of mutations involved in neurotransmitter fate determination, observing real time changes neurotransmitter expression, and as tools for monitoring and manipulating calcium spiking during embryonic development. In this chapter I describe the generation and experimental utilization of these reporter lines.

RESULTS AND DISCUSSION

Gad67

Previous studies demonstrated that 10.3kb of 5' upstream sequence in combination with the first exon and intron of the mouse GAD67 gene drives reporter expression that partially mimics the endogenous expression of GAD67 in transgenic mice (Katarova et al., 1998; Jin et al., 2001). We took a similar approach to generating a *X. tropicalis* GAD67 reporter. PCR was used to generate 5' upstream sequences of various lengths along with the first 14 amino acids of GAD67 fused in frame to eGFP. We used these constructs to generate transgenic *X. tropicalis*. We confirmed germline transmission in several of these lines and identified the reporter expression pattern. We successfully generated transgenic *X. tropicalis* driving eGFP expression in a

subset of neurons that appear to be GABAergic and tested this experimentally by doubly staining sections with antibodies to eGFP and to GABA (Figure 4.2).

ChAT

We developed constructs to mark cholinergic neurons by using variable amounts of genomic sequence upstream of choline acetyltransferase (ChAT) to drive eGFP (Figure 4.3). The cholinergic gene locus in *X. tropicalis* is similar to that of nematode, *Drosophila*, human, and mouse in that the vesicular acetylcholine transporter (VAChT) gene resides upstream of the ChAT gene in the same transcriptional orientation. Several cholinergic reporter lines have been created in mice, identifying a cholinergic-specific promoter/enhancer region located upstream of the ChAT gene that includes the VAChT coding exon (Eiden, 1998; Naciff et al., 1999; Schutz et al., 2000). We identified the corresponding upstream region in *X. tropicalis* that includes the coding exon for VAChT. We generated constructs that include 5' upstream sequences of increasing length along with the first 6 amino acids of the first ChAT coding exon fused in frame to eGFP, and generated transgenic *Xenopus* by injection of these constructs. We successfully generated transgenic *X. tropicalis* driving eGFP expression in a subset of neurons and tested the extent to which eGFP expression overlapped with VaChT expression by doubly staining sections with antibodies to eGFP and to VAChT (not shown).

Rohon Beard neurons (RB)

Analyses of the neurogenin 1 (ngn1) promoter in mice and zebrafish revealed conserved enhancer sequences that drive expression in the dorsal neural tube. Three regions 5' of the gene are conserved between human, mouse, and zebrafish (Blader et al., 2003). We analyzed this region in *X. tropicalis* and identified the same three conserved sequences (Figure 4.4): LSE, ANPE, and LATE. The LSE region has been characterized in zebrafish and drives expression in neural plate precursors to Rohon-Beard neurons (Blader et al., 2003). The LATE region drives expression in mature Rohon-Beard neurons of zebrafish and in the dorsal neural tube of mice (Blader et al., 2003; Nakada et al., 2004). We cloned the LSE and LATE enhancers from *X. tropicalis* genomic DNA and placed them upstream of the β-globin basal promoter (derived from BG-eGFP, (Timmer et al., 2001)). We generated several founder lines from the LSE transgene that transmitted to the F1 generation. These lines have not been further analyzed for reliability of reporter expression.

Motor Neurons (MN)

We generated transgenic lines expressing eGFP in motor neurons using a 125bp enhancer of the Hb9 gene recently demonstrated to be conserved in zebrafish, mouse, and humans (Tanabe et al., 1998). We identified this enhancer element in *X. tropicalis* and cloned it from genomic

DNA using PCR. To generate MN-specific expression of eGFP, the enhancer was placed upstream of the β-globin basal promoter driving eGFP. We made *X. tropicalis* lines using this transgene and demonstrated MN expression of eGFP (Figure 4.5).

We also made embryos expressing Hb9-eGFP in the presence or absence of flanking HS(4) insulator sequences (Sekkali et al., 2008). Transgenic *X. tropicalis* embryos with uninsulated transgenes exhibited heterogeneously variegated expression patterns of the eGFP reporter. This is due to positional effects commonly observed for randomly integrated transgenes in many different organisms. In contrast, addition of the HS(4) sequence refined the expression of the transgene and led to a more uniform and specific expression pattern. The inclusion of the insulator sequence resulted in a much more reproducible expression pattern between embryos.

In addition, we developed procedures for imaging motor neurons expressing Hb9-eGFP and extending axons *in vivo*. We visualized ventrolaterally located fluorescent cells extending axons posteriorly that exit the ventral aspect of the neural tube and later ramify on the surface of the trunk muscle (Figure 4.5). The spinal cord was isolated and eGFP-expressing motor neurons were positively identified by their ventrolateral position and posteriorly directed axon. Extension of axons was readily observed in the nascent ventrolateral fascicle as previously described from whole mount

preparations (Gomez and Spitzer, 1999) (Figure 4.5). Transgenic embryos were also used for live imaging of axon dynamics in specific cell types. We visualized axon outgrowth and electrical activity simultaneously through the use of D3cpv cameleon to image calcium dynamics (see below).

Monitoring and manipulating calcium activity using the Gal4/UAS system

We made several *X. tropicalis* lines carrying transgenes to monitor and manipulate calcium spikes *in vivo* under the control of Gal4-binding domains (UAS, Upstream Activating Sequence), as well as lines expressing Gal4 in Rohon-Beard neurons and motor neurons. To ease the process of generating these reagents we began by constructing template plasmids that contained either the UAS binding sites (ISceI UAS-CAR) or the Gal4 gene (ISceI Gal4-CAR), along with RFP under the control of the cardiac actin promoter (CAR), all flanked by I- SceI sites (Figure 4.6). The I SceI sites facilitate transgenesis (Grabher et al., 2004; Pan et al., 2006; Thermes et al., 2002) and the CAR element is used to identify transgenic animals by selecting animals expressing RFP in the musculature (Chae et al., 2002).

To monitor calcium activity *in vivo* we generated a UAS-D3cpv cameleon transgene (Figure 4.5, Figure 4.6) from the UAS template and made several UAS-D3cpv lines that transmitted to the F1 generation. D3cpv, kindly provided by Roger Tsien, was computationally redesigned to minimize

interactions with endogenous signaling pathways while maintaining a high dynamic range.

To determine whether the D3cpv cameleon construct worked in *Xenopus*, we injected D3cpv RNA into *X. laevis* embryos and plated neurons from stage 15 neural tubes. After 8-10 hours in culture we observed spontaneous calcium spikes in these neurons using ratiometric FRET imaging, demonstrating that D3cpv functions in *Xenopus* neurons and can be used to image calcium spiking (Figure 4.5).

To manipulate calcium spiking *in vivo* we generated several additional *X. tropicalis* lines. The first line (UAS-eGFPKir) contains eGFP followed in frame by the human Kir2.1 (hKir2.1) channel, all under UAS control. This line allows suppression of calcium spiking in specific subsets of spinal cord neurons. We also generated a control line (UAS-eGFPKir(mut)) that is identical to the first except the hKir2.1 channel has been mutated in the pore region to prevent ion conduction (Burrone et al., 2002). In addition, we generated a third line in which hKir2.1 is replaced with the rat brain sodium channel rNav2aα (UAS-eGFPNav), allowing enhancement of calcium spiking in specific subsets of neurons. Finally we generated a UAS-ChR2 (channelrhodopsin) line, which can be used to drive calcium spiking at specific frequencies.

To drive these UAS constructs in RB or MN neurons we cloned the promoter elements described above into the Gal4 transgene template. The Hb9- Gal4 line expresses in MN and the neurogenin -LSE- Gal4 line expresses in RB neurons.

METHODS

Phenylthiourea (PTU). Embryos were raised in 75 μM phenylthiourea (PTU) in 10% MMR (Marc's Modified Ringer's) in order to prevent development of pigmentation, allowing better visualization of eGFP and eCFP expression. This concentration of PTU effectively eliminates all pigmentation at stage 41 without affecting embryonic development or normal neurotransmitter specification.

Molecular Biology. Genomic DNA was isolated from stage 41 *Xenopus tropicalis* embryos and the described genomic region was amplified by PCR. Promoter and enhancer regions were cloned into the described vectors (see text).

Transgenesis. Transgenics were generated by the I-SceI meganuclease transgenesis procedure (Thermes et al., 2002; Grabher et al., 2004; Pan et al., 2006). Briefly, 2 nl of transgene reaction mix (20-40 ng/μl plasmid with 0.5 U/μl meganuclease I-SceI in 1x I-SceI Buffer) were injected into dejellied embryos prior to the first cell division. Transgenic embryos were selected by reporter expression at stage 41 using a fluorescence stereomicroscope.

For the generation of UAS and Gal4 transgenic lines, tadpoles were selected by RFP expression in the musculature driven by the cardiac actin promoter

(described in the text). Positive animals were grown to maturity and F1 offspring were used to identify germline transmission.

Immunocytochemistry. Embryos were fixed by 30 minute incubation in 4% PFA at 4°C and sectioned; sections were immunostained by incubation in a blocking solution of 2% fish gelatin for 1 hr at 22°C, followed by overnight incubation with primary antibody to GABA (Chemicon) or eGFP (Chemicon) at 4°C. Sections were incubated for 2 hr with fluorescently tagged secondary antibodies at 22°C. Immunoreactivity was examined on a Zeiss Axioscope with a 40x objective.

Neuronal culture and calcium imaging. *X. tropicalis* neuronal cultures and *in vitro* and *in vivo* calcium imaging were performed as previously described (Borodinsky et al., 2004; Chang & Spitzer, 2009).

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spontaneous Ca^{2+} transients upregulate GAD 67 transcripts in embryonic spinal neurons. *Mol Cell Neurosci 16*, 376-387. (2000).

FIGURES

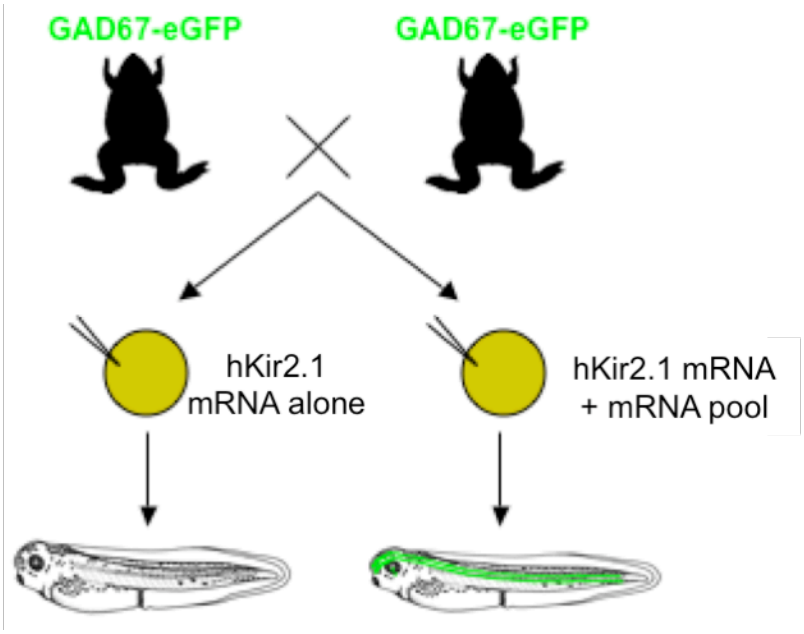

Figure 4.1 | Schematic of potential gain-of-function screen using transgenic *Xenopus.* GAD67-eGFP and hKir2.1 are used to illustrate the procedure, but a similar approach could be used for each of the neurotransmitter reporter lines and for rNa$_V$2aαß. Two homozygous GAD67-eGFP reporter frogs are mated to provide embryos for mRNA injection. Embryos are injected with either the hKir2.1 mRNA alone or the hKir2.1 mRNA plus a pool of eight mRNAs derived from the unique full-length cDNAs (described in chapter III). Injection of hKir2.1 causes a reduction in the incidence of cells expressing the GAD67-eGFP reporter. A fluorescence stereomicroscope is used to screen for mRNA pools that prevent this reduction or cause an alteration in the pattern of reporter expression. Individual clones from the positive pools are then re-screened using the same approach to identify individual genes involved in calcium-spike dependent neurotransmitter specification.

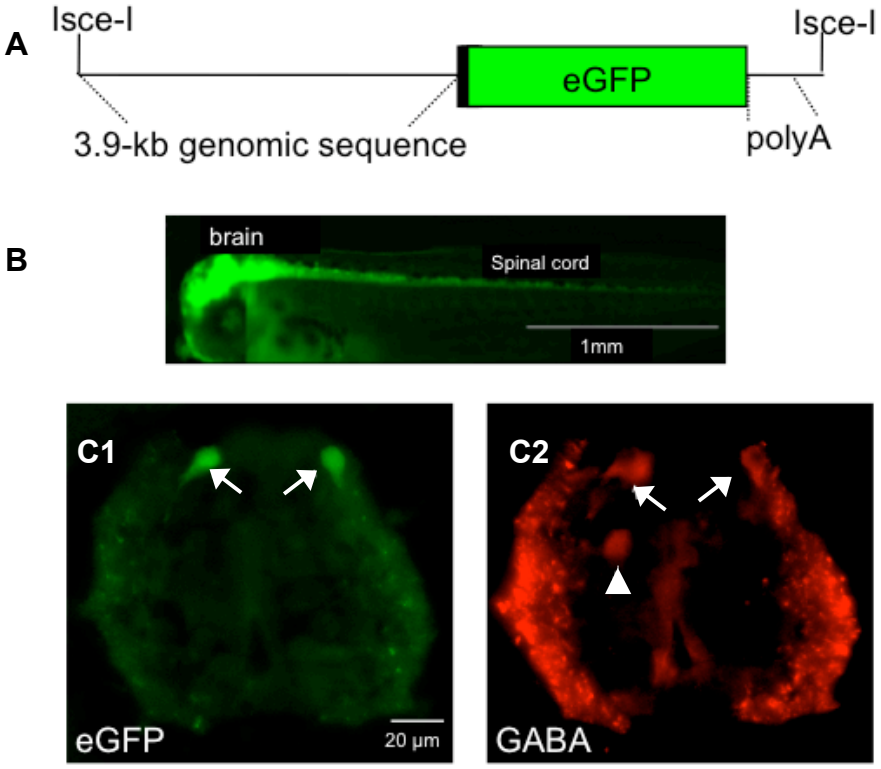

Figure 4.2 | Genomic sequence upstream of GAD67 drives eGFP expression in a pattern consistent with GABAergic neurons. A) Schematic representation of the transgenes used. Variable amounts of genomic sequence (ranging from 3.9-12kb) upstream of xtGAD67 and the sequence coding for the first 14 amino acids (black bar) of xtGAD67 were cloned in frame into a modified pEGFP-N1 vector. The black bar represents sequence coding for 5 amino acids of the vector prior to the eGFP start site. We generated transgenics from these constructs using the I-SceI meganuclease method **B)** A composite low magnification view of a stage 41 transgenic tadpole generated using 3.9kb of genomic sequence upstream of GAD67. Expression is present in the eye, brain, and single cells of the spinal cord. **C)** A transverse section of the spinal cord of a stage 41 transgenic tadpole (9kb of upstream sequence) stained with antibodies to GABA and eGFP (C1: eGFP, C2: GABA). The arrows indicate neurons that expressed eGFP and stained positive for GABA. The white arrowhead indicates a neuron that expressed GABA but was not positive for eGFP, indicating that this transgenic line labels a subset of GABAergic neurons.

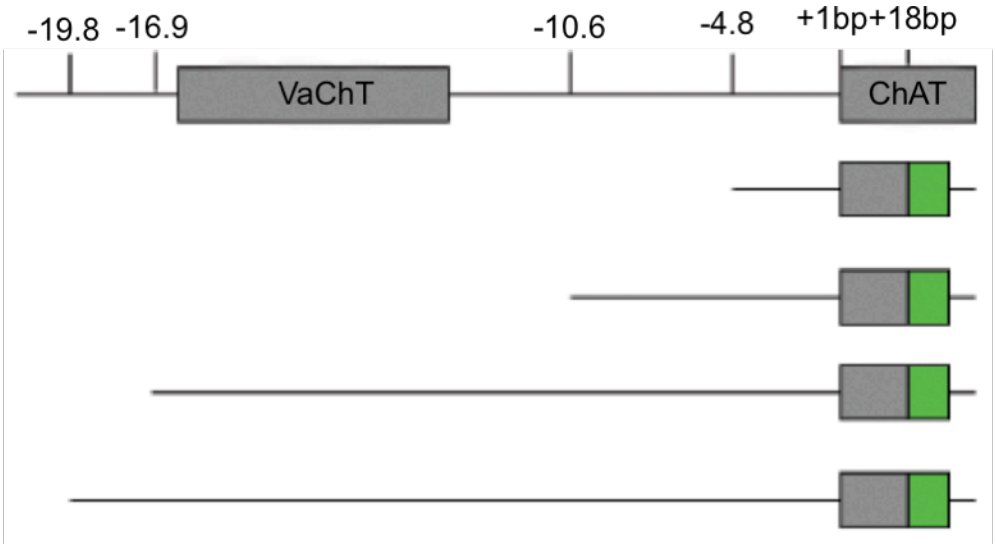

Figure 4.3 | Schematic representation of the 5' flanking region of the *X. tropicalis* cholinergic gene locus, including ChAT and VAChT. The putative ATG start codon is located at position +1, as predicted by JGI version 4.1 of the *X. tropicalis* genome. Protein sequence homology to mouse, human, and rat begins at position +1. Variable amounts of genomic sequence (4.8-19.8kb) upstream of ChAT and the sequence coding for the first six amino acids of ChAT were cloned in frame into a modified pEGFP-N1 vector. The grey box represents the first six amino acids of the vector prior to eGFP, represented by the green box.

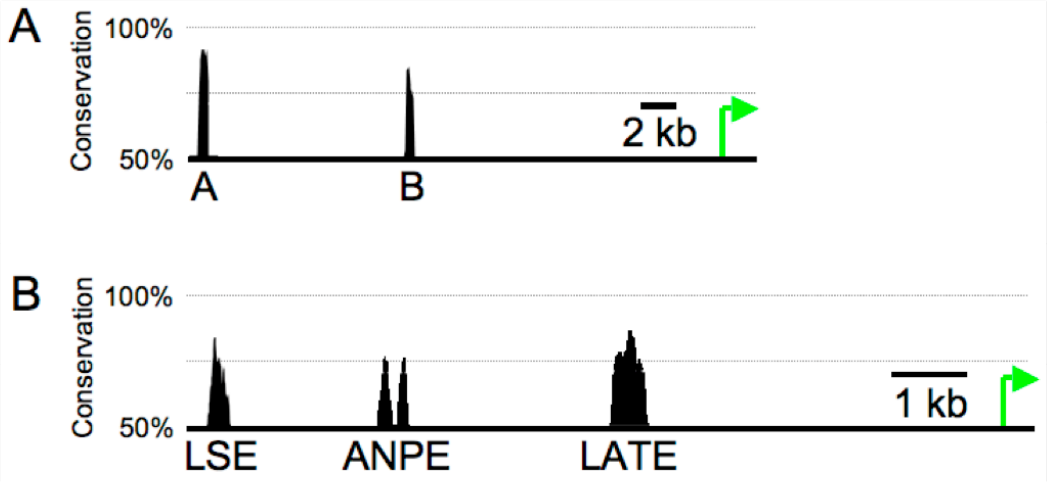

Figure 4.4 | Comparisons of *X. tropicalis* and human genomic sequence for the Hb9 and neurogenin 1 regions. A) Two regions upstream of the Hb9 gene (start site indicated by the green arrow) are highly conserved between *X. tropicalis* and human, as well as mouse and zebrafish (not shown). The region marked as B was shown in mice to drive motor neuron expression. We cloned the B region of the Hb9 locus along with the beta globin minimal promoter upstream of Gal4 to achieve motor neuron expression. **B)** Three regions upstream of the neurogenin 1 (ngn1) gene are highly conserved between human and *X. tropicalis*, as well as mice and zebrafish (not shown). LSE has been shown to drive expression in neural plate stage cells of zebrafish that mature into Rohon-Beard neurons. The LATE region has been shown to drive expression in mature Rohon-Beard neurons of zebrafish and in the dorsal spinal cord of mice. Adapted from JGI and the Vista Genome Browser.

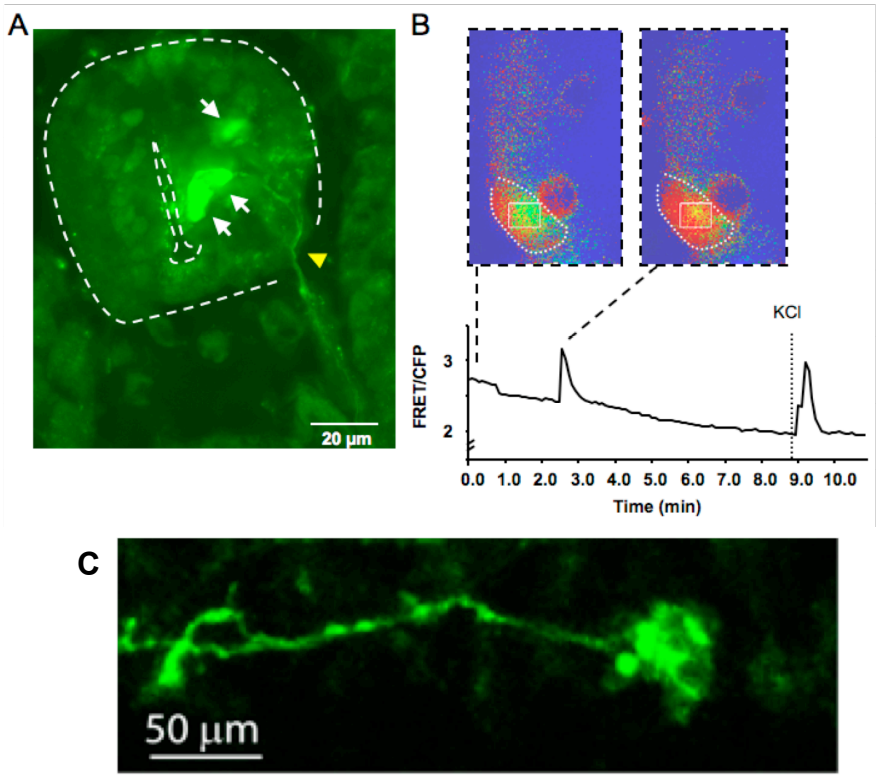

Figure 4.5 | Hb9-eGFP and D3cpv expression. A) Hb9-Bg-eGFP is expressed in motor neurons. Transgenic Hb9-Bg-eGFP embryos were identified by eGFP fluorescence in the spinal cord of stage 41 embryos. Transgenic embryos were fixed and 10 µm transverse sections were cut through the spinal cord and stained with an antibody to eGFP. A projection of a deconvolved z-stack through a section is presented showing three neurons (white arrows) with axons exiting the ventral root (yellow arrowhead). The spinal cord is delineated by the dashed white line. Although only two of the axons are visible in the projected image, focusing through the 3D stack clearly reveals all three axons. **B)** The D3cpv cameleon molecule was used to visualize calcium spikes. One-cell *X. laevis* embryos were injected with 500 pg of D3cpv mRNA. Spinal cord neurons were grown in vitro for 8-10 hours after dissection from stage 15 neural tubes and imaged on a Zeiss Axioskop 2 with a DualView beamsplitter (Optical Insights) to collect simultaneous images for both eCFP and FRET using Slidebook software (Intelligent Imaging Innovations). The resulting FRET/eCFP ratio trace is shown with a spontaneous calcium spike occurring at ~2.5 minutes. 50 mM KCl was added to the recording at ~9.0 minutes to induce calcium influx. Representative images from the baseline and the calcium spike are shown above the trace. **C)** Through-series projection of a confocal stack of images of a motor neuron from a stage 25 *X. tropicalis* embryo expressing Hb9-eGFP.

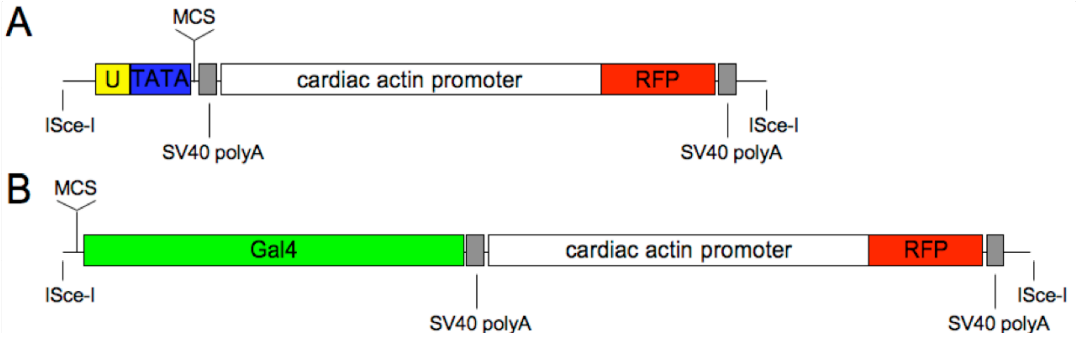

Figure 4.6 | UAS and Gal4 transgenes. A) The UAS template construct. Five Gal4 binding sites (UAS sites, labeled U in yellow) are followed by the 5' UTR sequence for beta globin and the TATA sequences (TATA in blue). The multiple cloning site (MCS) was used to insert any coding sequence of interest. This is followed by the SV40 polyA sequences. Finally, RFP is under the control of the cardiac actin promoter as a marker for transgenic animals. **B)** The Gal4 template construct. Gal4 is preceded by a multiple cloning site (MCS) to place Gal4 under the control of various enhancer/promoter sequences. Gal4 is followed by the SV40 polyA sequences and the cardiac actin-driven RFP as a marker for transgenic animals. Both constructs are flanked by ISce-I sites for making transgenics using the meganuclease procedure.

V. SUMMARY AND FUTURE DIRECTIONS

The experiments described in the preceding chapters further our understanding of the mechanism by which calcium activity in early embryonic development modulates neurotransmitter specification, and how this may enable regulation of subsequent neural activity in a changing external environment. I have demonstrated that Tlx3 acts as a calcium activity-dependent binary switch in selecting a glutamatergic over GABAergic phenotype, and that *tlx3* transcription is regulated in response to manipulations of calcium activity. In addition I have established that the regulation of *tlx3* transcription occurs through the interaction of cJun at the *tlx3* promoter, and that the activity of cJun at this promoter is dependent upon phosphorylation of specific amino acids in the cJun transactivation domain. I also described the design and implementation of two genetic screens for novel molecules involved in calcium spike-dependent neurotransmitter respecification, and the identification of Ptf1a as an activity-responsive regulator of GABAergic phenotype through the use of these screens. Finally, I described the development of transgenic *Xenopus tropicalis* lines for the purpose of screening, monitoring, and manipulating specific subsets of neurons in the embryonic nervous system.

This work contributes to our understanding of the ways in which developmental processes are regulated, and leads to several further questions.

While it is evident that cJun acts to regulate expression of *tlx3*, the complete pathway that links calcium activity to modulation of the *tlx3* promoter remains to be delineated. It is still unclear what occurs upstream of cJun phosphorylation and which pathways and kinases are responsible for its phosphorylation. Similarly, there are likely other molecules that bind to the *tlx3* promoter to act as transcriptional enhancers, activators or repressors, which may or may not act as binding partners with phosphorylated cJun. These molecules may be responsive to different patterns of calcium spike activity, acting as calcium effectors in response to specific coding of stimulation. This precise coding of activity has yet to be determined, and the described transgenic *Xenopus tropicalis* lines are expected to be useful in answering these questions.

In Chapter 3 I described the identification of Ptf1a as an activity-dependent regulator of GABAergic differentiation. It is not clear, however, how Ptf1a and other transcription factors (tlx3, for example) interact in a given progenitor cell to coordinate neurotransmitter specification. These transcription factors may act in at least partially overlapping pathways, and if so it will be interesting to determine which molecule lies upstream in this pathway, through knockdown and overexpression studies. Conversely, these genes may act in nonoverlapping pathways, due to spatial or temporal developmental separation, and this could be examined through protein localization experiments. In addition, it will be interesting to determine whether cJun or

another calcium-sensitive transcription factor acts at the *ptf1a* promoter to regulate transcription. It is possible that cJun acts in a ubiquitous manner to specify neurotransmitter phenotype, and that differences in neuronal differentiation are cell context-dependent. It will also be important to establish whether or not these transcriptional cascades are responsible for the calcium activity-dependence of neurotransmitter specification of other neurotransmitters, such as acetylcholine and glycine (Borodinsky et al., 2004), and whether they act to specify neurotransmitter phenotype in other neural regions as well. Likewise, it will be interesting to examine whether the same transcriptional cascades are responsible for neuronal phenotype specification in other organisms.

Further development of the described *Xenopus tropicalis* transgenic lines will be valuable in addressing these questions. As novel molecules are identified, it will be possible to generate transgenic *Xenopus* in which fluorescent markers are expressed in specific subsets of neurons expressing these novel molecules, enabling characterization of their function in regulating neuronal development.

Finally, it will be important to determine the extent to which these identified pathways are activated in disease states and cognitive disorders. Many disorders of human cognition present during prenatal and early postnatal development, as activity-dependent genes are induced and

repressed, remodeling synaptic connections in response to environmental stimuli. Mutations in activity-dependent pathways may therefore account for cognitive deficits that arise during development. Indeed, recent evidence suggests that mutations in activity-dependent genes are responsible for several of these disorders, including Cav1.2 (Timothy syndrome; Splawski et al., 2004), CBP (Rubenstein-Taybi syndrome; Petrij et al., 1995), and MeCP2 (Rett syndrome; Amir et al., 1999). The further characterization of these context-dependent selector genes and their roles in disease pathogenesis will be essential in developing therapeutic strategies for treatment of developmental disorders.

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