Transcription factor coexpression with GABAergic and glycinergic terminal differentiation genes

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Transcription factor coexpression with GABAergic and glycinergic terminal differentiation genes

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science in Biology from The College of William and Mary

by

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Abstract

The adoption of neurotransmitter phenotype is a crucial step in the development of the nervous system. Ca\(^{2+}\) transients have been shown to play a critical role by modulating neurotransmitter phenotype specification during differentiation. The mechanisms by which this occurs remain unclear, but given that Ca\(^{2+}\) affects terminal differentiation gene expression, transcription factors may be regulated by the Ca\(^{2+}\) transients. As a first necessary step in elucidating the effects of Ca\(^{2+}\) on transcription factors in neuronal development, we have characterized the coexpression patterns of transcription factors with terminal differentiation genes to provide a baseline for future studies. We have found that the transcription factors xDlx2 and xDlx5 colocalize extensively with both xGAD67 and xVIAAT in the telencephalon and diencephalon in very similar patterns. xPitx2 is coexpressed moderately with both xGAD67 and xGAT1 in the midbrain, and xPtf1a colocalizes moderately with xGAD67 in the retina. Xbh1 colocalizes extensively with xGlyT1 in the retina. Interestingly, xPitx2 is expressed mutually exclusive to xGAD67 and xGAT1 in the diencephalon with virtually no colocalization. Similarly, Xbh1 is expressed in a mutually exclusive pattern to xGAD67 and xVIAAT in the midbrain. All of these observations lead to several conclusions, among them that the similar coexpression patterns observed for xGAD67, xGAT1, and xVIAAT with several transcription factors suggest that the GABAergic terminal differentiation genes are all subject to similar transcriptional regulatory mechanisms. Furthermore, the mutually exclusive expression patterns observed suggest that either negative regulatory mechanisms or signaling mechanisms play a role in the specification of GABAergic terminal differentiation genes in certain regions of the central nervous system.
system. Finally, overlap observed in transcription factor expression patterns, a transcription factor binding site analysis, and the variability of transcription factor expression along the anterior-posterior axis suggest that inhibitory neurotransmitter phenotype specification is controlled by multiple transcription factors in a combinatorial fashion.
1. Introduction

The adult central nervous system is composed of neurons with hundreds of distinct types (Masland, 2004). Understanding how these neuronal fates are specified during development is a major question in neurobiology that has yet to be answered. Among the important aspects of a mature neuron’s phenotype is the neurotransmitter (or neurotransmitters) that it releases. The adoption of neurotransmitter phenotype is a critical step in the proper development of the nervous system and requires extensive coordination of gene expression to ensure that the appropriate terminal differentiation genes involved in neurotransmission are activated.

Several distinct but interacting mechanisms may play a role in the specification of neurotransmitter phenotype. Cell-cell interactions mediated by juxtacrine signaling mechanisms have been shown to play a role in cell type specification in neural development (Perron and Harris, 2000). The time of exit from the cell cycle may also control neurotransmitter phenotype specification (Ma, 2006). Perhaps the most pervasive effectors of neuronal development are transcription factors, which often act in cascades or combination to specify neuronal fate at the level of the individual neuron (Goridis and Brunet, 1999; Ma, 2006). While a number of transcription factors necessary for neurotransmitter phenotype specification have been identified, how these factors coordinate temporally and spatially in order to produce specific phenotypes is not well understood.

However, these “hard-wired”, genetically based mechanisms are not the only key regulators of neural identity—recent work has revealed that specific patterns of calcium ion (Ca^{2+}) activity in the developing nervous system may play a critical role in
development. Ca\textsuperscript{2+} signaling has been implicated in a broad range of processes from neural induction to neuronal specification and differentiation in multiple model organisms (Leclerc et al., 2000; Webb and Miller, 2006). More specifically, Ca\textsuperscript{2+} transients modulate the specification of neurotransmitter phenotypes in differentiating neurons, particularly the GABAergic and glutamatergic phenotypes (Spitzer et al., 2000; Watt et al., 2000; Borodinsky et al., 2004).

The mechanisms by which Ca\textsuperscript{2+} regulates neuronal specification remain unclear, although Ca\textsuperscript{2+} does appear to alter terminal differentiation gene expression (Watt et al., 2000). As such, the transcription factors that control the terminal differentiation genes could mediate the effects of Ca\textsuperscript{2+} on neuronal identity. This thesis addressed the first necessary step in determining if Ca\textsuperscript{2+} transients might be affecting the transcription factors regulating neurotransmitter phenotype differentiation genes. The major question it focused on was determining what the baseline, endogenous expression patterns of transcription factors involved in neurotransmitter phenotype specification are, as well as the colocalization patterns of these transcription factors with terminal differentiation genes. This will allow future work where Ca\textsuperscript{2+} concentrations are manipulated in the developing embryo followed by an analysis of transcription factor colocalization with terminal differentiation genes, to demonstrate if Ca\textsuperscript{2+} activity-dependent effects on neurotransmitter phenotype identity are mediated by Ca\textsuperscript{2+} regulation of transcription factor expression.
2. **Background**

2.1. **Inhibitory neurotransmitter phenotypes**

The most prevalent inhibitory neurotransmitter in the vertebrate nervous system is \(\gamma\)-aminobutyric acid (GABA) (Makinae et al., 2000). In addition to its long-established and broad role as an inhibitory neurotransmitter at the synapse in organisms as evolutionarily diverse as *Caenorhabditis elegans* and humans, GABA has been implicated in several developmental signaling roles prior to synapse formation in vertebrates: it appears to modulate DNA synthesis, neuronal migration, and neuronal growth and morphology in various neuronal precursors (reviewed in Ben-Ari et al., 2007). GABA also acts as the de facto excitatory neurotransmitter in early development, before glutamatergic neurotransmission is established (reviewed in Ben-Ari et al., 2007).

GABAergic neurons are characterized by a suite of terminal differentiation genes required for the synthesis, release, and reuptake of GABA. GABA is synthesized from glutamate by the enzyme glutamic acid decarboxylase (GAD), which exists as two distinct isoforms encoded by two different genes (Chattopadhyaya et al., 2007). GABA is then sequestered into vesicles that release it into the synaptic cleft by the vesicular inhibitory amino acid transporter (VIAAT) (McIntire et al., 1997). Sodium-coupled transport systems encoded by multiple GABA transporter genes (GATs) remove GABA from the synaptic cleft after synaptic transmission via uptake into the presynaptic space (Nelson et al., 1990).

Another important and prevalent inhibitory neurotransmitter in the vertebrate nervous system, particularly in the spinal cord, is the amino acid glycine. Like GABA, glycine has been implicated in a wide variety of developmental functions in addition to
its inhibitory synaptic role: it promotes interneuron differentiation (McDearmid et al., 2006), modulates glutamatergic neurotransmission by binding to N-methyl-D-aspartic acid (NMDA) receptors (reviewed in Betz et al., 2006), elevates intracellular calcium levels, and regulates synaptic remodeling of some inhibitory pathways (Kandler and Friauf, 1995). Glycine signaling also plays a major role in some motor functions, as it produces rhythmic swimming movements in lower vertebrates (Soffe et al., 2001).

Like GABAergic neurons, functional glycinergic neurons must express a set of terminal differentiation genes in order to release and reuptake glycine, though no unique synthetic enzyme is required to produce it, since uptake of extracellular glycine is sufficient for neurotransmission. Glycine is packaged into synaptic vesicles by the same protein required for packaging GABA, VIAAT (Wojcik et al., 2006). In addition, two differentially expressed glycine transporter proteins (GlyT1 and GlyT2) are responsible for the reuptake of glycine into the presynaptic neuron following neurotransmission (Betz et al, 2006).

### 2.2. $\text{Ca}^{2+}$ in neuronal specification

Many studies have illustrated the relationship between $\text{Ca}^{2+}$ activity and neural development, particularly relevant to GABAergic phenotypes. $\text{Ca}^{2+}$ activity in developing *Xenopus laevis* spinal neurons has been characterized by Gu et al. (1994) by imaging a fluorescent $\text{Ca}^{2+}$ indicator dye. These results show two kinetically distinct spontaneous fluctuations, or transients, in intracellular $\text{Ca}^{2+}$ during developmental stages—spikes and waves. $\text{Ca}^{2+}$ spikes involve calcium-dependent action potentials and can thus be propagated throughout a neuron, lasting ten seconds on average and occurring
at a frequency of one to ten times per hour. Pharmacological manipulations show that Ca\(^{2+}\) spikes depend on both influx of extracellular Ca\(^{2+}\) via voltage-gated channels and release of intracellular Ca\(^{2+}\) stores via ryanodine receptors (Spitzer et al., 2000). In contrast, Ca\(^{2+}\) waves do not involve action potentials, and thus occur locally with a distance-dependent decay in effect. They have a mean duration of 30 seconds and occur at a frequency of eight to nine times per hour with less amplitude than spikes. Like spikes, waves appear to depend upon both Ca\(^{2+}\) influx and release from intracellular stores (Spitzer et al., 2000).

Ca\(^{2+}\) waves and spikes appear to regulate different processes in the developing nervous system. Gu et al. (1994) observed that both removal of extracellular Ca\(^{2+}\) and reduction in intracellular Ca\(^{2+}\) promote neurite outgrowth, consistent with previous reports. This suggests that in the growth cone, waves regulate neurite extension. Spikes, unlike waves, significantly alter Ca\(^{2+}\) concentrations in the nuclear region and soma in general, leading Gu et al. (1994) to suggest that Ca\(^{2+}\) spikes could play a role in terminal differentiation by regulating calcium-dependent gene expression.

Studies of the relationship between Ca\(^{2+}\) transients and specific neurotransmitter phenotypes have focused on the effects of Ca\(^{2+}\) spike frequency on GABAergic specification. Gu and Spitzer (1995) imposed artificial patterns of Ca\(^{2+}\) transient activity on cultured *X. laevis* spinal neurons, observing that only patterns mimicking the endogenous frequencies of spikes resulted in normal neurotransmitter phenotype differentiation as measured by GABA immunoreactivity. More recent work shows that cultured neurons in the presence of extracellular Ca\(^{2+}\), which allows for spontaneous Ca\(^{2+}\) transients, express xGAD67 transcripts at a threefold greater level than in cultured
neurons in the absence of extracellular Ca\textsuperscript{2+}. Additionally, stimulation of Ca\textsuperscript{2+} spikes at frequencies similar to endogenous activity in cultured neurons causes an upregulation of \textit{xGAD67} transcripts (Watt et al., 2000). Borodinsky et al. (2004) observed that suppression of Ca\textsuperscript{2+} spike activity results in an increase in neurons expressing excitatory markers and a decrease in neurons expressing inhibitory markers, while enhancement of Ca\textsuperscript{2+} spike activity results in an increase in neurons expressing inhibitory markers and a decrease in neurons expressing excitatory markers. These results suggest a homeostatic mechanism of neurotransmitter phenotype regulation, where increased Ca\textsuperscript{2+} activity triggers increased inhibitory fate, and vice versa. Borodinsky et al. (2004) propose that activity and gene expression influence one another as the nervous system develops in a mechanism that stabilizes fluctuating neuronal identities as maturation occurs.

Despite the body of work connecting Ca\textsuperscript{2+} activity and neurotransmitter phenotype identity, the mechanism by which Ca\textsuperscript{2+} regulates this specification remains unclear. However, given that Ca\textsuperscript{2+} spikes can alter the expression of terminal differentiation genes such as \textit{GAD}, one possible mechanism is that Ca\textsuperscript{2+} spike activity directly or indirectly affects the transcription factors controlling terminal differentiation gene expression.

2.3. \textit{Mechanisms of Ca}\textsuperscript{2+} regulation of gene expression

As a result of the broad roles for Ca\textsuperscript{2+} signaling in cellular function, the mechanisms by which Ca\textsuperscript{2+} alters gene expression have been explored in several studies. Dolmetsch et al. (1998) used a Ca\textsuperscript{2+} clamp technique to examine the response of the pro-inflammatory transcription factors NF-AT, Oct/OAP, and NF-\kappaB in T lymphocytes to
varying Ca\(^{2+}\) transient frequencies. For all three transcription factors, Ca\(^{2+}\) oscillations decreased the activation threshold, but even more interestingly, an oscillation frequency-dependent response emerged—rapid Ca\(^{2+}\) oscillations stimulated NF-AT, Oct/OAP, and NF-\(\kappa\)B, but infrequent Ca\(^{2+}\) oscillations activated only NF-\(\kappa\)B. These results indicate that different transcription factors have varying Ca\(^{2+}\) sensitivities and kinetics in response to changes in intracellular Ca\(^{2+}\), providing a mechanism by which cells can respond to differing Ca\(^{2+}\) conditions. Remarkably, the observations of transcription factor Ca\(^{2+}\) dependence are consistent with the observed Ca\(^{2+}\) dependence of interleukin (IL)-2 and IL-8 expression. IL-2, which depends upon NF-AT and Oct/OAP, is activated by rapid Ca\(^{2+}\) transients, while IL-8, which depends on NF-\(\kappa\)B, is activated by infrequent Ca\(^{2+}\) transients (Dolmetsch et al., 1998). These results suggest that the Ca\(^{2+}\) sensitivity of IL-2 and IL-8 are a consequence of the Ca\(^{2+}\) sensitivity of the transcription factors which activate the interleukins. The current model suggested by Fisher et al. (2006) predicts that NF-AT activation only by high Ca\(^{2+}\) oscillation frequencies depends on calcineurin. High frequency Ca\(^{2+}\) activity activation of calcineurin maintains dephosphorylation of NF-AT, allowing it to remain in the nucleus. Conversely, NF\(\kappa\)B can be activated by low frequency oscillations because the degradation of I\(\kappa\)B, which allows translocation of NF\(\kappa\)B into the nucleus, is dependent only on the presence of Ca\(^{2+}\), not the frequency of oscillations (Fisher et al., 2006).

Another well-studied Ca\(^{2+}\)-dependent gene is brain-derived neurotrophic factor (BDNF), a small secreted protein critical to neuronal survival in the central nervous system (West et al., 2001). Recent work has identified some of the molecular mechanisms responsible for BDNF sensitivity to Ca\(^{2+}\) and suggests more general
mechanisms of Ca\textsuperscript{2+}-mediated changes in gene expression. West et al. (2001) reviewed three major components responsible for the Ca\textsuperscript{2+} dependence of BDNF: the route of Ca\textsuperscript{2+} influx, the phosphorylation pattern induced on the cAMP-response element (CRE), and the complement of transcription factors recruited to the BDNF promoter. Ca\textsuperscript{2+} influx through Ca\textsuperscript{2+} channels triggers a number of signaling molecules, including calcium-sensitive adenylate cyclase, calcium/calmodulin-activated kinases, and Ras, each of which initiates independent signaling cascades that ultimately affect CRE binding protein (CREB). It is the Ca\textsuperscript{2+}-sensitive modulation of CREB that directly affects BDNF transcription (West et al., 2001). This study provides a large framework of possible mechanistic components of Ca\textsuperscript{2+} dependence of transcription factor expression, some of which may be relevant to Ca\textsuperscript{2+}-sensitive neurotransmitter phenotype specification.

2.4. Transcription factors in inhibitory specification

In vivo and in vitro studies of GABAergic and glycinergic specification indicate that many possible transcription factors specify inhibitory phenotypes and could act as intermediates in the Ca\textsuperscript{2+}-mediated specification of neurotransmitter phenotype identity. Experimental evidence suggests involvement of the distal-less homeobox (Dlx) transcription factor family in specifying a GABAergic fate. Ectopic expression of Dlx2 and Dlx5 in mouse telencephalon slices has been shown to induce increased expression of both isoforms of GAD. In addition, Dlx family members may cross regulate themselves, as Dlx5 expression can be induced in slice cultures by ectopic expression of Dlx2 (Stühmer et al., 2002). A subsequent study established that Dlx5\textsuperscript{−/−} mice experience a prominent decrease in GAD67 expression in the olfactory bulb (Long et al., 2003).
Kuwajima et al. (2006) found that necdin, a paternally-expressed MAGE (melanoma antigen) protein family member, binds to a homologous MAGE protein MAGE-D1; the necdin-MAGE-D1 complex then associates with Dlx family transcription factors to form a ternary complex. When overexpressed in mouse forebrain slices, necdin significantly increased the population of cells expressing GABAergic markers GAD and calbindin D-28k, and enhanced the expression levels of these markers within cells. Further investigation revealed that in mutant mice lacking a paternal necdin allele, forebrain expression of calbindin D-28k, GAD, and Dlx5 were significantly reduced, while Dlx2 expression was unchanged; conversely, expression in the dorsal telencephalon was unchanged (Kuwajima et al., 2006). This suggests Dlx2 acts to induce GABAergic terminal differentiation genes and Dlx5, and is enhanced by necdin in a region-specific pattern.

In addition to the distal-less family, the paired-like homeodomain transcription factor 2 (Pitx2) appears to play a role in GABAergic specification. Eastman et al. (1999) have demonstrated that the UNC-30 homeodomain protein directly activates the expression of both *unc-25* (glutamic acid decarboxylase) and *unc-47* (GABA transporter) in type D motor neurons in *C. elegans*. Westmoreland et al. (2001) observed homeodomain similarity between *C. elegans* UNC-30 and mammalian Pitx2, a transcription factor associated with GABAergic neurogenesis in the mesencephalon and diencephalon. Subsequent experimentation established functional conservation between the UNC-30 and Pitx2 proteins, wherein Pitx2 rescued GABAergic differentiation defects in *unc-30* mutants and both Pitx2 and UNC-30 activated the mouse *Gad1* promoter. Martin et al. (2002) report that PITX2 colocalizes with GABA immunoreactivity in the
zona incerta of the diencephalon and specific neuroepithelial domains in the
mesencephalon. This evidence suggests that in some neurons, GABAergic differentiation
may be controlled by a strongly conserved developmental pathway characterized in
vertebrates by the transcription factor Pitx2.

Another transcription factor recently implicated in GABAergic and glycinergic
specification is Ptf1a, a member of the basic helix-loop-helix (bHLH) class of
transcription factors. Hoshino et al. (2005) showed that Ptf1a is expressed endogenously
in GABAergic (but not glutamatergic) precursors, and that introduction of Ptf1a to
 glutamatergic precursors causes a GABAergic morphological and migratory fate.
Additionally, Glasgow et al. (2005) reported that Ptf1a is necessary for the generation of
 a variety of GABAergic dorsal interneuron populations in the spinal cord dorsal horn. In
the absence of Ptf1a, not only is a complete loss of GABAergic interneurons observed,
but also an increase in glutamatergic neurons. Therefore, Ptf1a appears to be essential
for the specification of GABAergic over glutamatergic phenotypes in the spinal cord
(Glasgow et al, 2005). Additionally, Nakhai et al. (2007) showed that Ptf1a contributes to
the differentiation of both GABAergic and glycinergic amacrine cells in the mouse retina.

Finally, recent research suggests that the Bar homeobox family of transcription
factors play a role in glycinergic, but not GABAergic, terminal differentiation. Mo et al.
(2004) recently demonstrated that the mouse BarH-like 2 gene (Barhl2) is expressed in
postmitotic amacrine cells of the developing retina, and misexpression of Barhl2 directs
retinal progenitor cells to differentiate into glycinergic amacrine cells. Patterson et al.
(1999) have identified two distinct Bar homeobox genes, named Xbh1 and Xbh2, which
appear to be expressed in distinct regions of the developing central nervous system.
Furthermore, Poggi et al. (2004) demonstrated that Xbh1 acts as a transcriptional repressor downstream of the atonal genes Xath3 and Xath5, driving ganglion cell fate in the developing retina. The wide body of work on transcriptional regulation of inhibitory phenotypes provides an excellent framework of transcription factors to investigate in Ca\textsuperscript{2+} activity-dependent specification.

2.5. *Colocalization as an indicator of transcription factor function*

To accurately assess the possible role of transcription factors in mediating Ca\textsuperscript{2+}-dependent specification, a detailed baseline of transcription factor expression is needed for comparison with embryos cultured in differing Ca\textsuperscript{2+}-containing environments. A useful method of observing changes in transcription factor expression is analyzing transcription factor coexpression with terminal differentiation genes. Redestig et al. (2007) report that while transcription factor colocalization with a target gene cannot alone serve as an absolute indicator of a regulatory relationship, transcription factors do tend to coexpress with their target genes. So long as possible time shifts between a transcription factor and its target gene are accounted for, gene expression data can serve as a rough proxy for transcription factor activity (Redestig et al., 2007). Yu et al. (2003) caution that the expression relationships between transcription factors and their target genes are sometimes more complex than simple correlations, but in agreement with Redestig et al., acknowledge that a lack of colocalization is often only due to a time delay. Colocalization analyses should allow a correlational, if not functional, connection between manipulations in Ca\textsuperscript{2+} levels, changes in transcription factor gene expression, and changes in terminal differentiation gene expression.
Because the use of multiplex fluorescent in situ hybridization allows several transcripts to be detected simultaneously without interference, it is an ideal technique for performing a colocalization analysis between transcription factors and their terminal differentiation gene targets. Fluorescent signal is developed for each probe independently using tyramide signal amplification, allowing for dramatic amplification of signal intensity and preventing cross-deposition of fluorophores to multiple probes. Additionally, the fluorescent signal is detectable by confocal microscopy, allowing for single-cell signal resolution and true colocalization analysis (Denkers et al., 2004). Even if a correlation in the change of both a transcription factor and terminal differentiation gene target is not observed, the high resolution of the confocal microscopy provides an ideal platform to observe any changes in transcription factor expression, even minute, induced by manipulations in Ca\(^{2+}\) levels.

2.6. *Xenopus laevis* as a model system

*Xenopus laevis*, the African clawed frog, has been used for investigating numerous aspects of early neural development. The embryos produced by *X. laevis* are easily obtained with the use of artificially-induced matings and develop externally in simple salt solutions. This allows observation of early embryonic stages inaccessible in other organisms which develop internally. Additionally, due to the simple culture requirements and manipulability of the embryos, many of the studies on developmental roles of Ca\(^{2+}\) transients have been performed in *X. laevis* (Borodinsky and Spitzer, 2006). *X. laevis* has also been utilized extensively in research of the molecular and genetic aspects of early neural development, again due to the accessibility to early stages,
including neural induction and subsequent patterning (reviewed in De Robertis, 2006) and neurotransmitter phenotype determination and differentiation (Gamse and Sive, 2000). Given these advantages, *X. laevis* is an ideal organism in which to study transcription factor colocalization with multiple terminal differentiation genes, and will serve as an excellent model in future studies for examining the effects of Ca\textsuperscript{2+} on the expression of transcription factors controlling neuronal specification.
3. **Hypothesis and predictions**

Given the question of where transcription factors involved in GABAergic and glycinergic neurotransmitter phenotype specification are expressed, the existing research suggests that transcription factors responsible for driving GABAergic and glycinergic terminal differentiation will likely be coexpressed with the terminal differentiation genes they control. This predicts that since the *Dlx2, Dlx5, Pitx2, Ptf1a, Xbh1*, or *Xbh2* transcription factors are responsible for driving GABAergic or glycinergic differentiation, these transcription factors will be coexpressed with the terminal differentiation genes (*xGAD67, xGlyT1, xGlyT2*, or *xVIAAT*) in a given tissue of the central nervous system. Given the variable nature of the transcription factor sets required for expression of the same phenotype in different areas along the anterior-posterior axis, it is likely that the set of transcription factors that overlap the spatial expression and precede or overlap the temporal expression of a given terminal differentiation gene will vary significantly along the anterior-posterior axis of *X. laevis* embryos. These data provide a strong foundation on which to begin a comparative study where Ca$^{2+}$ levels are manipulated and changes in transcription factor expression and coexpression are observed.
4. Materials and methods

4.1. Animal use

Embryos for experimentation were obtained using human chorionic gonadotropin (hCG)-induced natural matings of albino *X. laevis* frogs as described in Sive et al. (2000). Animal care and use were performed in adherence to the regulations established by the Institutional Animal Care and Use Committee at the College of William and Mary.

4.2. Cloning and sequence analysis

Total RNA was extracted from *X. laevis* embryos using the RNeasy Maxi kit (Qiagen) according to the manufacturer’s protocol with one exception. Rather than disrupting the tissue samples with a mortar and pestle in liquid nitrogen, tissue samples were disrupted with a mortar and pestle quickly at room temperature. Swimming tadpole stage embryos were used for all extractions due to reports that *xPtf1a, Xbh1*, and *Xbh2* were expressed at high levels at these stages (Afelik et al., 2006; Patterson et al., 2000). cDNA was synthesized using the iScript cDNA Synthesis kit (BioRad) according to manufacturer’s protocol with one exception. Instead of a 30 minute 42°C reverse transcription step, the time for this step was extended to 60 minutes to improve yield at the suggestion of a different manufacturer’s kit. cDNA was purified with the QIAquick PCR Purification kit (Qiagen) according to the manufacturer’s protocols. Polymerase chain reaction (PCR) was performed using Taq DNA polymerase (New England Biolabs).

For *xPitx2, xPtf1a, Xbh1*, and *Xbh2*, published *X. laevis* mRNA sequences in GenBank (accession numbers AF077767, DQ007931, NM_001088552, and AF283692,
respectively) were used for primer design. Full mRNA sequences were processed by Primer3 primer design software (http://frodo.wi.mit.edu; Rozen and Skaletsky, 2000) with selection criteria for product lengths greater than 600 base pairs and less than 1300 base pairs, primer lengths of 20 base pairs, and primer melting temperatures between 54.0°C and 60.0°C. For each transcript, the two distinct primer pairs producing the longest products encompassing the full coding sequences and including portions of the untranslated region were selected for use. Primers were used for PCR amplification using X. laevis cDNA, with conditions as shown in Table 1.

PCR products were initially analyzed with agarose gel electrophoresis to confirm appropriate sizes, cloned into pCRII-TOPO (Invitrogen), and transformed into One Shot TOP10 chemically competent E. coli or One Shot MAX Efficiency DH5α-T1R E. coli (Invitrogen) according to the manufacturer’s protocols. Transformed colonies were grown on LB-agar media at 37°C and selected for by addition of ampicillin to the plate. Selection for recombinant plasmids was carried out by addition of X-Gal to the plates according to standard methods (Sambrook and Russell, 2001). Individual colonies were picked from plates and cultured overnight in LB liquid media, and plasmids were isolated from the cultures using the Wizard Plus SV Miniprep kit (Promega). Purified plasmid DNA was analyzed for the correct insert size using restriction digestion with EcoRI (Promega) and agarose gel electrophoresis.

Plasmids containing the appropriate size insert were prepared for sequencing with the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). The reaction consisted of 2.0 µl ABI Terminator Ready Reaction mix, 200–500 ng plasmid DNA template, 3.2 pmol of M13(forward) or M13(reverse) sequencing primer, and nuclease-
Table 1. PCR primers and conditions for cloning transcription factors $xPitx2$, $xPtf1a$, $Xbh1$, and $Xbh2$.

The successful primers used for cloning of $xPitx2$, $xPtf1a$, $Xbh1$, and $Xbh2$. Included are the NCBI Accession Numbers for the sequences used to design the primers, the forward and reverse primer sequences, the positions of the primers in the sequences used for primer design, melting temperatures of the primers, annealing temperatures used during the PCR reaction, and amplicon length as determined by sequencing following cloning into pCRII-TOPO.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Num.</th>
<th>Primers (position in seq.)</th>
<th>$T_m$</th>
<th>$T_{annealing}$</th>
<th>Amplicon Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>xPitx2</td>
<td>AF077767</td>
<td>Forward 5'-GGCTGGGGAGTAGAGTTGCTG-3' (118-137)</td>
<td>57.6°C</td>
<td>60.3°C</td>
<td>748 base pairs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5'-AGGGAAGGGGTGCTGAGATT-3' (845-864)</td>
<td>56.2°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>xPfl1a</td>
<td>DQ007931</td>
<td>Forward 5'-TTCCCCGTACTTGTAGTGGG-3' (97-116)</td>
<td>54.2°C</td>
<td>59.0°C</td>
<td>624 base pairs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5'-GAGAGAGAGGATGTCCCGCAAG-3' (701-720)</td>
<td>55.3°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xbh1</td>
<td>NM_001088552</td>
<td>Forward 5'-CCATCTTCCGCCCATATCAGT-3' (234-253)</td>
<td>54.4°C</td>
<td>60.0°C</td>
<td>1014 base pairs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5'-TCTCCGTTTCTGATGTCCTT-3' (1228-1247)</td>
<td>55.5°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xbh2</td>
<td>AF283692</td>
<td>Forward 5'-ACCCCGTTATCGATCTCCAC-3' (152-171)</td>
<td>55.9°C</td>
<td>57.3°C</td>
<td>1002 base pairs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5'-ATACGGGGAAGGAACATCC-3' (1134-1153)</td>
<td>54.4°C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
free water to bring the reaction to 10 µl total volume. This mixture was heated to 96°C for one minute and cycled 25 times at 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. Once the reaction was complete, 2.5 µl of 125 mM ethylene diamine tetraacetic acid (EDTA) disodium salt and 30 µl of absolute ethanol were mixed into the reaction, which was incubated at room temperature, spun in a microcentrifuge for 20 minutes at 4°C, washed with 150 µl 70% ethanol, spun again for 5 minutes at 4°C, and dried for 2 minutes in a Savant Speedvac. The pellet was resuspended in 30 µl Hi-Di Formamide (Applied Biosystems) and the resuspension was incubated at 95°C for 5 minutes before loading into the ABI 3100 Avant automated sequencer (Applied Biosystems). Resulting sequence data were analyzed using Vector NTI Advance 10 software (Invitrogen) and publicly available BLAST searches against the NCBI nucleotide collection (NCBI, http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) to confirm the identity of each clone.

\(x\text{GlyT2}, x\text{VIAAT},\) and other neurotransmitter phenotype markers were obtained as described in Wester et al. (2008). Clones of \(x\text{Dlx2}\) and \(x\text{Dlx5}\) partial coding sequences were generously provided by Robert M. Grainger (University of Virginia).

4.3. Antisense RNA probe generation

Plasmid DNA was obtained using the Quantum Prep Plasmid Midiprep kit (BioRad) according to the manufacturer’s protocol with two exceptions. First, instead of growing bacteria in a 50 ml LB liquid media culture until turbid and then pelleting into an Oak Ridge tube using a single 10 minute, 5000 RPM spin in a Sorvall RC5B refrigerated centrifuge, bacteria were grown in a 150 ml culture and pelleted using a series of 10
minute, 7000 RPM spins. Secondly, instead of performing the final elution with 600 µl of sterile, deionized, distilled water, elution was performed with 300 µl of sterile, deionized, distilled water in order to maximize the final plasmid concentration. Probes were then linearized by restriction digestion to produce a linear template for in vitro transcription using the restriction enzymes shown in Table 2. After digestion, the linear DNA was isolated by successive phenol/chloroform/isoamyl alcohol (25:24:1) extraction, chloroform/isoamyl alcohol (24:1) extraction, and ethanol precipitation. The linear DNA was verified to be the appropriate size using agarose gel electrophoresis.

Linear DNA was transcribed in vitro using standard methods with a 13:7 ratio of unlabeled UTP to labeled UTP. For chromogenic in situ hybridization, digoxigenin-11-UTP (Roche) was used as the probe label, while for multiplex fluorescent in situ hybridization, dinitrophenol-11-UTP (Perkin-Elmer) and fluorescein-12-UTP (Roche) were used in addition. After transcription with either T7 or SP6 RNA polymerase (Promega) to produce the antisense RNA strand (Table 2), the template in the reaction was destroyed with RQ1 DNase (Promega). Presence of the RNA transcript was confirmed using agarose gel electrophoresis, and the RNA was then purified using the RNeasy MinElute Cleanup kit (Qiagen) according to the manufacturer’s protocol and confirmed once again using agarose gel electrophoresis. The RNA was then diluted with in situ hybridization buffer and stored at -20°C until used.

4.4. **Chromogenic in situ hybridization**

*X. laevis* embryos obtained from hCG-induced natural matings were fixed in 1X MEMFA (3.7% (v/v) formaldehyde, 100 mM 3-morpholinopropane-1-sulfonic acid
Table 2. Linearization and transcription parameters for production of antisense RNA probes used for in situ hybridization.

The restriction enzymes used to linearize plasmids containing cDNA inserts of xDlx2, xDlx5, xPitx2, xPtf1a, Xbh1, and Xbh2 for in vitro transcription, and the RNA polymerase used during the transcription reaction to produce the antisense RNA fragment.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Restriction Enzyme</th>
<th>RNA Polymerase</th>
</tr>
</thead>
<tbody>
<tr>
<td>xDlx2</td>
<td>EcoRI</td>
<td>T7</td>
</tr>
<tr>
<td>xDlx5</td>
<td>EcoRI</td>
<td>T7</td>
</tr>
<tr>
<td>xPitx2</td>
<td>BamHI</td>
<td>T7</td>
</tr>
<tr>
<td>xPtf1a</td>
<td>BamHI</td>
<td>T7</td>
</tr>
<tr>
<td>Xbh1</td>
<td>EcoRV</td>
<td>SP6</td>
</tr>
<tr>
<td>Xbh2</td>
<td>EcoRV</td>
<td>SP6</td>
</tr>
</tbody>
</table>
(MOPS), 2 mM ethylene glycol tetraacetic acid (EGTA) disodium salt, and 1 mM magnesium sulfate in sterile, deionized, distilled water) at room temperature for 90 minutes and stored in absolute ethanol at -20°C until use. Sterile, deionized, distilled water was prepared for all aqueous solutions by autoclaving (20 minutes at 121°C and 20 psi) water ultrapurified using a distillation, deionization, UV sterilization, and particulate filtration system to 18.2 MΩ-cm resistance (Thermo Fisher Scientific).

For in situ hybridization, a significantly modified protocol based on Harland (1991) was used. Briefly, embryos were rehydrated, permeabilized with proteinase K, treated with acetic anhydride, re-fixed, and prehybridized. Then, embryos were hybridized with antisense RNA probe, washed, RNase treated, and incubated with an antibody–alkaline phosphatase conjugate to detect the labeled RNA probe. Finally, unbound antibody was washed out and alkaline phosphatase substrate added to form a visible signal.

In detail, fixed embryos were rehydrated and permeabilized by successive five minute washes at room temperature in absolute ethanol, 75% ethanol (aqueous), 50% ethanol (aqueous), 25% ethanol in PTw, and finally, four separate washes in PTw. PTw was made up as a solution of 1X phosphate-buffered saline (PBS—2.7 mM potassium chloride, 137 mM sodium chloride, 2 mM monobasic potassium phosphate, and 10 mM dibasic sodium phosphate in sterile, deionized, distilled water and pH adjusted to 7.5) with 0.1% (v/v) Tween-20. Embryos were then further permeabilized by treatment with 10 μg/ml proteinase K in sterile, deionized, distilled water for 30 minutes at room temperature. Following permeabilization, embryos were washed three times for five minutes each in 0.1 M triethanolamine, treated for five minutes with 0.25% (v/v) acetic
anhydride, and treated for five minutes with 0.5% (v/v) acetic anhydride to acetylate amine groups, thus reducing background signal. Embryos were then washed twice in PTw, re-fixed in 4% paraformaldehyde in PTw, and washed three times in PTw to remove residual paraformaldehyde. Embryos were introduced to a 20% hybridization buffer in PTw mixture to equilibrate and then placed in hybridization buffer and incubated for 10 minutes at 60°C. The hybridization buffer was replaced with fresh hybridization buffer and the embryos were incubated at 60°C for at least six hours to prehybridize and reduce nonspecific binding upon addition of RNA probe. Following prehybridization, hybridization buffer was replaced with a 1:10 dilution of the stock antisense RNA probe in hybridization buffer. The embryos were then incubated at 60°C for approximately 12 hours to allow the probe to hybridize to transcripts.

Following hybridization, the probe was recovered and washed out of the embryos by successive 20 minute washes in 2X SSC (30 mM sodium citrate and 300 mM sodium chloride in sterile, deionized, distilled water and pH adjusted to 7.0) at 60°C. Remaining unbound probe was hydrolyzed by a 30 minute treatment with 20 µg/ml RNase A in 2X SSC at 37°C. RNase A was removed by two 10 minute washes in 2X SSC at room temperature and two 30 minute washes in 0.2X SSC at 60°C, after which the embryos were washed twice for 15 minutes each in maleic acid buffer (MAB—100 mM maleic acid and 150 mM sodium chloride in sterile, deionized, distilled water and pH adjusted to 7.5). Embryos were then blocked with 2% (w/v) blocking reagent (Roche) in MAB at room temperature for one hour. To detect labeled probe, anti-digoxigenin–alkaline phosphatase antibody conjugate (Roche) was diluted 1:2000 in 2% (w/v) blocking
reagent in MAB, and 500 µl of the diluted antibody was placed into each vial containing embryos. Antibody detection was carried out at 4°C for approximately 12 hours.

Unbound antibody was washed out by five successive washes in MAB of at least one hour each. Following these washes, embryos were washed twice for five minutes each in alkaline phosphatase buffer (100 mM tris(hydroxymethyl)aminomethane (tris), 50 mM magnesium chloride, 100 mM sodium chloride, 2 mM tetramisole hydrochloride, and 0.1% (v/v) Tween-20 in sterile, deionized, distilled water). An alkaline phosphatase color reaction mixture was prepared by adding 4.5 µl of nitro-blue tetrazolium (NBT) and 3.5 µl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) to 1 ml alkaline phosphatase buffer. This mixture (1 ml) was added to each vial containing embryos and incubated at room temperature with no agitation until visible color signal developed. The time until the first visible signal ranged from 20 minutes to several hours depending on transcript levels and probe strength. Approximately half of the embryos were fixed once strong signal developed but before background increased substantially, rendering them suitable for whole mount photography. The remaining embryos were allowed to continue reacting for up to several days regardless of background, rendering them suitable for histological analysis. To stop the color reaction, embryos were placed in 1X MEMFA and fixed overnight at 4°C. Following fixation, embryos were washed for five minutes in 1X PBS and then stored in fresh 1X PBS at 4°C. All in situ hybridization experiments were conducted with 30 or more embryos, and each individual experiment was replicated at least twice. All embryos were examined under the microscope in detail. In situ hybridization experiments using sense probes were performed as controls to exclude the possibility of nonspecific probe binding and verify authentic signal for antisense probes.
4.5. Paraffin embedding and sectioning

Embryos processed with chromogenic in situ hybridization were embedded in paraffin and sectioned for histological analysis. Following fixation and storage in 1X PBS, embryos were dehydrated by successive 15 minute washes in 25% ethanol in 1X PBS, 50% ethanol in 1X PBS, 75% ethanol in 1X PBS, and absolute ethanol. Embryos were then washed for 15 minutes each in 50% xylenes (mixture of o-, p-, and m- isomers) in ethanol and then 100% xylenes. The embryos were then washed for 15 minutes in 50% paraffin in xylenes at 62°C, and then washed two times for one to two hours each in paraffin at 62°C. Finally, embryos were placed in fresh paraffin and oriented such that the anterior end faced downward in the embedding boats as the paraffin hardened. Blocks were allowed to harden for at least 12 hours overnight before proceeding to sectioning.

The blocks were trimmed around the embedded embryo and 10-µm sections were taken of the embryos. Paraffin sections were placed onto Mayer’s albumin adhesive-treated slides covered with sterile, deionized, distilled water and allowed to dry on a slide warmer at 40°C. Once dried, slides were rinsed in Citrisolv (Fisherbrand) for three to five minutes, dried, and cover slipped with Permount (Fisherbrand). Three drops of Permount were applied to the slide surface and a single glass cover-slip was slowly lowered onto the slide from one end to the other to minimize bubble formation. Any apparent bubbles over sections were gently pressed to the edge of the slide and excess Permount removed. Cover-slipped slides were allowed to dry at room temperature and stored until photographed.
4.6. Fluorophore-tyramide synthesis

Fluorophore-tyramide conjugates for use during the fluorophore deposition of fluorescent in situ hybridization were synthesized in a conjugation reaction between fluorophore functionalized into a reactive N-hydroxysuccinimide (NHS) ester and tyramine hydrochloride. Fluorophore NHS esters used were Cy3 NHS ester (GE Life Sciences), Cy5 NHS ester (GE Life Sciences), and NHS-fluorescein (Pierce Biotechnology). A solution (TEA-DMF) of 1 part anhydrous triethylamine (Sigma) in 100 parts anhydrous dimethyl formamide (Acros Organics) was prepared by mixing. Tyramine hydrochloride (Sigma) was then dissolved to a concentration of 10 mg/ml in the TEA-DMF. Fluorophore NHS ester was brought up to a concentration of 10 mg/ml in anhydrous dimethyl formamide. To conjugate the tyramine to the fluorophore, 33 µl of tyramine hydrochloride solution was added to 100 µl of fluorophore-NHS solution, inverted to mix well, and incubated at room temperature in the dark for two hours. Following this reaction, the fluorophore-tyramide conjugate was diluted in 1.2 ml of absolute ethanol, aliquoted, and stored at -20°C (Appendix 1 for detailed protocol).

4.7. Multiplex fluorescent in situ hybridization

Multiplex fluorescent in situ hybridization was performed identically to the chromogenic in situ hybridization protocol except for the modifications described here, adopted from Davidson and Keller (1999). For hybridization, instead of a single probe, 500 µl of each individual probe were mixed to make the combination probes. When making combinations, the transcript most weakly expressed was paired with a digoxigenin-labeled probe, since digoxigenin labeled probes yielded the highest signal-
to-noise ratio. Probes were allowed to hybridize at 60°C for between 12 and 16 hours. Following RNase treatment and SSC washes, embryos were washed twice in PTw at room temperature for 15 minutes each. Endogenous peroxidases were inactivated with a one hour wash in 2% hydrogen peroxide (made by diluting 30% hydrogen peroxide in PTw) at room temperature. Embryos were then washed twice in TBST for 15 minutes each at room temperature. TBST was made up as a solution of 1X tris-buffered saline (TBS—12 mM tris and 150 mM sodium chloride, in sterile, deionized, distilled water and pH adjusted to 7.5) with 0.1% (v/v) Tween-20. The embryos were then blocked for approximately five minutes with 2% (w/v) blocking reagent in MAB at room temperature. Probes were detected sequentially, starting with the digoxigenin-labeled probe. To detect the probe, anti-digoxigenin–horseradish peroxidase antibody conjugate (Roche) was diluted 1:1000 in 2% (w/v) blocking reagent in MAB, and 500 µl of the diluted antibody was placed into each vial containing embryos. Antibody detection was carried out at 4°C for approximately 12 hours.

Unbound antibody was washed out by five successive washes in TBST of at least one hour each. Following these washes, the embryos were washed twice in PTw for 10 minutes each. A dilution of fluorophore-tyramide (described above) was made in PTw in the dark to prevent photobleaching—these were optimized empirically by qualitatively comparing signal-to-noise ratios among depositions with different fluorophore dilutions. For Cy3-tyramide, the optimal dilution was 1:25; for Cy5-tyramide, the optimal dilution was 1:10; for fluorescein-tyramide, the optimal dilution was 1:200. For the anti-digoxigenin–horseradish peroxidase conjugate, Cy3-tyramide was used so that fluorescein-tyramide could be used for any anti-fluorescein–horseradish peroxidase
conjugate deposition in subsequent steps, to prevent antibody binding of the anti-fluorescein to any deposited fluorescein-tyramide. PTw in the vials was replaced with 1 ml of dilute fluorophore-tyramide and the vials were nutated vertically for 20 minutes to allow the fluorophore to penetrate the embryo. To catalyze the deposition of the fluorophore, 3.3 µl of 0.3% hydrogen peroxide (made by diluting 30% hydrogen peroxide (aqueous) in PTw) was added to each vial for a final hydrogen peroxide concentration of 0.001%, and the vials were nutated vertically for 40 minutes. During this incubation, the horseradish peroxidase catalyzed the formation of a covalent bond between the fluorophore-tyramide and tyrosine residues of proteins close enough to the antibody conjugate for the horseradish peroxidase to act on them (Figure 1). Following deposition, embryos were washed for 15 minutes in TBST.

To detect a second (or third) probe, embryos were then washed twice in PTw for 15 minutes each, and antibody–peroxidase conjugates were inactivated by a mechanism-based inactivation process achieved with a one hour wash in 2% hydrogen peroxide. Embryos were then washed twice in TBST for 15 minutes each at room temperature and blocked for approximately five minutes with 2% (w/v) blocking reagent in MAB at room temperature. To detect the second (or third) probe, anti-dinitrophenol–horseradish peroxidase antibody conjugate (Perkin-Elmer) or anti-fluorescein–horseradish peroxidase antibody conjugate (Roche) was diluted 1:1000 in 2% (w/v) blocking reagent in MAB, and 500 µl of the diluted antibody was placed into each vial containing embryos. Antibody incubation was carried out at 4°C for approximately 12 hours.

As was done after the first antibody detection, after the second (or third) antibody detection, embryos were washed five times in TBST for one hour or more each and
Figure 1. Proposed mechanism for the horseradish peroxidase-catalyzed reaction of fluorophore-tyramide with tyrosine residues.

The theoretical mechanism by which fluorophore-tyramide forms a covalent bond with tyrosine residues during the fluorophore deposition step of multiplex fluorescent in situ hybridization. In the presence of a catalytic amount of hydrogen peroxide, horseradish peroxidase initiates radical formation on the phenolic oxygens of fluorophore-tyramide molecules and tyrosine residues in the cell. The radicals isomerize and terminate the radical chain reaction by forming a covalent bond between the two rings. The keto-phenolic rings enolize, forming the final reaction product in which fluorophore-tyramide is covalently bound to tyrosine in the cell.
washed twice in PTw for 10 minutes each. A fluorophore dilution in PTw and subsequent fluorophore deposition was carried out as described above. To detect a third uniquely-labeled probe and deposit a third fluorophore, the same procedure as used for the second detection and deposition was followed. Once all fluorophore depositions were completed, embryos were washed for at least 24 hours in TBST with several solution changes to wash out unbound fluorophore. Cy3 and fluorescein depositions could be checked by observation with an epifluorescent stereoscope, though Cy5 could not be verified until observed using the confocal microscope with a 633-nm laser excitation. Embryos were washed in TBST continuously and observed two to three times daily with an epifluorescent stereoscope until no further increase in signal-to-noise ratio was observed, after which embryos were fixed in 1X MEMFA and stored in the same manner as described for chromogenic in situ hybridization. All fluorescent in situ hybridization experiments were conducted with 30 or more embryos, and each individual experiment was replicated at least twice. All embryos were examined under the microscope in detail.

4.8. Frozen sectioning

Embryos processed with multiplex fluorescent in situ hybridization were cryosectioned for histological analysis, as paraffin embedding diminished the signal-to-noise ratio in the sections. Following fixation and storage in 1X PBS, embryos with the highest signal-to-noise ratios were selected for sectioning and incubated overnight in 1.6 M sucrose in sterile, deionized, distilled water at 4°C to cryoprotect the tissue. Embryos were placed in TBS tissue-freezing medium and frozen at -40°C until fully hardened, equilibrated to -20°C in the cryostat chamber, and 16-µm sections were taken and placed
on gelatin-coated slides. Slides were allowed to dry fully and were then rinsed for approximately five to ten minutes in 1X PBS. Finally, slides were cover-slipped using VectaShield HardSet fluorescent-protecting mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) nuclear stain at 1.5 µg/ml (Vector Laboratories). Two drops of medium were applied to the slide surface and a single glass cover-slip was slowly lowered onto the slide from one end to the other to minimize bubble formation. Excess medium was absorbed from the edges of the slide, after which the slide was allowed to harden and stored at 4°C.

4.9. Imaging

Images of representative embryos processed by whole mount in situ hybridization were taken after fixing and storing in 1X PBS as described above. In order to fully visualize chromogenic signal, whole embryos were cleared by dehydrating in methanol (three washes of 10 minutes each) and clearing in a 2:1 mixture of benzyl benzoate and benzyl alcohol (BB:BA). Embryos were viewed in glass dishes in 2:1 BB:BA using an Olympus SZH10 research stereoscope, and photographs were taken using an Olympus DP71 digital camera.

Images of representative paraffin-sectioned embryos from chromogenic in situ hybridization were viewed on an Olympus BX60 upright compound light microscope, and photographs were taken using a Media Cybernetics QCapture digital camera. All images were taken at the highest practical optical magnification available and slides were photographed to include a representative series of photographs along the anterior-posterior axis.
Cryosectioned embryos from multiplex fluorescent in situ hybridization were initially viewed on an Olympus BX60 upright compound epifluorescent microscope, and images for colocalization analysis were taken using a Carl Zeiss LSM510 laser scanning confocal and multiphoton microscope paired with a Carl Zeiss 20X apochromatic water immersion objective. The entire anterior-posterior axes of multiple embryos from a given multiplex fluorescent in situ hybridization experiment were examined. Representative images were taken based upon this examination. Briefly, this protocol was as follows. The neural tube was centered and magnified to fill the field of view, and plane-of-focus adjusted to the center of the Z-axis of the section. Cy3, Cy5, and fluorescein signal or Cy3, DAPI, and fluorescein signal were visualized simultaneously using multi-track recording. Each track was adjusted independently to maximize the dynamic range of the image by altering the detector gain such that the maximum signal was at the saturation limit, and by altering the amplifier offset such that the background was at the detection limit.

All images were manipulated for minor color, contrast, and brightness adjustment, and all figures were compiled and processed in Adobe Photoshop CS3.

4.10. Transcription factor binding site analysis

Coding sequences for xGAD67, xGAT1, and xVIAAT (GenBank Accession Numbers NM_001085801, NM_001095684, and BC057733, respectively) were located in the X. tropicalis genome by BLAST search (Joint Genome Institute, http://genome.jgi-psf.org/Xentr4/Xentr4.home.html). The 10 kbp upstream and downstream regions of each of these genes in the X. tropicalis genome were downloaded and screened for
transcription factor binding sites using MatInspector (Genomatix Software; Cartharius et al., 2005). Minimum core binding site similarity was restricted to 0.70 and greater, overall matrix similarity set to be optimized for function, and vertebrate transcription factor binding site weight matrices used for screening. Binding sites identified for Dlx1/2/5 and Ptf1a were recorded, as weight matrices for Pitx2 and BarH are not yet available for MatInspector.
5. **Results**

5.1. **xPitx2, xPtf1a, Xbh1, and Xbh2 clones**

In order to obtain partial clones for *X. laevis* paired-like homeodomain transcription factor 2 (*xPitx2*), pancreas-specific transcription factor 1a, bar homeobox protein 1, and bar homeobox protein 2, RT-PCR was employed using the primers described in the previous chapter and Table 1. For *xPitx2*, a fragment 748 bp in length was amplified that was found to share 99.6% identity with the published *X. laevis* *Pitx2* mRNA sequence (GenBank Accession Number AF077767). For *xPtf1a*, the 624 bp fragment produced shared 99.7% identity with the published *X. laevis* *Ptf1a* mRNA sequence (GenBank Accession Number DQ007931). The *Xbh1* fragment produced was 1014 bp in length and had a 96.9% nucleotide identity with the published *X. laevis* *Xbh1* mRNA sequence (GenBank Accession Number NM_001088552). Finally, for *Xbh2*, a fragment 1002 bp in length was amplified that shared 98.3% identity with the published *X. laevis* *Xbh2* mRNA sequence (GenBank Accession Number AF283692) (Appendix 2 for sequences for all clones).

5.2. **Expression patterns of xDlx2, xDlx5, xPitx2, xPtf1a, Xbh1, and Xbh2**

Whole mount chromogenic in situ hybridization followed by histological analysis was used to determine the expression patterns of *xDlx2, xDlx5, xPitx2, xPtf1a, Xbh1,* and *Xbh2* throughout development and compare them to those of the GABAergic and glycineric terminal differentiation genes *xGAD67, xGlyT1, xGlyT2,* and *xVIAAT.* *xDlx2* transcripts are first detected early in embryogenesis, and by neurula and early tailbud stages, are found in the branchial arches (Fig. 2A). In agreement with previous reports
Figure 2. Spatial and temporal expression patterns of \textit{xDlx2} and \textit{xGAD67} in developing embryos.

(A–H) Whole mount in situ hybridization; embryos are viewed laterally, with anterior to the left and dorsal to the top.  (A,E) Early tailbud stage embryos.  (B,F) Late tailbud stage embryos.  (C,G) Hatching stage embryos.  (D,H) Swimming tadpole stage embryos.  (I–T) Transverse 10-µm sections of swimming tadpole stage embryos processed with whole mount in situ hybridization, with dorsal to the top.  Schematic diagrams to the left of sections indicate the approximate plane of sectioning for each panel.  (A–D,I–N) \textit{xDlx2} expression.  (E–H,O–T) \textit{xGAD67} expression.  Abbreviations: br, branchial arch; cg, cement gland; di, diencephalon; ey, eye; hb, hindbrain; mb, midbrain; no, notochord; ot, otic vesicle; sc, spinal cord; te, telencephalon.  Scale bars represent 1.0 mm.
(Papalopulu and Kintner, 1993), xDlx2 is expressed in the telencephalon, diencephalon, cement gland, otic vesicle, and branchial arches by late tailbud stages (Fig. 2B). At hatching and swimming tadpole stages, xDlx2 mRNA is strongly expressed in the ventral telencephalon (Fig. 2C–D,I), medial diencephalon (Fig. 2C–D,J), retina (Fig. 2C–D,K), branchial arches (Fig. 2C–D,I–L), otic vesicle (Fig. 2C–D,L), and cement gland (Fig. 2C–D,I). The telencephalic and diencephalic expression, as well as the retinal expression, appears to occur in the same regions as that of xGAD67 in tailbud, hatching, and swimming tadpole stages (Fig. 2F–H,O–Q).

xDlx5 is expressed first in the presumptive forebrain, otic vesicle, and cement gland during neurula and early tailbud stages (Fig. 3A). As previously reported (Papalopulu and Kintner, 1993), by late tailbud stages, xDlx5 mRNA is found in the telencephalon, diencephalon, branchial arches, otic vesicle, and cement gland (Fig. 3B). This pattern persists and intensifies through swimming tadpole stages, ultimately being found in the ventral telencephalon (Fig. 3D,I), medial diencephalon (3D,J), branchial arches (Fig. 3D,I–K), otic vesicle (Fig. 3D,L), cement gland (Fig. 3D,I–J), and proctodeum (Fig. 3D,N). As with xDlx2, xDlx5 appears to be expressed in the same regions as xGAD67 in the telencephalon and diencephalon (Fig. 3F–H,O–P).

xPitx2 mRNA expression is first observed in the cement gland and presumptive diencephalon in early neurula stages. By early tailbud stages, in agreement with previous reports (Campione et al., 1999; Essner et al., 2000), xPitx2 is found in the cement gland, head mesenchyme, diencephalon, midbrain, retina, spinal cord, and left lateral plate mesoderm (Fig. 4B). This expression pattern continues through hatching stages (Fig. 4C). At swimming tadpole stages, xPitx2 expression is no longer observed in the head
Figure 3. Spatial and temporal expression patterns of $xDlx5$ and $xGAD67$ in developing embryos.

(A–H) Whole mount in situ hybridization; embryos are viewed laterally, with anterior to the left and dorsal to the top. (A,E) Early tailbud stage embryos. (B,F) Late tailbud stage embryos. (C,G) Hatching stage embryos. (D,H) Swimming tadpole stage embryos. (I–T) Transverse 10-µm sections of swimming tadpole stage embryos processed with whole mount in situ hybridization, with dorsal to the top. Schematic diagrams to the left of sections indicate the approximate plane of sectioning for each panel. (A–D,I–N) $xPitx2$ expression. (E–H,O–T) $xGAD67$ expression. Abbreviations: cg, cement gland; di, diencephalon; ey, eye; hb, hindbrain; hm, head mesenchyme; lp, lateral plate mesoderm; mb, midbrain; no, notochord; ot, otic vesicle; pr, proctodeum; sc, spinal cord; te, telencephalon. Scale bars represent 1.0 mm.
Figure 4. Spatial and temporal expression patterns of *xPitx2* and *xGAD67* in developing embryos.

(A–H) Whole mount in situ hybridization; embryos are viewed laterally, with anterior to the left and dorsal to the top. (A,E) Early tailbud stage embryos. (B,F) Late tailbud stage embryos. (C,G) Hatching stage embryos. (D,H) Swimming tadpole stage embryos. (I–T) Transverse 10-µm sections of swimming tadpole stage embryos processed with whole mount in situ hybridization, with dorsal to the top. Schematic diagrams to the left of sections indicate the approximate plane of sectioning for each panel. (A–D,I–N) *xDlx5* expression. (E–H,O–T) *xGAD67* expression. Abbreviations: br, branchial arch; cg, cement gland; di, diencephalon; ey, eye; hb, hindbrain; mb, midbrain; mc, myocardium; no, notochord; ot, otic vesicle; sc, spinal cord; te, telencephalon. Scale bars represent 1.0 mm.
mesenchyme, but is present in the cement gland (Fig. 4D), ventral and medial diencephalon (Fig. 4D,J), ventral midbrain (Fig. 4D,K), otic vesicle (Fig. 4D,L), and spinal cord (Fig. 4D,M–N). Additionally, xPitx2 transcripts are present in the outermost layers of the retina, possibly corresponding to photoreceptor or pigmented epithelial cells (Fig. 4D,J), and unilaterally in the left myocardium and lateral plate mesoderm, but not in the right (Fig. 4D,K–L). The xPitx2 expression observed in the medial diencephalon and ventral midbrain occurs in the same regions as xGAD67 expression, and transcripts observed in the spinal cord could overlap with xGAD67 or xGlyT2 expression.

In agreement with Afelik et al. (2006), xPtf1a transcripts are first observed at early tailbud stages in the presumptive hindbrain (Fig. 5A). By late tailbud stages, expression extends posteriorly into the anterior spinal cord and appears in the retina (Fig. 5B). At hatching stages, xPtf1a transcripts are found in the hindbrain, anterior spinal cord, retina, and dorsal and ventral pancreatic buds (Fig. 5C), a pattern that persists through swimming tadpole stages (Fig. 5D,I–K). The neural expression domains appear to overlap with xGAD67 expression in similar regions, especially considering the established role of xPtf1a in GABAergic retinal development (Dullin et al., 2007) (Fig. 5E–H,M–O).

Xbh1 expression, as previously reported (Patterson et al., 2000), is found in the presumptive diencephalon in late neurula and tailbud stages (Fig. 6A,B), developing to robust expression in the medial diencephalon, dorsal midbrain, and retina by swimming tadpole stages (Fig. 6D,N,O). Weak expression is also detected in the dorsal hindbrain by swimming tadpole stages (Fig. 6D,P). The expression in the diencephalon and midbrain may overlap with xGAD67-positive neurons; the retinal expression appears in
Figure 5. Spatial and temporal expression patterns of *xPtf1a* and *xGAD67* in developing embryos.

(A–H) Whole mount in situ hybridization; embryos are viewed laterally, with anterior to the left and dorsal to the top. (A,E) Early tailbud stage embryos. (B,F) Late tailbud stage embryos. (C,G) Hatching stage embryos. (D,H) Swimming tadpole stage embryos. (I–P) Transverse 10-μm sections of swimming tadpole stage embryos processed with whole mount in situ hybridization, with dorsal to the top. Schematic diagrams to the left of sections indicate the approximate plane of sectioning for each panel. (A–D,I–L) *xPtf1a* expression. (E–H,M–P) *xGAD67* expression. Abbreviations: di, diencephalon; dp, dorsal pancreatic buds; ey, eye; hb, hindbrain; mb, midbrain; no, notochord; ot, otic vesicle; sc, spinal cord; te, telencephalon; vp, ventral pancreatic buds. Scale bars represent 1.0 mm.
Figure 6. Spatial and temporal expression patterns of Xbh1, xGAD67, and xGlyT2 in developing embryos.

(A–L) Whole mount in situ hybridization; embryos are viewed laterally, with anterior to the left and dorsal to the top. (A,E,I) Early tailbud stage embryos. (B,F,J) Late tailbud stage embryos. (C,G,K) Hatching stage embryos. (D,H,L) Swimming tadpole stage embryos. (M–X) Transverse 10-µm sections of swimming tadpole stage embryos processed with whole mount in situ hybridization, with dorsal to the top. Schematic diagrams to the left of sections indicate the approximate plane of sectioning for each panel. (A–D,M–R) Xbh1 expression. (E–H,S–U) xGAD67 expression. (I–L,V–X) xGlyT2 expression. Abbreviations: di, diencephalon; ey, eye; hb, hindbrain; mb, midbrain; no, notochord; ot, otic vesicle; sc, spinal cord; te, telencephalon. Scale bars represent 1.0 mm.
the same areas as \(xGAD67\) and \(xGlyT1\) expression; and the hindbrain expression could overlap with that of \(xGlyT2\) (Fig. 6F–H,J–L,T–V). \(Xbh2\) mRNA is found in similar regions and agrees with Patterson et al. (2000) (Fig. 7). Transcripts are expressed in the diencephalon, dorsal midbrain, and dorsal hindbrain by swimming tadpole stages (Fig. 7D), possibly overlapping with \(xGAD67\) in the diencephalon and midbrain, and \(xGlyT2\) in the hindbrain (Fig. 7F–H,J–L,T–V).

5.3. **Coexpression of transcription factors with terminal differentiation genes**

To assess the coexpression of transcription factors with terminal differentiation genes, whole mount multiplex fluorescent in situ hybridization (FISH) was performed. Combinations of various transcription factors and terminal differentiation genes were selected based upon possible overlap in expression as indicated by chromogenic in situ hybridization. Experiments and results are summarized in Table 3.

Whole mount multiplex FISH was used to examine colocalization between the \(xDlx2\) and \(xDlx5\) transcripts and the GABAergic marker \(xGAD67\). In the telencephalon and diencephalon, virtually all cells expressing \(xGAD67\) (Cy3, red) also show expression of \(xDlx2\) (fluorescein, green); however, the expression pattern of \(xDlx2\) is more extensive (Fig. 8A–F). Additionally, though \(xGAD67\) expression is weak in the retina at hatching stages, it appears to colocalize moderately with \(xDlx2\) (Fig. 8G–I). Likewise, virtually all cells in the telencephalon and diencephalon expressing \(xGAD67\) (Cy3, red) also express \(xDlx5\) (fluorescein, green) (Fig. 8J–O). \(xDlx5\) shows a more extensive expression pattern than \(xGAD67\) in these regions. It thus appears that virtually all cells in the forebrain expressing \(xGAD67\) also express both \(xDlx2\) and \(xDlx5\). Whole mount multiplex FISH
Figure 7. Spatial and temporal expression patterns of $Xbh2$, $xGAD67$, and $xGlyT2$ in developing embryos.

(A–L) Whole mount in situ hybridization; embryos are viewed laterally, with anterior to the left and dorsal to the top. (A,E,I) Early tailbud stage embryos. (B,F,J) Late tailbud stage embryos. (C,G,K) Hatching stage embryos. (D,H,L) Swimming tadpole stage embryos. (M–X) Transverse 10-$\mu$m sections of swimming tadpole stage embryos processed with whole mount in situ hybridization, with dorsal to the top. Schematic diagrams to the left of sections indicate the approximate plane of sectioning for each panel. (A–D,M–R) $Xbh2$ expression. (E–H,S–U) $xGAD67$ expression. (I–L,V–X) $xGlyT2$ expression. Abbreviations: di, diencephalon; ey, eye; hb, hindbrain; mb, midbrain; no, notochord; ot, otic vesicle; sc, spinal cord; te, telencephalon. Scale bars represent 1.0 mm.
Table 3. Summary of colocalization analyses performed and results obtained.

Colocalization between transcription factors and terminal differentiation genes. Boxes indicate results for the central nervous system along the anterior–posterior axis, except for grayed boxes, which indicate that the particular combination was not performed. “No coloc.” designates a lack of any colocalization in the particular region, while bolded results indicate either colocalization or mutually exclusive expression. Colocalization is shown as a pair of percentages, where the first number is the approximate percentage of transcription factor-expressing cells also expressing the terminal differentiation gene, and the second number is the approximate percentage of terminal differentiation gene-expressing cells also expressing the transcription factor. “Anti” indicates mutually exclusive expression between the two transcripts in a single region with no significant colocalization. fb-tel, forebrain–telencephalon; fb-di, forebrain–diencephalon; mb, midbrain; hb, hindbrain; sc, spinal cord.
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Figure 8. Colocalization analyses of transcription factors (*xDlx2* and *xDlx5*) and *xGAD67* in the developing CNS of hatching stage embryos using whole mount fluorescent in situ hybridization.

All images are transverse sections viewed using confocal microscopy, with dorsal to the top. Each group of three images from left to right shows the red channel (Cy3), green channel (fluorescein), and a composite image of the red, green, and transmitted light channels where yellow represents overlap. Schematic diagrams to the left of the sections indicate the approximate plane of sectioning for a given set of images. (A–C) *xDlx2* and *xGAD67* in the telencephalon. (D–F) *xDlx2* and *xGAD67* in the diencephalon. (G–I) *xDlx2* and *xGAD67* in the eye. (J–L) *xDlx5* and *xGAD67* in the telencephalon. (M–O) *xDlx5* and *xGAD67* in the diencephalon. Scale bars represent 100 µm.
was also employed to observe colocalization between \(x\text{Pitx2}\) transcripts and the GABAergic markers \(x\text{GAD67}\) and \(x\text{GAT1}\). In the midbrain, \(x\text{Pitx2}\) (Cy3, red) and \(x\text{GAD67}\) (fluorescein, green) show moderate amounts of colocalization, but also have distinct, non-colocalized regions of cells that express one or the other (Fig. 9D–F). The same observation was made when comparing \(x\text{Pitx2}\) and \(x\text{GAT1}\) expression in the midbrain. However, in the diencephalon, \(x\text{Pitx2}\) (Cy3, red) and \(x\text{GAD67}\) (fluorescein, green) exhibit expression patterns that occupy the same region, but show virtually no cellular coexpression (Fig. 9A–C). This mutually exclusive pattern was also observed when examining \(x\text{Pitx2}\) and \(x\text{GAT1}\) expression in the diencephalon. In the spinal cord, \(x\text{Pitx2}\) is expressed too dorsally to overlap with either \(x\text{GAD67}\) or \(x\text{GAT1}\) expression (Fig. 9G–I).

The colocalization between \(x\text{Ptf1a}\) or \(x\text{Xbh1}\) and the GABAergic marker \(x\text{GAD67}\) was then analyzed. Whole mount multiplex FISH showed that in the retina, most cells expressing \(x\text{GAD67}\) (fluorescein, green) also express \(x\text{Ptf1a}\) (Cy3, red), while only a small fraction of the cells expressing \(x\text{Ptf1a}\) also express \(x\text{GAD67}\) (Fig. 9J–L). In the remainder of the central nervous system, \(x\text{Ptf1a}\) is expressed in regions dorsal to \(x\text{GAD67}\) expression (Fig. 9M–O). \(x\text{Xbh1}\) expression (Cy3, red) was found in a pattern mutually exclusive to \(x\text{GAD67}\) (fluorescein, green) in the midbrain (Fig. 9P–R).

Possible coexpression of \(x\text{Ptf1a}\) or \(x\text{Xbh1}\) with the non-neural glycinergic marker \(x\text{GlyT1}\) was then assessed given the possible overlapping patterns indicated by chromogenic in situ hybridization, and both genes’ possible roles in glycinergic amacrine cell specification. No colocalization was found between \(x\text{Ptf1a}\) (Cy3, red) and \(x\text{GlyT1}\) (fluorescein, green)—both are expressed in distinct domains in the central nervous
Figure 9. Colocalization analyses of transcription factors ($xPitx2$, $xPtf1a$, and $Xbh1$) and $xGAD67$ in the developing CNS of hatching stage embryos using whole mount fluorescent in situ hybridization.

All images are transverse sections viewed using confocal microscopy, with dorsal to the top. Each group of three images from left to right shows the red channel (Cy3), green channel (fluorescein), and a composite image of the red, green, and transmitted light channels where yellow represents overlap. Schematic diagrams to the left of the sections indicate the approximate plane of sectioning for a given set of images. (A–C) $xPitx2$ and $xGAD67$ in the diencephalon. (D–F) $xPitx2$ and $xGAD67$ in the midbrain. (G–I) $xPitx2$ and $xGAD67$ in the anterior spinal cord. (J–L) $xPtf1a$ and $xGAD67$ in the eye. (M–O) $xPtf1a$ and $xGAD67$ in the anterior spinal cord. (P–R) $Xbh1$ and $xGAD67$ in the midbrain. Scale bars represent 100 µm.
system and do not overlap in the eye (Fig. 10A–C). However, all cells in the retina expressing Xbh1 (Cy3, red) also show xGlyT1 expression (fluorescein, green) (Fig. 10D–F). Because the expression pattern of Xbh1 is more extensive than that of xGlyT1, only a moderate fraction of cells expressing xGlyT1 also express Xbh1.

Next, whole mount multiplex FISH was used to determine possible transcription factor colocalization with the neural glycinergic marker xGlyT2. xPitx2 showed no colocalization with xGlyT2 in the spinal cord, as the xPitx2 expression was always more dorsal (Fig. 10G–I). Similarly, neither Xbh1 nor Xbh2 colocalize with xGlyT2 in the hindbrain, as the transcription factor expression in both cases was dorsal to that of xGlyT2 (data not shown). In summary, none of the transcription factors analyzed appear to colocalize with xGlyT2.

Finally, the possible coexpression of transcription factors with xVIAAT was assessed, since extensive overlap of some transcription factors and xGAD67 was observed. In the telencephalon and diencephalon, the colocalization patterns between xDlx2 and xVIAAT appear identical to those for xDlx2 and xGAD67 (Fig. 10J–L). The same observation was made when observing colocalization between xDlx5 and xVIAAT (data not shown). Finally, mutually exclusive expression observed between Xbh1 and xVIAAT in the midbrain, identical to that observed between Xbh1 and xGAD67 (Fig. 10M–O). Therefore, it appears that xDlx2, xDlx5, and Xbh1 colocalize with the same frequencies with xVIAAT as they do with xGAD67 in the telencephalon, diencephalon, and mesencephalon.
Figure 10. Colocalization analyses of transcription factors (\textit{xPitx2}, \textit{xPtf1a}, and \textit{Xbh1}) and \textit{xGlyT1}, \textit{xGlyT2}, and \textit{xVIAAT} in the developing CNS of hatching stage embryos using whole mount fluorescent in situ hybridization.

All images are transverse sections viewed using confocal microscopy, with dorsal to the top. Each group of three images from left to right shows the red channel (Cy3), green channel (fluorescein), and a composite image of the red, green, and transmitted light channels where yellow represents overlap. Schematic diagrams to the left of the sections indicate the approximate plane of sectioning for a given set of images. (A–C) \textit{xPtf1a} and \textit{xGlyT1} in the eye. (D–F) \textit{Xbh1} and \textit{xGlyT1} in the eye. (G–I) \textit{xPitx2} and \textit{xGlyT2} in the posterior spinal cord. (J–L) \textit{xDlx2} and \textit{xVIAAT} in the telencephalon. (M–O) \textit{Xbh1} and \textit{xVIAAT} in the midbrain. Scale bars represent 100 µm.
5.4. **Transcription factor binding site analysis**

In order to further examine the role of these transcription factors in inhibitory specification using available in silico technologies, transcription factor binding site analysis was performed using MatInspector (Genomatix Software; Cartharius et al., 2005). Unfortunately, the MatInspector transcription factor weight matrix database is not a complete database of all vertebrate transcription factors, and thus only screening for Dlx1/2/5 and Ptf1a sites was possible with the software. Mat Inspector was chosen because the other transcription factor binding site analysis programs available for free academic use, including rVista 2.0 (NCBI), TFBIND (University of Tokyo), TFSEARCH (Computational Biology Research Center), and OFTBS (Tsinghua University), all screened for fewer transcription factors.

For each GABAergic terminal differentiation gene (\textit{xGAD67}, \textit{xGAT1}, and \textit{xVIAAT}), 10 kb upstream and downstream regions from the \textit{X. tropicalis} genome, which is over 95\% complete and very similar to the \textit{X. laevis} genome, were screened for binding sites. This analysis showed flanking regions of GABAergic terminal differentiation genes contain numerous transcription factor binding sites. \textit{xGAD67} has five upstream Ptf1a binding sites, six downstream Ptf1a binding sites, and one downstream Dlx1/2/5 site. Similarly, \textit{xGAT1} is flanked by three upstream and three downstream Ptf1a binding sites, and two downstream Dlx1/2/5 sites. Finally, \textit{xVIAAT} has four upstream Ptf1a binding sites, one upstream Dlx1/2/5 site, 12 downstream Ptf1a binding sites, and one downstream Dlx1/2/5 site. Perhaps most interestingly, the region between 9 and 10 kb downstream of \textit{xVIAAT} contains many of these sites—a cluster containing seven regularly spaced Ptf1a binding sites and a Dlx1/2/5 site is present (Table 4).
Table 4. Transcription factor binding sites in the upstream and downstream regions flanking the coding sequences of GABAergic terminal differentiation genes.

Transcription factor binding sites found by MatInspector. Position indicates the number of base pairs upstream or downstream of the coding sequence where the transcription factor binding site is located. Strand indicates whether the binding site is located on the coding strand (+) or template strand (-). Core identity is the sequence identity of the core binding sequence, and matrix identity is the sequence identity of the full binding matrix sequence.
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6. **Discussion**

6.1. *Similar colocalization patterns suggest common regulatory mechanisms*

We have observed that colocalization patterns of the GABAergic terminal differentiation genes (*xGAD67*, *xGAT1*, and *xVIAAT*) and several transcription factors exhibit substantial similarity in certain regions of the developing nervous system. As described in the results, the colocalization observed between *xDlx2* and *xGAD67* in the telencephalon and diencephalon appears to be identical with that observed between *xDlx2* and *xVIAAT* in the same regions. A similar phenomenon is observed in the telencephalon and diencephalon when examining the colocalization pattern of *xDlx5* and *xGAD67* and comparing it to that of *xDlx5* and *xVIAAT*.

Likewise, coexpression pattern similarity is observed between *xGAD67* and *xGAT1*. *xPitx2* expressing neurons colocalize with both *xGAD67* and *xGAT1* in the midbrain, and are mutually exclusive of both *xGAD67* and *xGAT1* in the diencephalon. This similarity in mutual exclusivity of expression within a region is also observed with *Xbh1* transcripts, which coregionalize without colocalizing with both *xGAD67* and *xVIAAT* in the midbrain.

The similarity in colocalization patterns between various transcription factors and the three primary GABAergic terminal differentiation genes suggests that *xGAD67*, *xGAT1*, and *xVIAAT* could be controlled by common regulatory pathways involving these transcription factors. Yu et al. (2003) report that 3.3% of target gene pairs regulated by the same transcription factors are coexpressed, a percentage four times greater than expected for a random pair of genes. Indeed, previous reports indicate that in these regions of the brain, the expression patterns of *xGAD67* and *xVIAAT* are very similar, not
surprising when considering that GAD67 is required for GABA synthesis and VIAAT is required for synaptic release of GABA (Wester et al., 2008). Additionally, xGAT1 has been found to colocalize extensively with xGAD67 throughout the central nervous system (Daniel Teasley, unpublished). This indicates that the three primary GABAergic terminal differentiation genes, merely by their coexpression with one another, may be regulated by a common factor or factors.

Given that all three exhibit similar coexpression patterns with multiple transcription factors, as well as mutual exclusivity with others, it appears even more likely that GABAergic terminal differentiation is controlled by a set of transcription factors that regulate xGAD67, xGAT1, and xVIAAT. This is further supported by the observation that all three genes share binding sites in the flanking noncoding regions for Ptf1a and Dlx1/2/5. Since the terminal differentiation genes colocalize with these transcription factors, it is possible that they are directly regulating transcription of GABAergic terminal differentiation during development. Although transcription factor binding site analysis is not available yet for the BarH family of transcription factors, the extensive colocalization of Xbh1 and xGlyT1 in the retina indicates that Xbh1 may be playing a direct regulatory role in glycinergic amacrine cell specification. Finally, while this study did not identify any transcription factors that colocalize with xGlyT2 transcripts, it is likely that studies of more transcription factors would identify possible candidates as has been done for the GABAergic terminal differentiation genes and the glycinergic amacrine cells.
6.2. Mutually exclusive expression patterns

Two of the transcription factors examined—\textit{xPitx2} and \textit{Xbh1}—exhibited a phenomenon where the transcription factor was expressed in the same region but in a mutually exclusive pattern to GABAergic terminal differentiation genes, resulting in virtually no colocalization. For \textit{xPitx2}, this occurred in the diencephalon with \textit{xGAD67} and \textit{xGAT1}, while for \textit{Xbh1}, it was observed in the midbrain with \textit{xGAD67} and \textit{xVIAAT} (Table 3). Such a pattern is suggestive of two possible regulatory modes by which these transcription factors might be acting—either negative or inhibitory regulation, where the transcription factor downregulates the expression of GABAergic terminal differentiation genes, or signaling pathways, where a transmembrane or secreted ligand causes adjacent cells to upregulate the expression of GABAergic terminal differentiation genes.

Inhibitory mechanisms of regulation have been previously found to be involved in neuronal specification with the \textit{Tlx3} post-mitotic selector gene. \textit{Tlx3} has been found to regulate the glutamatergic neurotransmitter phenotype by inhibiting GABAergic terminal differentiation genes (Cheng et al., 2004). In \textit{Tlx3} null mice, GABAergic markers are derepressed in prospective glutamatergic neurons. Furthermore, ectopic expression of \textit{Tlx3} in the chick spinal cord is able to repress most GABAergic differentiation and induce formation of excess glutamatergic populations (Cheng et al., 2004). Taken together, \textit{Tlx3} appears to regulate glutamatergic specification by inhibiting GABAergic neurotransmitter phenotype specification. The expression patterns seen for \textit{Tlx3} compared to GABAergic markers are similar to those observed here between \textit{xPitx2} or \textit{Xbh1} and GABAergic markers. It is possible that these patterns are observed because
expression of \textit{xPitx2} in the diencephalon and expression of \textit{Xbh1} in the midbrain inhibit expression of \textit{xGAD67}, \textit{xGAT1}, and \textit{xVIAAT}.

Juxtacrine mechanisms of regulation, specifically the Notch signaling pathway, have also been implicated in neurotransmitter phenotype specification. It has been shown that the Notch pathway plays a critical role in neurotransmitter phenotype specification in some systems (Perron and Harris, 2000). Yun et al. (2002) have found that bHLH and homeobox transcription factors can act in concert to regulate Notch signaling activity in telencephalon precursors, though the components of the pathway which are being regulated have not yet been identified. In the absence of bHLH transcription factor \textit{Mash1}, Notch signaling was compromised, resulting in an increase of \textit{DLX2}, \textit{Dlx5}, and \textit{GAD67} expressing cells (Yun et al., 2002). In contrast, \textit{Dlx1/2} mutants show increased Notch signaling and a resultant decrease in neuronal differentiation, suggesting that \textit{Mash1} and \textit{Dlx1/2} act in a homeostatic manner to upregulate and downregulate Notch signaling during development, ultimately controlling neuronal phenotypes (Yun et al., 2002). Kabos et al. (2002) have also found that inhibition of Notch signaling increases GABAergic progenitors, finding that suppression of \textit{HES1}, a target transcription factor of the Notch intracellular domain, results in increased GABAergic proliferation. Given the mutual exclusivity of expression between \textit{xPitx2} and GABAergic terminal differentiation genes, as well as \textit{Xbh1} and GABAergic terminal differentiation genes, it is possible that these transcription factors could be modulating a Notch signaling pathway component, resulting in adjacent cells transcribing GABAergic terminal differentiation genes.

Other signaling pathways could also be responsible for the observed phenomenon of mutually exclusive expression. Gulasci and Lillien (2003) found that sonic hedgehog
(Shh) induces GABAergic interneuron production in dorsal telencephalic progenitors, while bone morphogenetic protein (BMP) inhibits GABAergic interneurons. Furthermore, Shh may be promoting interneuron fate by blocking BMP signaling (Gulasci and Lillien, 2003). Epstein et al. (2000) found that Sim2, a bHLH transcriptional regulator, activates Shh. It is possible that that xPitx2 and Xbh1 act similarly to upregulate a signaling molecule like Shh, triggering expression of GABAergic terminal differentiation genes in nearby cells.

6.3. Transcription factors may act in combination to regulate specification

The overlap in transcription factor expression patterns and colocalization patterns suggest that inhibitory neurotransmitter phenotype specification occurs via combinatorial mechanisms in which multiple transcription factors are necessary for appropriate patterning. First, as noted by Yu et al. (2003), target genes of transcription factors are significantly more likely to colocalize when the targets are controlled by multiple common transcription factors. Since significant colocalization is observed between the major three GABAergic terminal differentiation genes, it is much more likely that multiple transcription factors regulate them than a single transcription factor.

Just as telling is the overlap between the transcription factors themselves. xDlx2 and xDlx5, which have been shown to cross-regulate one another and both of which are implicated in inhibitory neurotransmitter phenotype specification (Stühmer et al., 2002), show significant overlap in expression patterns in the telencephalon and diencephalon. xDlx2 and xDlx5 also show distinct but very similar coexpression patterns with GABAergic terminal differentiation genes, suggesting that the two may be acting in
concert to regulate GABAergic specification in the forebrain. Additional evidence is observed in the diencephalon, where \textit{xPitx2} is expressed mutually exclusive to GABAergic terminal differentiation genes. Thus, in the diencephalon, \textit{xDlx2}, \textit{xDlx5}, and \textit{xPitx2} could all be regulating GABAergic specification together. In the midbrain, \textit{xPitx2} exhibits moderate colocalization with GABAergic terminal differentiation genes, while \textit{Xbh1} shows mutually exclusive expression to the same genes. Regardless of whether \textit{Xbh1} is acting via an inhibitory or juxtacrine mechanism, it is possible that both \textit{xPitx2} and \textit{Xbh1} act in concert to regulate GABAergic specification in the midbrain.

Finally, the transcription factor binding site data confirm the possibility of combinatorial regulation by transcription factors. Binding sites for multiple transcription factors, and significantly, for Dlx1/2/5 and Ptf1a, are found in the flanking regions of GABAergic terminal differentiation genes in the \textit{X. tropicalis} genome. Given the high levels of conservation between the \textit{X. laevis} and \textit{X. tropicalis} genomes, these results are indicative of binding sites in the \textit{X. laevis} genome. While additional evidence in the form of in vivo promoter analysis is necessary to confirm the validity of the expression and transcription factor binding site data, it seems likely given both sets of data that in at least some of the central nervous system, the transcription factors analyzed are acting in a combinatorial mechanism to regulate GABAergic specification.

### 6.4 Future directions

Several possibilities exist for future studies based upon this research. Our original primary question was to examine if transcription factors mediated the effects of Ca$^{2+}$ on neurotransmitter phenotype identity. Ca$^{2+}$ manipulations can be accomplished in \textit{X.}
laevis simply by adjusting the amount of Ca\(^{2+}\) available in the culture medium. By varying Ca\(^{2+}\) concentration, the frequency and amplitude of Ca\(^{2+}\) transients can be modulated—if no Ca\(^{2+}\) is available in the extracellular space, Ca\(^{2+}\) spikes and waves will not occur. Performing the analysis conducted in this thesis on embryos cultured in varying Ca\(^{2+}\) concentrations and observing perturbations in the transcription factor expression patterns and coexpression with terminal differentiation genes will provide evidence for or against transcription factor-mediated Ca\(^{2+}\) effects on neurotransmitter phenotype. Another interesting, if more difficult, step would be correlating these data directly with Ca\(^{2+}\) activity by electrophysiological or Ca\(^{2+}\)-imaging studies on Xenopus embryos or single cells. However, as this study has demonstrated, the coexpression patterns of transcription factors, while consistent, implicate several more complex transcriptional regulatory mechanisms in neurotransmitter phenotype specification. Elucidating how Ca\(^{2+}\) activity regulates a more complex regulatory pathway, such as the mediation of signaling pathways or combinatorial transcription factor activity, may be difficult.

The scope of this study could also be expanded to include other transcription factors and phenotypes. For instance, examining \(xTlx1\) and \(xTlx3\) and their roles in specifying the glutamatergic terminal differentiation gene \(xVGlut1\) would be a valuable step in understanding the relationships between transcription factors and terminal differentiation genes (Cheng et al., 2004; Gleason et al., 2003). This study was unable to identify transcription factors that colocalize with the neural glycinegic marker \(xGlyT2\), which have yet to be identified in the literature. An analysis of more transcription factors
could identify factors that could be involved in glycinergic specification, a relatively unexplored field.

Finally, and perhaps most interestingly, examining the combinatorial roles of the transcription factors in vivo would be a logical next step. Previous work in this lab has identified a 4 kb region upstream of \textit{xGAD67} that drives reporter gene expression in a tissue-specific pattern nearly identical to the \textit{xGAD67} endogenous expression pattern using transgenesis in \textit{X. laevis} (Conor Sipe and David Solomon, unpublished). Using the bioinformatic and expression data gathered here, regions containing binding sites for transcription factors that coexpress with terminal differentiation genes could be analyzed in vivo for sufficiency in driving reporter gene expression. Such a study could determine more conclusively which transcription factors are involved in specification by including regions containing binding sites for specific factors only in the analysis. Additionally, morpholino knockdown and mRNA overexpression experiments in \textit{X. laevis} could clarify any transcription factors’ roles in specifying a given terminal differentiation gene by monitoring the terminal differentiation gene expression following knockdown and upregulation.
Appendix 1: Fluorophore-tyramide synthesis protocol

This protocol details the preparation of the fluorescent-tyramide conjugate solution for use in a fluorescent in situ hybridization. The chemical reaction will only work under anhydrous conditions, so reagents should be as fresh as is reasonable. Dimethyl formamide absorbs water once opened, so we use a stock bottle of “extra dry dimethyl formamide” from Acros Organics with a rubber septum to prevent moisture entry. The dimethyl formamide must be dispensed by piercing the septum with the needle on a syringe, tipping the bottle sideways, and drawing out the needed volume. Dimethyl formamide should be transferred to a microcentrifuge tube and measured for the reaction with a pipetman, as the syringe does not accurately measure volume. Dimethyl formamide and triethylamine (the latter in particular) are toxic and flammable, and should be handled and dispensed only in the hood. Fluorophore NHS esters (Cy3-NHS, Cy5-NHS, and fluorescein-NHS) are extremely expensive and light sensitive, so all work should be performed with the lights out! Note that the quantities are NOT interchangeable for Cy3- or Cy5- verses fluorescein-tyramide synthesis, but that for a given fluorophore, the reaction may be scaled to make more or less final product.

Cy3- or Cy5-tyramide

1. Make DMF-TEA. Combine the following in a microcentrifuge tube:
   - 1 ml dimethyl formamide (Acros Organics #326871000)
   - 10 µl triethylamine (Sigma #T0886)

2. Make tyramide solution. Combine the following in the vial in which the tyramine hydrochloride is shipped:
   - 10 mg tyramine hydrochloride (in the vial) (Sigma #T2879-10mg)
   - 1 ml DMF-TEA

3. Make a 10 mg/ml stock of Cy3-NHS (or Cy5-NHS) in dimethyl formamide. Remember to transfer the dimethyl formamide from the bottle to a microcentrifuge tube with a syringe, and measure the amount to add to the Cy3-NHS (or Cy5-NHS) with a pipetman! Combine the following in the vial in which the Cy3-NHS (or Cy5-NHS) is shipped:
   - 1 mg Cy3-NHS (or Cy5-NHS) solid (in the vial) (GE Healthcare #PA13101 for Cy3-NHS, #PA15101 for Cy5-NHS)
   - 100 µl dimethyl formamide

4. Perform the final reaction in a microcentrifuge tube:
   - 100 µl Cy3-NHS in dimethyl formamide
   - 33 µl tyramide solution

   Mix the tube well by inverting. Cover the tube with aluminum foil and incubate at room temperature for 2 hours.
5. Dilute and aliquot. Add 1.2 ml of 100% ethanol to the reaction and mix well. Separate into 200 µl aliquots and store at -20°C protected from light. Dispose of the extra tyramide solution into a designated hazardous waste container.

**Fluorescein-tyramide**

1. Make DMF-TEA. Combine the following in a 15 ml Falcon tube:
   - 2 ml dimethyl formamide (Acros Organics #326871000)
   - 20 µl triethylamine (Sigma #T0886)

2. Make tyramide solution. Combine the following in a 15 ml Falcon tube:
   - 20 mg tyramine hydrochloride (weigh carefully on the balance) (Sigma #T2879)
   - 2 ml DMF-TEA

3. Make a 10 mg/ml stock of fluor-NHS in dimethyl formamide. Combine the following in a 15 ml Falcon tube:
   - 40 mg fluor-NHS solid (weigh carefully on the balance) (Pierce Biotechnology #46100)
   - 4 ml dimethyl formamide

4. Perform the final reaction in a 15 ml Falcon tube:
   - 4 ml fluor-NHS in dimethyl formamide
   - 1.37 ml tyramide solution

   Mix the tube well by inverting. Cover the tube with aluminum foil and incubate at room temperature for 2 hours.

5. Dilute and aliquot. Add 4 ml of 100% ethanol to the reaction and mix well. Separate into 1 ml aliquots and store at -20°C protected from light. Dispose of the extra tyramide solution into a designated hazardous waste container.
Appendix 2: Transcription factor clone sequences

**xPitx2 full sequence:**

1. GGCTGGAGGT AGAGTTGCTG CAGCTTCCTC CCATCCACAC CACATCATC ACCACCCAGA CACAGAGTG
2. CCGACCCCTA TCTCAAGCAG GTAAGAGAGA GGTAGGTGTT GTGTAGTATG TTGTTGGTCTG GTCGTCAC
3. ACGCTTGTAT CACAGCTCCT CCTATTGCTG CATGGCTCAG CAGGTGCACT CACGAGTCAG
4. TGAGAGCTCAG GCACTCCAGA CAAACAGAC TCAAGCTAAC CAGGGAAAGA ATGCGAGTAC
5. GCCTGGCTGG GTGGCAGGTC GATGCTTCTA GTT GCCCTTTCTGAGC
6. AAAGGAGCGA CAGAGACCTT CAGTATGTA AAGAAGAGTG
7. CTTGACGCCTG CTGCTCTGAG AAGAGACCTT CAGTATGTA AAGAAGAGTG
8. GCTGCTGGCTGG CTGCTCTGAG AAGAGACCTT CAGTATGTA AAGAAGAGTG

---

**xPtf1a full sequence:**

1. TTGCCTTGCA TTTGATGAAG AGATTTTCTT CACGGATCATC TCTCTCTAGGG ACCTCCAGCA CCGACAGAC
2. AAGGCCAATG AAATCTCCCT TCTCAAGGAG AAGGTCTAGT ACGAGATCCTT CCGTGGAGCT GCATTCCGCT
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XbaI sequence from M13(forward) sequencing primer (5' end of XbaI)*:

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211  GAGACGCAAA CCCCCTCGCTG CCGGCCGCCG GCAGACACA CAGCTTCTCT CAGCTTCTCA CACTCCAAAA
    CCGTCTGTTT GGACCCGAGC GCACCGGGGG CATGCTGCAG TGCCAAGGGAA GAGGAGATGT GTGGAGGTGT
281  CGGAGAGCCA ATGGCTCTTC GCAGAATGCT CCGCCCAAAC TAGAACAGGA GTAGCAAGAT GCCAAGGGGA
    GACCTCCTGC TACCCAGAGG CCTCTGAGAG GGCAGGGTGG ACTCTTGCCT CTAGTTTCTA CGGTTTCCCT
351  AGTGAAGACA GATCCGGGAA GACTTCGATA CAGACTTCAA AGGGCAAG
    TCCATCTGTT CTACGCGCTTC ATGAGCAGAT GTCTGAAGTT TCCCGTGC
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XbaI sequence from M13(reverse) sequencing primer (3' end of XbaI)*:

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71  ATAGATAGTG TTTTCACGGG TGGGAGGTTG ACCTCGGATT GGGTTTTCAAC GAGGGTTGAC AGCTGTTGCTC
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141  CCCCCGCGCC CGCAGACATG GATAAGTACC CTGGGGACCA AGGGCTCTCT CATTGTTTGGG TGGGGGGAGC
    GGGGACCCCG GTGTGGCTAC CTATTCTAGG GACCCTGGTCC TCCCCAGAGAC ATCAAGACCC ACCCCCGTC
211  GGGAGACTCC CCCCCTCAGG
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*Due to a clone length of over 1000 bp, full sequences were unable to be obtained without sequencing using internal primers. As such, the sequences obtained from sequencing primers in the vector are reported here.
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*Due to a clone length of over 1000 bp, full sequences were unable to be obtained without sequencing using internal primers. As such, the sequences obtained from sequencing primers in the vector are reported here.*
References


