UNIVERSITY OF CALIFORNIA, SAN DIEGO

Screening for Molecules Essential in Activity-Mediated Neurotransmitter Specification

A thesis submitted in partial satisfaction of the

requirements for the degree of Master of Science

in

Biology

by

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2009

The thesis of Jennifer Jeeye Su is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2009

I would like to dedicate this thesis to my family and friends. Thank you for reminding me of God's unfailing love for us.

Your love, oh Lord

Reaches to the heavens

Your faithfulness stretches to the sky

Your righteousness is like the mighty mountains

Your justice flows like the ocean's tide

I will lift my voice

To worship You, my King

I will find my strength

In the shadow of your wings

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ABSTRACT OF THE THESIS

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Professor Nicholas Spitzer, Chair

Neurotransmitter specification, which is vital for appropriate communication between neurons, is developmentally regulated by the combinatorial expression of genes and calcium spike activity. In order to identify molecules that may be involved in activity-dependent neurotransmitter

specification, I performed a genetic screen using 1156 non-redundant full-length cDNAs from the Emb7 library. The Emb7 library is a good starting point for such a screen because these clones are expressed during stages 20-27 when calcium spiking occurs. For both the pilot screen and the main screen, clones were combined in pools of 8 and transcribed into mRNA. The pools of mRNA along with mRNA encoding hKir2.1 were then injected into *Xenopus tropicalis* embryos at the 1 cell stage. Larvae were fixed, sectioned and stained with GABA antibodies. The GABA-IR cells were counted per 100 μM of the embryo spinal cord.

The pilot screen of transcription factors identified 3 individual clones that appeared to occlude the effect of hKir2.1 on calcium spike suppression. Morf4l2, which is expressed in the *Xenopus* spinal cord and related to the MRG family of transcription factors was of particular interest. MRG15 has been shown to be involved in synaptic plasticity and in guiding other transcription factors. In the main, unbiased, screen, 6 clones were of high interest because they are expressed in *Xenopus* spinal cord and have known functions. Of the 6 clones, Brain acid soluble protein 1 (Basp1) and Tetraspanin 7 (Tspan7) are potential candidates for further study. Identifying these types of molecules is vital to understand how neurotransmitter specification is regulated by both gene expression and calcium signaling.

INTRODUCTION

The differentiation of neurons in the brain and spinal cord is regulated by both gene expression and electrical excitability. Sequential expression of genes defines default neuronal phenotypes that are then modified and reshaped by electrical activity, often via regulation of gene expression. The specification of the neurotransmitters used by different classes of neurons is of particular interest, because chemical synapses appear to be the primary basis for communication between neurons. Thus neurotransmitter specification is important for the proper functioning of neuronal networks.

Genetic determination of neurotransmitter phenotype

Neurotransmitters can be excitatory, increasing the production of action potentials, or inhibitory, decreasing the production of action potentials. Glutamate and GABA are the most common excitatory and inhibitory neurotransmitters in the central nervous system. The initial default transmitter phenotype is established by dorso-ventral gradients of signaling molecules that lead to expression of a combinatorial code of transcription factors. Expression of the transcription factor Motor Neuron Restricted 2 (MNR2) in neural cells induces motor neuron differentiation and the expression of acetylcholine as the

neurotransmitter in somatic motor neurons (Tanabe et al., 1998). Transcription factors also act as switches in determining transmitter phenotype: Tlx3 acts to select for expression of glutamate while Lbx1 selects for the GABAergic phenotype (Cheng et al., 2004; Cheng et al., 2005).

Activity-dependent neurotransmitter specification

Along with genetic specification of neurotransmitter phenotype, neuronal activity also plays a key role. When the NMDA class of glutamate receptors was blocked in cultured hypothalamic neurons, normally glutamatergic neurons acquired a cholinergic phenotype (Belousov et al., 2001; Belousov et al., 2002). Significantly, the activity generated by activation of NMDA receptors is calcium-dependent. Activity during early development of petrosal ganglion sensory neurons is required in addition to the presence of the Phox2a/2b homeodomain transcription factor for the normal expression of tyrosine hydroxylase (TH), the enzyme necessary for dopamine synthesis in these neurons (Brosenitsch and Katz, 2002).

During a critical window in the early development of *Xenopus* embryos, calcium spikes regulate neuronal specification both *in vitro* and *in vivo*. Suppressing calcium spike activity increases the number of neurons expressing excitatory neurotransmitters while decreasing the number of neurons expressing inhibitory transmitters. Conversely, the enhancement of calcium spike activity

produces the opposite effect. Ion channel misexpression and pharmacological agents were used to change activity. Importantly, the use of markers of cell identity revealed that cell type does not change when neurotransmitter switches take place following changes in activity (Borodinsky et al 2004). However, the events that follow Ca spiking to determine neurotransmitter phenotype are still unclear. Previous work with cells of the immune system has provided insights into the mechanisms by which calcium transients produce their effects. The expression of transcription factors NF-κB and NFAT is encoded by the frequency of these transients. In contrast, the way in which calcium spikes encode expression of transcription factors and other molecules in *Xenopus* embryos is not yet well understood.

Xenopus tropicalis **as a model system**

Many developmental biological studies have been performed using *Xenopus laevis,* because external fertilization allows access to early stages of development and their large eggs make embryos easy to dissect. Nonetheless the allotetraploid genome of this species has made it difficult to apply genetic tools. *Xenopus tropicalis* was introduced to solve this problem because of its diploid genome. *X. tropicalis* comes originally from the genus *Silurana* because of its unique diploidy. The morphology of *X. laevis* is similar to that of X. tropicalis, but it is smaller in size.

While most of the research regarding calcium signaling comes from studies of *X. laevis*, *X. tropicalis* and *X. laevis* are comparable, specifically during stages 20 to 28 (21.75-32.5 hr of development), in several respects. The rate of neurite outgrowth of embryonic neurons cultured from *X. tropicalis* shows the same dependence on extracellular calcium as neurites of neurons cultured from *X. laevis*. The incidence and frequency of spontaneous calcium spiking in the intact spinal cord of X. tropicalis is similar to that in *X. laevis* (Chang and Spitzer, 2009). Furthermore, experimental alterations in calcium spiking in *X. tropicalis* neurons cause changes in neurotransmitter phenotype as in neurons from *X. laevis*. These similarities show that *X. tropicalis* can be used for studying calcium activity in the same way as *X. laevis*.

Performing a gain of function screen

In order to identify molecules potentially involved in neurotransmitter specification, a gain of function screen was performed. Previous work showed that injection of hKir2.1 mRNA leads to suppression of calcium spike activity that decreases the number of GABAergic neurons present in the spinal cord (Borodinsky et al., 2004). To screen for molecules that might be involved in specification of GABAergic neurons, embryos were co-injected with hKir2.1 mRNA and pools of mRNA encoding transcription factors. The number of GABAergic neurons in these embryos was then scored. These experiments

aimed to test the hypothesis that one or more of these transcription factors are involved in specification of GABAergic neurons in an activity-dependent manner.

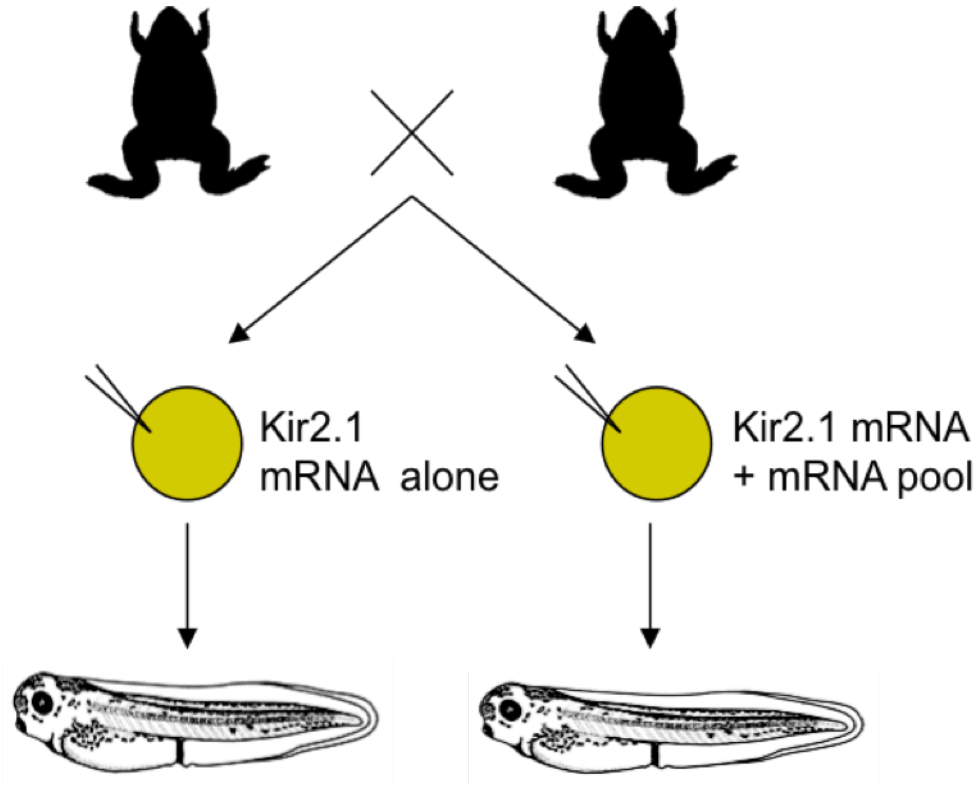


Figure 1. Schematic diagram of hKir2.1 mRNA injections and hKir2.1 mRNA co-injection with mRNA pools into 1-cell stage *Xenopus tropicalis* embryos.

The genes tested were selected from the 24 384-well plates of full-length clones from several cDNA libraries prepared from embryos at the stages during which spontaneous calcium spiking occurs (the Emb7 library and Christof Niehr's stage 10-30 library). Specific clones were chosen either because the transcription factor domains were known or because they were previously found to be molecules involved in neurotransmitter specification.

Table 1. 13 Libraries containing full-length non-redundant cDNAs derived from the time period before and during calcium spiking.

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

RESULTS

In an initial pilot screen, 74 clones encoding transcription factors were selected, inoculated into 200 μL of LB broth and ampicillin on a 96-well plate, and left on a shaker for ~15-18 hr at 37°C. After combining 8 clones together into an eppendorf tube, a total of 10 of these pools were centrifuged and purified with a Qiagen Mini-prep kit. Once resuspended in 30 μL of water, the DNA was digested with NOT 1 restriction enzyme, purified with Qiaquick Gel Extraction Kit and finally resuspended in 30 μL of DNase free water. 1 μg of DNA was transcribed into RNA using a Megascript SP6 RNA transcription kit. 1.6 ng from each pool was then co-injected with 250 pg of hKir2.1 into each of 15-20 *X. tropicalis* embryos at the one-cell stage. The embryos that survived until stage 41 from 7 pools (2, 3, 4, 6, 8, 9 and 10) were fixed, sectioned, stained for GABA and scored for GABA-immunoreactive (GABA-IR) cells per 100 μm length of spinal cord. Three pools of embryos (5, 7, and 11) did not survive to stage 41.

Table 2. Results of the pilot screen with the number of GABA-IR neurons/100 μM for wildtype, hKir2.1 mRNA injected and pool mRNA plus hKir2.1 mRNA co-injected embryos. * indicates significantly different from Kir; $p<0.05$

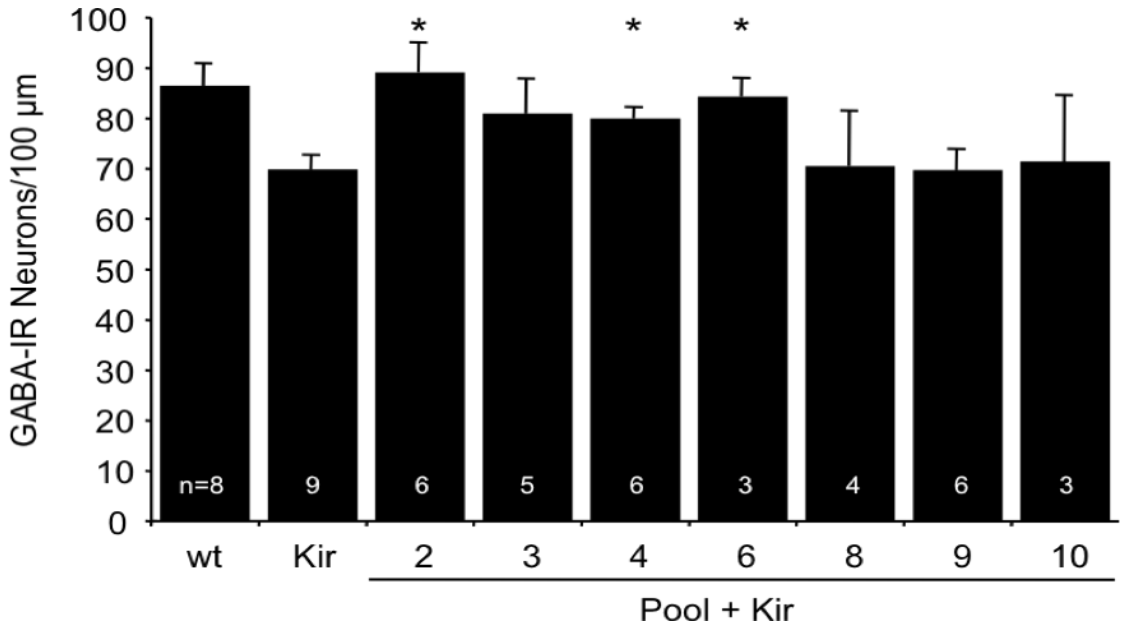
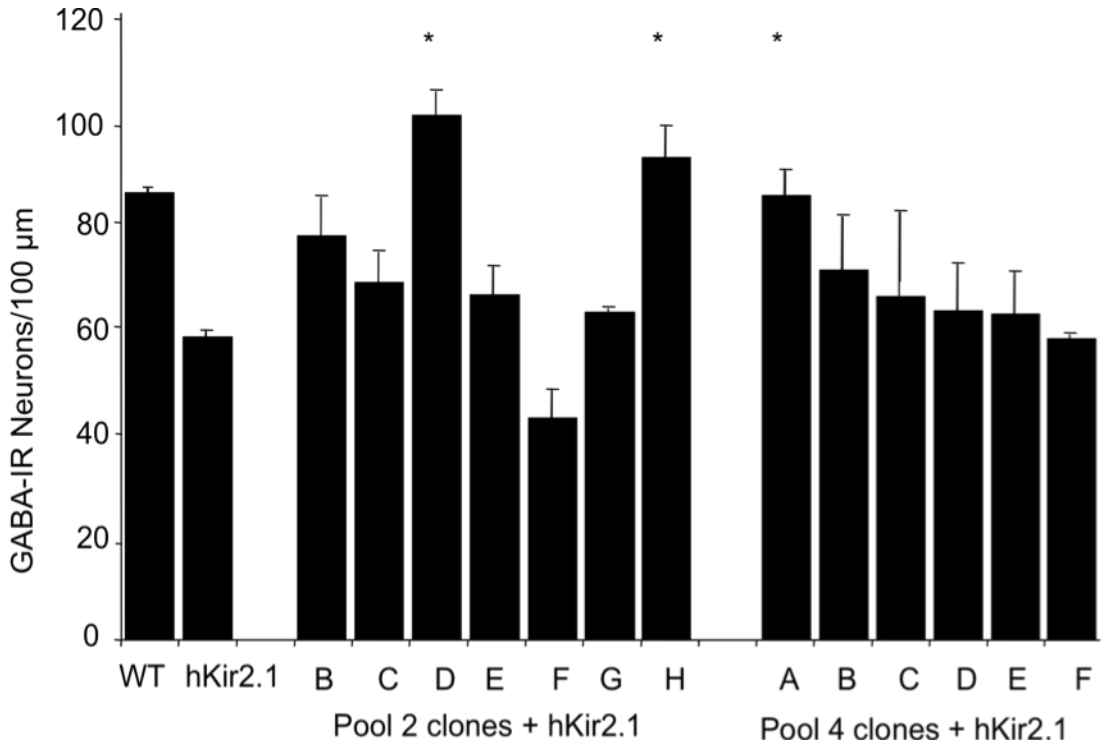


Table 2 shows that pool 2 yields a significantly larger number of GABA-IR neurons than the WT (118%), suggesting that the presence of a transcription factor or factors in this pool that blocks the effect of hKir2.1. This transcription factor may act either upstream or downstream of calcium spiking activity. Pools 4 and 6 also yielded a higher number of GABA-IR cells than produced by injection of hKir2.1 mRNA alone, but still lower than WT numbers.

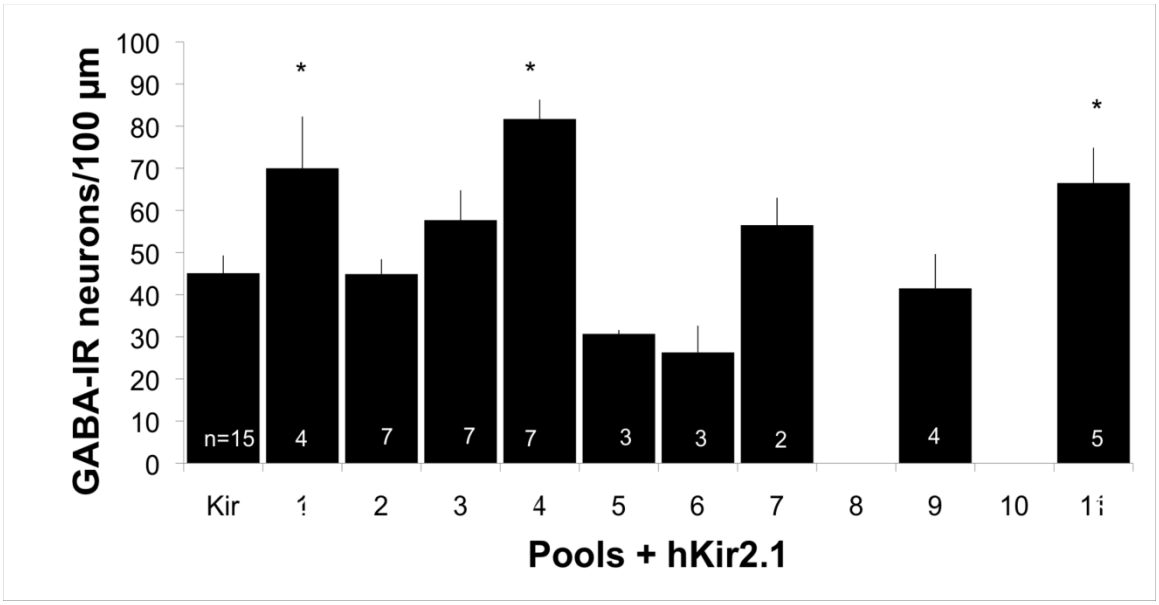
To further examine these specific pools of interest, the individual clones in pools 2 and 4 were selected and cultured in 200 μL of LB broth and ampicillin. The DNA was purified, digested with NOT 1 restriction enzyme, and transcribed into mRNA. 200 pg of the individual clones were co-injected with 250 pg of hKir2.1 mRNA at the 1-cell stage. After the embryos were fixed, sectioned, and stained, the GABAergic cells were counted. The number of GABA-IR neurons in these embryos was compared with the number of GABA-IR neurons resulting from injecting embryos with hKir2.1 mRNA alone.

Table 3. Results of the pilot screen with the number of GABA-IR neurons/100 μM for wildtype, hKir2.1 mRNA injected and clone mRNA plus hKir2.1 mRNA co-injected embryos. * indicates significantly different from Kir; $p<0.05$



Clones from pools 2 (2D and 2H) and pool 4 (4A) were sequenced and blasted. Clone 2D (TGas066p02) was identified as cDNA encoding mortality factor 4 like 2 (mof4l2) that is a part of the MORF-related gene (MRG) family of transcription factors. Clone 2H (TTpA022n08) was identified as a homolog of twist1, while clone 4A (TTpA009c08) was identified as a LIM homeobox (lhx8) transcription factor.

Table 4. Results of the pilot screen with the number of GABA-IR neurons/100 μM for wildtype, hKir2.1 mRNA injected and clone mRNA plus hKir2.1 mRNA co-injected embryos. * indicates significantly different from Kir; $p<0.05$



Following up the initial pilot screen, we continued the gain-of-function screen with another set of clones. 88 randomly selected clones were taken from 2 separate 384-well plates, inoculated onto a 96-well plate and left on the shaker for 15-18 hr at 37°C. The DNA was combined into 11 pools, purified with the Qiagen Mini-prep kit and digested with NOT 1 restriction enzyme for 2 hr. After digestion, the DNA was cleaned with the Qiaquick Gel Purification Kit and transcribed into mRNA *in vitro* using the SP6 RNA Transcription Kit.

In this experiment, we injected 2-5 pools and hKir2.1 at the one-cell stage into 20-30 more embryos than in the pilot screen (total of 40-50 embryos) in a series of 4 experiments. 1.6 ng for each pool were co-injected with 250 pg of hKir2.1 into the embryos and stage 41 surviving embryos were sunk in 30% sucrose, fixed with 4% paraformaldehyde and sectioned. The slides were stained with Abcam rabbit GABA primary antibody and GABA-IR cells were blind counted per 100 μm length of spinal cord.

With the exception of pools 8 and 10, injected embryos in pools 1-7, 9, and 11 survived ($n \geq 2$ for each pool) through 4 experiments. Table 4 shows pools 1, 4, and 11 with 155%, 181%, and 147%, respectively, greater number of GABA-IR cells and are statistically significant when compared to hKir2.1 mRNA-injected embryos. These pools potentially contain transcription factors that occlude the effect of expression of hKir2.1 mRNA alone.

In contrast, pools 5 and 6 show considerably lower number of GABA-IR neurons compared to the number of GABA-IR neurons in hKir2.1 mRNA-injected

embryos and WT embryos, indicating that these pools 5 and 6 may contain molecules that enhance the activity of hKir2.1.

DISCUSSION

The results of this screen begin to identify molecules that potentially play a role in calcium spike activity-dependent neurotransmitter specification. Given the central role of transcription factors in transmitter specification and the results from other studies of this problem (Marek et al., in review), a transcription factor screen was an appropriate starting point for the pilot screen. The main screen was not limited to identifying transcription factors, but included genes encoding other classes of molecules that could regulate Ca spike activity and transmitter specification.

After the pilot screen, 3 clones (2D, 2H and 4A) were sequenced to identify the known functions of these transcription factors and their potential roles in calcium signaling and transmitter specification in *X. tropicalis*. Clone 2D (TGas066p02) showed the largest increase in number of GABA-IR cells, at 118% that of WT embryos. Its sequence contained the full-length cDNA of mortality factor 4 like 2 (morf4l2). Morf4l2 is related to the MRG family of transcription factors, and in particular to MRG15. In previous studies, MRG15 was found to play a role in synaptic plasticity (Matsuoka et al., 2002) and in directing other transcription factors (Bowman et al., 2006). Clone 2H, known as a homolog of twist1 with respect to basic helix-loop-helix structure, is involved in cell fate determination but is not expressed in the spinal cord. Clone 4A was identified as

LIM homeobox 8 (lhx8), which is involved in differentiation of GABAergic neurons and functions to decrease the expression of GABA in the forebrain, but is not expressed in the spinal cord (Bachy et al., 2006 Manabe et al., 2005). Although Lhx8 is not expressed in the spinal cord, it remains interesting due to its regulation of GABAergic differentiation and is a clone of interest for later studies. Because Morf4l2 is found in the spinal cord, it could be selected as a transcription factor worthy of further study. Overexpression, loss-of-function using morpholinos and *in situ* hybridization experiments would test its role in specification of the GABAergic phenotype.

In subsequent experiments, we changed the protocol by injecting only 2-5 pools instead of 10 pools, and injecting 20-30 more embryos (for a total of 40-50 embryos) for each round. The protocol was changed to accommodate processing up to 10 pools at a time and to generate a larger n. Difficulties were experienced using the Chemicon guinea pig GABA primary antibody that was used in the pilot screen. In efforts to produce better staining, we changed from a Chemicon primary to an Abcam rabbit GABA primary antibody. Of the 11 pools injected throughout the 4 experiments, 3 pools (1, 4, and 11) showed significant increases in the number of GABA-IR cells compared to hKir2.1 mRNA-injected embryos. In particular, pool 4 had a 155% increase in the number of GABA-IR cells compared to hKir2.1 injected embryos, suggesting the presence of a molecule that may block the effect of hKir2.1. In order to identify clones of

interest from pools 1, 4, and 11, the 24 individual clones within those pools should be injected and embryos should be stained for GABA-IR cells.

Table 5. Potential clones of interest, their expression and function, from the pools that showed positive hits compared to hKir2.1 injected embryos in the experimental results.

CLONES	NAME	DESCRIPTION	EXPRESSION IN XENOPUS	EXPRESSION IN OTHER ANIMALS	FUNCTION	LEVEL OF INTEREST
TTpA003e07	myl1	myosin, light polypeptide 1, alkali; skeletal, fast	somite			+
TTpA003e10	dscr1	Down syndrome critical region gene 1			regulator of calcineurin	+
TTpA006l10	slc10a7	solute carrier family 10 member 7,	plasma membrane	Mice, rats	membrane transporter	+
TTpA003g23	rcp9	calcitonin gene-related peptide-receptor component protein			modulates CGRP responsiveness in different cell types	+
TTpA012c17	mbtd1	mbt domain containing 1	unknown		can't find	
TTpA009f19	api5	apoptosis inhibitor 5	unknown		anti-apoptotic protein	+
TTpA012e21	gapdh	glyceraldehyde 3 phosphate dehydrogenase	spinal cord		a receptor-mediated protein	++++
TTpA009h23	pin1	peptidylprolyl cis/trans isomerase, NIMA-interacting 1	unknown		mitotic regulator	+
TTpA007h13	ola1	Obg-like ATPase 1	unknown		involved in nucleotide binding and hydrolsis of ATP	+
TTpA010d13	ca2	carbonic anhydrase II	spinal cord	weak expression in mouse spinal cord E14.5	Ion transporter	++++
TTpA010d21	cops5	COP9 constitutive photomorphogenic homolog subunit 5 (Arabidopsis)	unknown	mouse spinal cord E13.5		+
TTpA012l24	tspan7	tetraspanin 7	spinal cord	mouse brain E14.5 and E16.5	organizers that modulate other molecules	++++
TTpA010e20	pitx1	paired-like homeodomain 1	hindlimbs		hindlimbs of mice, chick and fish	+
TTpA010f20	sdc2	syndecan 2	ectoderm	chick primitive streak stage 1 and 3	transmembrane heparan sulfate proteoglycan	+
TTpA012n03	basp1	Brain acid-soluble protein	spinal cord	rat hippocampus E18	modulates neurite outgrowth,	++++
TTpA010f21	gnl2	guanine nucleotide binding protein-like 2 (nucleolar)			endoplasmic reticulum-Golgi vesicular trafficking	+
TNeu079a21	fbl	fibrillarin	unknown	unknown		+
TEgg092f15	b3gnt5	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5	unknown	Mouse spinal cord E11, cerebellar purkinje cells	invovled in embryonic development, cell adhesion and differentiation	+
THdA035f15	YARS	tyrosyl-tRNA synthetase	unknown	mouse spinal cord E14.5		+
TTpA009d04	slc30a6	solute carrier family 30 (zinc transporter), member 6	spinal cord	rat	removes excess zinc into trans-gogi-network	++++
TGas137j24	dnaja1	DnaJ (Hsp40) homolog, subfamily A, member 1	unknown	unknown	Chaperone proteins	++++
TNeu105b18	hdac1	histone deacetylase 1	unknown	unknown	regulation of gene expression	+
TNeu112g02	krt	keratin	unknown	unknown	unknown	+
TGas077p15			unknown	unknown	unknown	+

Preliminary examination of the clones in these 3 pools in BLAST searches on Xenbase and in the literature on Pubmed was intriguing. Of the clones in pools 1, 4, and 11, 6 clones show high level of interest because they are expressed in *Xenopus* spinal cord and have a known function. Two of the 6 clones, tetraspanin 7 (Tspan-7) and brain acid soluble protein 1 (Basp1), are expressed in the spinal cord. The family of tetraspanins are 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. Both Tspan-7 and Basp-1 are plausible candidates to drive occlusion of the effect of hKir2.1. To identify specific molecules within these pools that block the effect of hKir2.1 mRNA expression and cause increases in the number of GABA-IR neurons, individual clones from pools 1, 4 and 11 should be selected, transcribed into mRNA and injected into embryos.

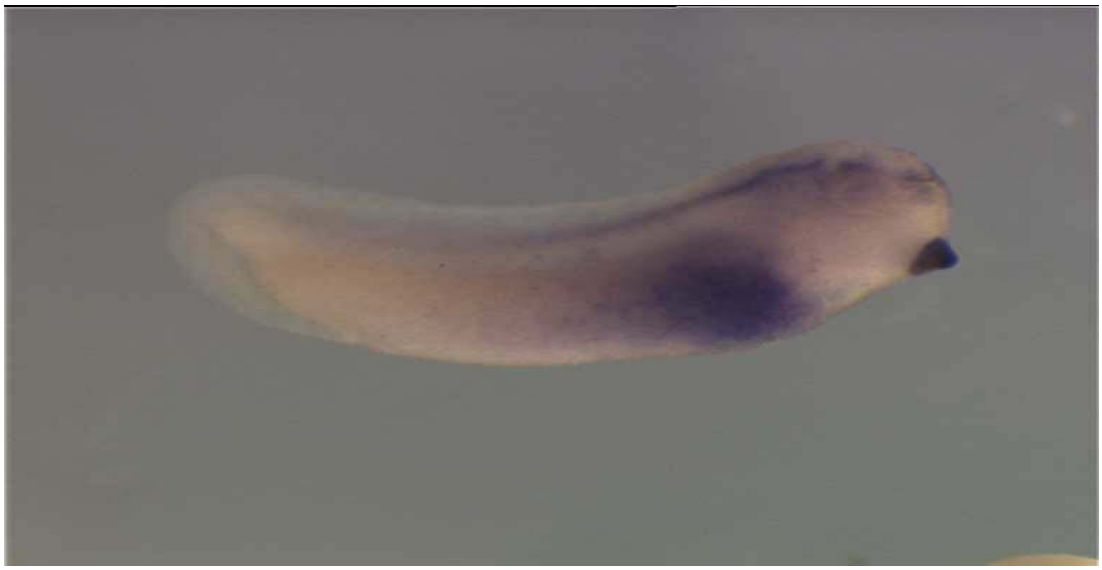


Figure 2. Images of in situ hybridizations for brain acid soluble protein 1 (above) and tetraspanin 7 (below) expression in *Xenopus* spinal cord.

In the same way, pools 5 and 6 may contain transcription factors that modulate the effect of hKir2.1 mRNA expression to decrease the number of GABA-IR cells. Since n is only 3 for both of these pools, a total of 5-7 embryos should be injected to confirm these results. If further data substantiate these preliminary findings, individual clones should be selected, transcribed into mRNA and injected into embryos.

FUTURE PROJECTS

The purpose of the screen was to identify molecules that are involved in calcium activity-dependent neurotransmitter specification. The next step is to identify further pools of interest, grow up the individual clones, and inject mRNA into embryos and identify the effects of individual genes on transmitter phenotype. Just as the effective cDNAs were sequenced and blasted in the pilot screen, the same should be done for newer hits until the screen is completed and all the clones are tested.

When the hits are identified, the next step is to sequence the clones from the plasmid prep DNA to compare these sequences with the information provided by the library. BLAST searches can be performed to reveal similar sequences and homologs of the molecules. To better understand endogenous expression in the spinal cord, we should look to the literature for information about these or related molecules.

In the case in which a clone of interest is found to be expressed endogenously in the spinal cord, there are several methods to validate the hits. Overexpressing a clone is one way to confirm the function of that particular transcription factor. Another approach is to perform a loss-of-function experiment with morpholinos to knockdown expression of the gene. For instance, translation of the transcription factor morf4l2 could be prevented with a morpholino. After

the morpholino has been injected, the significance of morf4l2 would be assessed by scoring the number of GABA-IR neurons.

Furthermore, if loss-of-function experiments reveal that transcription factors are necessary for the increase in the number of GABA-IR cells, their localization could be determined immunocytochemically. When antibodies are not available, as appears to be the case for morf4/2, in situ hybridization experiments could be performed. These approaches allow one to follow changes in expression when calcium spike activity is altered.

While this screen was designed to identify molecules that occlude the effect of hKir2.1, the results may reveal molecules that appear to be hits even though they are not involved in calcium activity-mediated neurotransmitter specification. These molecules may regulate neurotransmitter specification downstream of calcium signaling or may work entirely independent of calcium spiking through an unknown pathway. *In vivo* calcium imaging can provide insight into the action of specific molecules and whether or not they influence Ca spike activity.

MATERIALS AND METHODS

Clone selection, DNA purification, and mRNA transcription

Transcription factors were selected from a 384-well plate and inoculated onto a 96 well plate. Each well contained 200 μL of LB broth and 0.05% Ampicillin. The 96 well plate was incubated and shaken ~15-18 hr at 37°C. After growing the bacteria, clones were combined to make pools. A Qiagen mini-prep kit was used to purify the DNA, which was then digested with NOT 1 restriction enzyme for 2 hr in a 37°C water bath. DNA was purified with a Qiaquick Gel Extraction Kit and ethanol-precipitated to increase the concentration to 1 μg per 6 μL of DNA. The DNA was transcribed into mRNA *in vitro* with the Ambion Megascript Sp6 kit, TURBO DNase treated for 15 min at 37°C and precipitated with 30 μL of lithium chloride solution overnight at -20°C. The mRNA was pelleted after centrifugation and then washed with 70% ethanol and centrifuged again. Finally, the 70% ethanol was removed and the mRNA was resuspended in 20μL of RNase-free water.

Injection of embryos

4 female and 4 male frogs were primed 24 hr before injections with 20 U and 10 U of human chorionic gonadotrophin (HCG). 3-4 hr prior to injections, the frogs were boosted with HCG at 200 U and 100 U for females and males, respectively. The frogs were left to mate in buckets containing 10% MMR. 0.75 mm capillary needles (Fred Haier Co.) and 1% agarose plates (60 x 15 mm) with 10% MMR were prepared. After frogs mated and fertilization was accomplished, eggs were treated with 2% cysteine to remove the jelly coats and placed in injection dishes with 6% Ficoll with a glass pipet. 1.6 ng of each pool of mRNA in addition to 0.25 ng of hKir2.1 mRNA were injected at the 1 cell stage into each of 15-20 embryos in the pilot screen experiments and into 30-40 embryos in the later experiments.

Sinking, fixation, embedding in OCT, and sectioning

After injections, embryos were sorted and ones with morphological defects were discarded. Embryos that survived to reach stage 41 (approx. 65 hr after injection) were sunk in 30% sucrose for ~4-6 hr at 4°C and fixed with 4% paraformaldehyde with 0.05 % glutaradehyde in 0.1X phosphate-buffered saline (PBS) for 2 hr at 4°C. Following fixation, the embryos were sunk in 30% sucrose for 5-7 hr then embedded in molds with Optimal Cutting Temperature embedding medium (O.C.T.) to be frozen at -20°C. Embedded embryos were then sectioned

at 10 μM using a Leica CM 1900 cryostat and placed on Fischer Scientific microscope slides.

Immunocytochemistry

After sections were dried and the edges of the slides were made water-repellent with a pap pen (Daido Sangyo Co.), slides were washed in in 0.1X PBS, 1X PBS with 5% Triton X-100 and left in 2% fish gelatin blocking buffer for 2 hr. Following 2 5-min washes in 1X PBS, sections were incubated overnight in primary antibodies to GABA at 1:100 and 1:500 with Chemicon or Abcam antibodies respectively in moist chambers at 4°C. After 3 10-min washes in 1X PBT and 2-10-min washes in 1X PBS, sections were incubated in A594-anti-guinea pig or A488 Cy3 Alexa-tagged secondary antibodies at 1:300 for 2 hr at room temperature (21°C). Stained slides were given 3 10-min washes in 1X PBT and 2 10-min washes in 1X PBS. Lastly, slides were mounted with Fluoromount, DAPI (for nuclear staining) and a coverslip.

Counting

Slide labels were covered with colored tape and randomly rearranged to assure blind counting. Using a Zeiss Axioscope with 10X and 40X objective lenses, the number of GABA-IR cells were counted per 10 sections of the spinal cord starting at the rostral end of the spinal cord for each embryo. The number of GABAergic cells was averaged per embryo.

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