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The Role of Spontaneous Intracellular Calcium Transients in Neurotransmitter Phenotype Specification in Xenopus laevis

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The Role of Spontaneous Intracellular Calcium Transients in Neurotransmitter Phenotype Specification in *Xenopus laevis*

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

by

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Abstract

Spontaneous intracellular calcium activity has been implicated in a host of processes related to nervous system development, including neural induction, neural tube closure, and synaptogenesis. One of these calcium-influenced processes, neurotransmitter phenotype specification, involves the acquisition of the correct balance and patterning of excitatory and inhibitory neurons, and its regulation is vital to proper nervous system functionality. While a high frequency of intracellular calcium transients in presumptive neurons during development has been correlated with an inhibitory fate, the persistence of this phenomenon in in vitro models has not been conclusively demonstrated. Additionally, we believe that current methods of calcium activity analysis, which are limited to counting fluorescent indicator spikes above a particular threshold, is limiting. To this end, we employed Xenopus laevis presumptive neural tissue as an in vitro model system, imaging calcium activity in developing neural cells. This data was analyzed via a novel pipeline that uses fluorescence trace entropy as a comparative metric rather than relying on predetermined parameters to define particular features (i.e., spikes or waves). Use of this analysis method revealed differences in calcium activity across development, with cells dissected from younger embryos displaying more entropic calcium activity that gradually decreased across development. Relatively small differences were found between cells positive for the expression of particular neural marker genes and cells that did not express these genes, and these differences varied across developmental time points. Most notably, cells positive for different specific neural marker genes displayed significantly different levels of calcium activity entropy from one another. As a whole, these results provide support for the hypothesis that particular patterns of calcium dynamics are associated with the expression of particular genes involved in the neuronal differentiation process.
1. Introduction

1.1. Overview of the Problem

Within the embryonic vertebrate nervous system, neural progenitor cells must undergo the highly-regulated process of neuronal differentiation, developing from precursor stem cells into mature neurons expressing distinct neurotransmitter phenotypes. Inappropriate regulation of this process can result in improper expression of GABAergic and/or glutamatergic neurons, disrupting the precisely-controlled balance between inhibitory and excitatory neuronal phenotypes. The proper acquisition of this phenotypic balance is vital to nervous system function, with disruptions implicated in disorders ranging from schizophrenia to autism (Kehrer et al., 2008; Hussman, 2001). However, the mechanisms by which this balance is acquired remain incompletely understood.

Significant attention has been paid to the role of ‘hard-wired’ transcriptional cascades in the terminal neural differentiation process (Feng et al., 2013; Rossignol, 2011). However, activity-dependent mechanisms must also be investigated. One promising activity-dependent mediator of fate determination is calcium – specifically, low-frequency calcium transients that occur spontaneously throughout the developing nervous system. These transients are observed beginning at fertilization (Halet et al., 2003), persist throughout embryonic development, and have been implicated in neural induction, neurogenesis, and neurotransmitter phenotype specification (Blankenship and Feller, 2010). These calcium waves, mediated by voltage-gated calcium channels (VGCCs) have been shown to modulate protein expression by coordinating protein-protein interactions and influencing phosphorylation states (Spitzer, 2006). Calcium activity has also been
linked to transcriptional regulation (Spitzer et al., 2000). More specifically, the proposed homeostatic model links increased calcium spike frequency during a critical developmental period with an eventual decreased expression of excitatory neurons and, inversely, decreased spike frequency with increased expression of excitatory neurons. (Spitzer et al., 2005).

Manipulation of this calcium activity at key developmental stages has been shown to impact neurotransmitter phenotype, even to the point of overriding other co-existing developmental cues (Borodinsky et al., 2004). However, many of these experiments have been limited both in their ability to directly trace the identity of a particular cell and in their ability to analyze this calcium activity in a nuanced way. The goal of this project, then, is to use in vitro cell culture methods to study the relationship between intracellular calcium transients and neurotransmitter phenotype on a single-cell level. *Xenopus laevis* has been used as a model organism for this research, which explores the existence of differential calcium activity between cells expressing different neural marker genes through novel feature-independent methods of calcium data analysis.

### 1.2. Review of the Literature

#### 1.2.1. Neural Development and Neurotransmitter Phenotype Specification

Neurotransmitter phenotype specification is a complex process that involves significant interplay between electrical activity, trophic factors, and morphogenetic proteins (Borodinsky and Belgacem, 2015). However, this terminal differentiation is a relatively late step in a more extensive process of neural development that begins during
gastrulation and continues, in *Xenopus laevis*, through a poorly-characterized phase of secondary neurogenesis occurring at the beginning of the larval period (Wulliman et al., 2005). These processes must regulate acquisition of specific neural identity, as well as large-scale morphological migrations. The following review will discuss these processes of neural development primarily as understood in *Xenopus laevis*; however, these mechanisms are largely conserved across vertebrate species.

Patterning begins at blastula stages, as β-Catenin becomes localized to the dorsal side of the embryo (Heasman et al., 1994). This leads to high expression levels of Nodal-related genes (*Xnr1*, *Xnr2*, *Xnr4*) in these cells, inducing formation of the Spemann Organizer at the dorsal blastopore lip (Agius et al., 2000). The Spemann Organizer expresses *Noggin* and *Chordin*, both of which function to antagonize bone morphogenetic proteins (BMPs) by blocking them from interacting with their receptors (Piccolo et al., 1996; Zimmerman et al., 1996). BMPs, most notably BMP-4, serve to repress neural development; therefore, the quenching of these signals by noggin and chordin proteins allows neural induction to progress. Severe disruption of notochord and forebrain development in *chordin;noggin* double-homozygous mutant mice is further evidence for the vital role of these genes in patterning the developing embryo (Bachiller et al., 2000).

Fibroblast growth factor (FGF) signaling also plays a key role in the mesodermalization of appropriate tissues. In *Xenopus* embryos, expression of a nonfunctional mutant FGF receptor leads to a failure of mesoderm induction, as well as subsequent irregularities during gastrulation (Amaya et al., 1991). Additionally, microinjection of synthetic *XbFGF* mRNA has been shown to induce ectopic mesoderm
formation in animal cap explants (Kimelman and Maas, 1992). A cadre of other proteins secreted by the Spemann organizer, including Frzb-1, Dkk1, Cerberus, and Follistatin, also contribute to proper dorsal-ventral and anterior-posterior patterning (Carron and Shi, 2016).

The modulation of Wnt signaling has also been implicated in this patterning and differentiation process. For example, expression of xNorrin has been demonstrated to promote anterior neural tissue formation by both activating canonical Wnt signaling and inhibiting BMP/Nodal signaling (Xu et al., 2012). Wnt also functions to inhibit Sonic hedgehog (Shh) pathways, with antagonistic interactions between the two establishing a dorsal-ventral pattern within presumptive neural tissue (Ulloa and Marti, 2009).

This combination of BMP, FGF, and Wnt signaling pathways is necessary for proper definition of the neural plate (Meulemans and Bronner-Fraser, 2004). The neural plate border (NPB) serves to separate neural and non-neural ectoderm, with neural ectoderm induced to express the transcription factors Sox2, Zicr-1, and Zic3 (Mizuseki et al., 1998; Nakata et al., 1997). Zicr1 has been shown to be sufficient to induce expression of Xngn-1 and initiate neuronal differentiation. Xngn-1, or neurogenin, has been implicated in neuronal fate determination, inducing expression of the proneural genes neuroD and delta1 (Ma et al., 1996). Both Neurogenin and NeuroD are bHLH transcription factors that promote the expression of a core transcriptional network of targets, which then mediate neurogenesis by regulating transcription, signal transduction, and cytoskeletal rearrangements (Seo et al., 2007).
As development proceeds into the neurula stages, the sheet of neural ectoderm that composes the neural plate begins to invaginate to form the neural tube. This process involves a highly-coordinated series of morphological movements that form, shape, and bend the neural plate, eventually resulting in a fused neural tube that will give rise to the central nervous system (Colas and Schoenwolf, 2001). Neural progenitors are initially restricted to a single cell layer within the neural tube. However, two additional zones are formed during development, segregating radial glial cells and postmitotic neurons (Leclerc et al., 2012). These radial glia can divide symmetrically to renew progenitors or asymmetrically to produce a neural progenitor and a neuron (Fish et al., 2008).

Within the ventral neural tube, differentiation is modulated by a sonic hedgehog (Shh) gradient that exists along the dorsal-ventral axis. As the result of interactions between Class I (repressed by Shh) and Class II (activated by Shh) proteins, progenitor domain boundaries become specified (Dessaud et al., 2008). Due to differential sensitivity to Shh concentration, relevant genes become activated and repressed to different degrees along the D-V axis. In a specific example, a Pax6 gradient becomes established along the neural tube, which then inhibits Nkx2.2 expression to establish the p3-pMN boundary (Ericson et al., 1997). Similarly, the expression domain of homeobox gene Nkx6.1 restricts expression of Dbx2, Gsh1, and Gsh2, with Nkx6.1-expressing cells ultimately giving rise to MN (motor), V2, and V3 neurons (Sander et al., 2000).

Simultaneously, roof-plate-dependent BMP and Wnt signals establish distinct classes of dorsal progenitors, with cells expressing Math1, Ngn1/2, and Mash1 giving rise to d1, d2, and d3-5 interneurons, respectively (Chizhikov and Millen, 2005). Furthermore,
β-Catenin/Tcf activity serves to regulate Gli3 expression, which in turn functions to repress the Shh gradient within dorsal tissues (Alvarez-Medina, et al., 2008). Purinergic receptors and voltage-gated calcium channels have also been shown to influence neuronal fate determination (Glaser et al., 2013; Lewis et al., 2014).

The process of neurotransmitter phenotype specification is intimately related to the larger process of neuronal differentiation, with the expression of various neurotransmitters tightly regulated within various cells and populations. Transcription factors Tlx3 and Tlx1 are post-mitotic selector genes that induce a glutamatergic neuronal fate in the dorsal spinal cord (Cheng et al., 2004). Additional evidence suggests that, while homeobox gene Lbx1 defines a basal GABAergic state, Tlx3 antagonizes this function to induce glutamatergic differentiation (Cheng et al., 2005). Conversely, Ptf1a expression within the neural tube is required for Pax2 expression, which is ultimately associated with suppression of Tlx3 and expression of a GABAergic phenotype (Glasgow et al., 2005; Maricich and Herrup, 1999). This Pax2 expression is additionally activated and maintained by Lhx1 and Lhx5, specifically as established in dorsal horn interneurons (Pillai et al., 2007). Although the majority of characterized transcriptional regulation is mediated by Pax2, Evx1 and Evx2 have been shown to act independently of Pax2 to induce a glutamatergic phenotypic in commissural ascending (CoSA) interneurons (Juarez-Morales et al., 2016). In addition to these ‘hard-wired’ transcriptional cascades, activity-dependent mechanisms also influence this neurotransmitter phenotype acquisition process. These mechanisms, which are primarily mediated by calcium signaling, will be discussed in subsequent sections.
1.2.2. Roles of Calcium in Neural Development

While calcium serves as the basis for numerous signaling pathways within the developing embryo, its importance in the developing nervous system is particularly crucial. Calcium activity has been implicated in neural induction, neuronal differentiation, neuro-glial switching, and the formation of functional neuronal circuits (Leclerc et al., 2012). This activity has been broadly categorized into pulses, waves, and steady gradients, with more specific classification based on both spatial extent of the activity (localized vs. pan-embryonic) and – for wave-like activity – speed of propagation (Jaffe, 1999).

Particular attention has also been paid to calcium spikes, spontaneous transients that increase intracellular calcium concentration over a period $10^4$ times longer than that of an action potential (Gu and Spitzer, 1995).

The importance of calcium in neural induction was first suggested by the observed increase in calcium concentration within salamander embryos during gastrulation (Stableford, 1967). Use of a fluorescent reporter revealed anterior-dorsal-localized patterns of calcium activity in gastrulating Xenopus embryos, which can be blocked by pharmacological inhibition of L-type voltage-gated calcium channels. The inhibition of this calcium activity was found to cause severe morphological defects in the anterior nervous system, as well as downregulation of the neuralizing genes Zic3 and geminin (Leclerc et al., 2000). It has been suggested that neural inducer noggin initiates this activity by activating DHP-sensitive L-type calcium channels, allowing calcium influx of 15% above resting level over a 10-20 minute period (Leclerc et al., 1999; Leclerc et al., 2006).
Functionally, this increase in intracellular calcium has been suggested to activate calcineurin, a calcium-dependent phosphatase that functions to inhibit epidermal fate, and CaM kinase III, a neural fate activator (Leclerc et al., 2001). In *Xenopus*, arginine methyltransferase xPRMT1b has been identified as responsive to increases in calcium concentration, acting to link calcium activity to downstream neural induction through chromatin remodeling capabilities (Moreau et al., 2008). Independent of calcium’s affect on cell fate during neural induction, experiments inhibiting calcium activity in the dorsal marginal zone (DMZ) with thapsigargin or benzohydroquinone demonstrate the necessity of calcium dynamics for convergent extension (Wallingford et al., 2001).

These calcium transients peak in frequency and intensity at mid-gastrula stage, and are spatially restricted to the dorsal ectoderm. This further suggests a role in neural induction, which occurs exclusively in dorsal rather than ventral ectoderm (Leclerc et al., 2006). In addition to propagating calcium waves, which have a characteristic velocity of \( \sim 5.0 \text{ \mu m/s} \) and propagate 5-20 cell diameters away from the point of initiation, non-propagating single-cell calcium spikes are also noted (Webb et al., 2005). Non-neuronal cell types have been shown to induce specific cell responses in response to calcium activity of differential amplitude and duration during these early developmental stages, suggesting that these distinct classes of calcium activity encode distinct information (Dolmetsch et al., 1997).

Calcium activity has also been shown to play a major role in neural plate and neural tube stages. In chick embryos, multi-pass transmembrane protein Calfacilitin binds to and slows inactivation of L-type CaV1.2 channels. The resultant influx of calcium leads
to increased expression of geminin and sox2, both markers of neural progenitor populations (Papanayotou et al., 2013). T-type calcium channels are necessary for proper closure of the neural tube during neurulation, with TTCC mutants demonstrating inappropriate upregulation of ephrinA-d causing aberrant openings in the anterior neural tube (Abdul-Wajid et al., 2015). Pharmacological inhibition of this calcium activity with levetiracetam (shown to inhibit 18-40% of high-voltage calcium channel currents) leads to similar defects in the sealing of neural folds (Ozgural et al., 2015).

The first implication of calcium activity in neurotransmitter phenotype specification came with the observed responses of sympathetic rat neurons to increased or decreased calcium concentration. Neurons cultured in high-calcium media were shown to be less responsive to cholinergic factors, allowing their development into adrenergic neurons. Conversely, neurons exposed to pharmacological inhibitors of this calcium influx were more likely to become ultimately cholinergic (Wallicke and Patterson, 1981). Specific types of calcium activity were established as sufficient for specific aspects of neuronal maturation, with disruption of spikes affecting neurotransmitter phenotype assumption and disruption of wave-like activity affecting neurite extension (Gu and Spitzer, 1995).

Additionally, distinct patterns of calcium spiking were observed in sensory Rohon-Beard neurons (glutamatergic), dorsolateral interneurons (glycinergic), ventral interneurons (GABAergic) and motor neurons (cholinergic), suggesting a relationship between activity profile and neurotransmitter phenotype (Spitzer et al., 2004).

*Xenopus* neurons cultured in high-calcium media are shown to express higher transcription of xGAD67 (which codes for the rate-limiting enzyme of GABA synthesis),
associating this increased spike activity with an eventual inhibitory phenotype (Watt et al., 2000). Experimental manipulation of embryonic Xenopus spinal cord neurons established a homeostatic paradigm, in which artificially increased levels of spiking correlated with the acquisition of an inhibitory neurotransmitter phenotype, while artificially suppressed spike frequency was associated with an excitatory phenotype (Borodinsky et al., 2004). A non-cell-autonomous mechanism for this phenomenon has been proposed, linking spike-induced BDNF release to ultimate regulation of a glutamate/GABA selector gene (tlx3), this is not sufficient to explain the persistence of this spike/phenotype relationship in dissociated cell culture (Guemez-Gamboa et al., 2014).

In addition to acquisition of a neurotransmitter phenotype, neuronal maturation involves migration, the development of axonal and dendritic structures, and synapse formation. Calcium activity has been implicated in all of the processes. Post-migratory neurons, for example, have been shown to exhibit a higher rate of spontaneous calcium spiking than do migrating neurons, with artificial increase in calcium activity resulting in hindered migration and premature process branching (Bando et al., 2016). Just as different patterns of calcium activity have been noted as characteristic to particular neurotransmitter phenotypes, distinct patterns of calcium activity are exhibited by extending and retracting axonal processes. These observations are supported by pharmacological experiments, in which repression of calcium activity severely reduced differential process outgrowth (Hutchins and Kalil, 2008). In dendrite formation, calcium has been found to function both by inducing cytoskeletal rearrangements and by initiating specific transcriptional programs, specifically through CAMKIV (Konur and Ghosh, 2005).
Finally, reduction of intrinsic calcium oscillations via S(+)-ketamine results in the down-regulation of synapsin, disrupting the ability of neurons to form functional synapses (Sinner et al., 2011).

### 1.2.3. Calcium and Gene Expression

While calcium’s ability to influence cellular phenomena has been demonstrated holistically, it has also been investigated from a mechanistic perspective. Much of this activity occurs through proteins with distinct calcium-binding domains, many of which exhibit EF-hand binding motifs. This EF-hand ‘super-family’ includes a group of calcium sensor proteins, which are characterized as having relatively low affinity for calcium, yet undergo significant conformational change upon calcium binding (Mikhaylova et al., 2011). The most universal of these is calmodulin (CaM), an adaptor protein that binds calcium, inducing a conformational change that exposes hydrophobic residues. This then allows calmodulin to bind to various target proteins by wrapping around amphipathic regions (Clapham, 2007). In one common pathway, calmodulin binds to a CaM binding site within the autoregulatory domain of calmodulin kinase (CaMK), suppressing autoinhibition (Simon et al., 2015).

Another relevant member of this family of neuronal calcium sensors is the Downstream Regulatory Element Antagonist Modulator, or DREAM. Binding of calcium to this protein functions to stabilize an unfolded intermediate conformation, preventing repressive interactions with target DRE sites on DNA (Pham et al., 2015; Carrion et al., 1999). Transgenic mice expressing a calcium-insensitive DREAM variant express
significantly less GABA receptors than do wild-type mice, with associated impairments in both learning and memory (Mellström et al., 2013).

In addition to these proteins, which represent potential mechanisms for the translation of calcium signals into transcriptional effects, direct and specific links have been found. One of these mechanisms for connecting calcium signaling and neuronal development is the prmt1b gene, an arginine methyltransferase whose expression is (a) directly up-regulated by increase in calcium concentration and (b) directly correlated with increased expression of neural marker zic3 during neural induction stages. The PRMT1 family methylates histone H4, potentially regulating the transcriptional state of relevant proneural genes. An alternative hypothesis proposes that prmt1b is recruited by transcription factor YY1 to form an active transcriptional complex (Batut et al., 2005). In addition to this specific mechanism, approximately 30 other genes have been identified as calcium-dependent and restricted to pre-neural regions during this neural induction phase. These genes include a bHLH transcription factor inhibitor, an RNA-binding protein involved in pre-mRNA splicing, and a substrate for protein kinase C (Moreau et al., 2016). Some of these genes are probably downstream results of prmt1b activity, while other may represent independent mechanisms by which calcium influences gene expression.

A specific signaling cascade has also been hypothesized to explain the role of calcium in activity-dependent neurotransmitter respecification in Xenopus spinal neurons. In this model, calcium spikes regulate release of BDNF, which then initiates a TrkB/MAPK signaling pathway. MEK and JNK are phosphorylated; The former phosphorylates ERK1/2, which then phosphorylates cJun, while the latter phosphorylates cJun directly. P-cJun
serves to repress \( tlx3 \) by binding to an upstream CRE element. Since \( tlx3 \) generally functions to promote a glutamatergic cell fate, its repression by P-cJun favors a GABAergic state (Guemez-Gamboa et al., 2014; Marek et al., 2010). However, it is important to note that this pathway is exclusively modulated by global suppression or increase of calcium spiking, rather than by suppression or increase in the activity of a single cell, leaving it an unsatisfactory explanation for the cell-autonomous response to calcium spike frequency observed by Borodinsky et al. (2004).

Calcium channels themselves may also provide additional mechanisms for this response. L-type voltage-gated calcium channel \( Ca_{\text{v}}2.1 \), for example, is cleaved to release a C-terminal domain that translocates to the nucleus and functions as a transcription factor (Gomez-Ospina et al., 2006). Smad dephosphorylation has also been linked to calcium activity, specifically during gastrulation; calcium-dependent phosphatase calcineurin may phosphorylate Smad to inhibit the anti-neural BMP-4 signaling pathway (Moreau et al. 2008). Furthermore, calcium-dependent electrical activity has been shown to influence motoneuron differentiation, implying the additional importance of calcium in electrical phenotype acquisition (Xie et al., 1995; Borodinsky et al., 2015).

### 1.2.4. Methods of Calcium Activity Analysis

As demonstrated in the preceding literature review, an expansive breadth of research is concerned with calcium patterns and dynamics. As such, categories have been developed to describe and classify this activity in both qualitative and quantitative ways. However, these classes differ meaningfully between research groups, and even between
different papers published by the same research group. Therefore, while a review of
current methods for analyzing calcium data is certainly useful in its own right, it is more
broadly indicative of an overarching lack of nuanced, unbiased methods.

Whole-embryo patterns of calcium activity can be categorized in a broad,
qualitative sense as pulses, waves, and steady gradients (Webb and Miller, 2006). These
patterns are not mutually exclusive. For example, during gastrulation, the vegetal portion
of *Xenopus* embryos exhibits numerous fast calcium spikes on top of an underlying single
slow rise in calcium concentration (Webb et al., 2005). Even in experiments with cultured
embryonic spinal cord neurons, 70% of neurons were found to exhibit calcium transients
(spikes), 75% exhibited slower-form calcium waves, and 55% exhibited both types of
activity over a 7-to-12-hour period (Gu and Spitzer, 1995).

Waves can be classified as ultraslow, slow, fast, and ultrafast based upon
propagation speeds at room temperature (Jaffe, 1999). Spatially, signals can be described
as planar (traveling across an anterior-posterior axis) or vertical (traveling between
ectoderm and mesoderm) (Webb and Miller, 2006). Wallingford, et al. divide these waves
further into two categories: large, which propagate ~15 cell diameters at a speed of
~5μm/s, and small, which propagate ~5 cell diameters at a speed of ~2 μm/s (2001).

Within individual cells, waves and spikes can be differentiated both by time scale
and spatial extent. Waves have a duration of 33±4 seconds, while spikes have a duration
of 9±1 seconds. Additionally, waves decay spatially (so that a wave in the neuronal soma
may not be detected at the growth cone), while spikes extend across an entire cell (Gu
and Spitzer, 1995; Glaser et al., 2013). Both, however, seem to arise stochastically with respect to timing and location (Webb et al., 2005).

The kinetics of the activity have also been used to differentiate spikes from waves. Spikes are described as reaching peak calcium concentration in ~1 second, followed by double exponential decay (time constants of 10 seconds and 3 minutes). Waves are categorized by longer rise and decay times, although their specific kinetic parameters have not been as explicitly determined (Spitzer and Gu, 1997). This distinction has been further complicated by the observation that ~20% of spikes do not show this supposedly distinctive double decay (Gu et al., 1994).

The most ambiguous aspect of this analysis across literature is the intensity of fluorescence necessary for classification as a spike or wave. Gu and Spitzer set the lowest threshold, calling any increase in fluorescence above 150% of baseline significant enough to be classified (1995). However, earlier work characterized spikes as exhibiting increases in fluorescence of at least 400% over baseline, with even waves exhibiting a 200% increase (Gu et al., 1994). A ‘spike threshold’ of three times baseline is also commonly applied (Ciccolini et al., 2003). Alternative methods have defined a spike as an increase of more than twice the standard deviation of the baseline during the previous 10-minute time frame (Guemez-Gamboa et al., 2014).

These guidelines provide a workable framework for distinguishing single-cell spikes and waves as biologically distinct activities. Waves have been primarily implicated in neurite extension in growth cones, and seem to occur independently of voltage-gated calcium channels. Conversely, spiking activity involved both voltage-gated calcium
channels and calcium release from intracellular stores, and has been most extensively linked with neurotransmitter phenotype specification (Spitzer and Gu, 1997). However, the aforementioned frameworks provide no meaningful way to make distinctions within these broad classes based on potentially relevant variables (spike amplitude, time to peak, area under the spike, etc.). Additionally, no methods have been developed to analyze the longitudinal profile of calcium activity within a single cell. Since the homeostatic paradigm postulates that a cell’s neurotransmitter phenotype is linked to the patterns of calcium activity it exhibits (Spitzer et al., 2005), there is reason to be interested in developing these sorts of analytical tools.

More elegant analyses have been more recently developed to investigate calcium waves across an entire cell population. Automated image processing frameworks can extract amplitude, decay rate, time of excitation, and propagation point from calcium images. This advancement produces more reliable statistic results than manual methods, and, when applied to monolayer rat epithelial cell samples, revealed propagation velocity differences between leptin-containing, adiponectin-containing, and control samples that were not detected via manual techniques (Milovic et al., 2013).

Some of these analysis and detections methods are applicable to cell-culture experiments, yet pose significant limitations. In studies of action potential dynamics in rat cell culture, Patel et al. demonstrated the shortcomings of both percentile-based thresholds (which could not consistently identify both small and large amplitude transients) and the continuous waveform transform method traditionally applied to EEG data (which had limited temporal resolution and a prohibitively high false-positive rate)
Patel et al. favor a template-matching algorithm developed by Schultz et al. (2009) that compares the profile of a particular calcium event to a database of known and characterized transients, which is well-suited for relatively stereotyped phenomena. While this avenue could provide a defensible methodology for discerning calcium spikes from calcium waves, it would still be limited in its ability to detect more nuanced patterns and correlations.

1.3. Experimental Objectives and Hypotheses

As a review of the literature demonstrates, calcium transients and patterns of calcium activity are of particular interest to the developmental neurobiologist, as they have been implicated in a wide array of processes vital to the creation of a functional nervous system. Specifically, frequency of calcium spikes in neural progenitor cells has been linked via a homeostatic model to eventual neurotransmitter phenotype, with the postulate that a high frequency of calcium spiking is correlated with an inhibitory/GABAergic phenotype and that a low frequency of calcium spiking is correlated with an excitatory/glutamatergic phenotype (Spitzer et al., 2005).

However, several aspects of this model have yet to be explored. Foremost, while early literature held that this relationship was maintained in dissociated cell culture (i.e., is cell-autonomous), this has not been supported by later experiments. Earlier literature attempted to test this autonomy by artificially imposing particular spike frequencies on cultured neurons and found that imposition of high-frequency spikes was correlated with suppressed incidence of Glu+/VGluT+ and ChAT+ neurons. The authors note that
susceptibility to this stimulation is affected by intrinsic cell identity, and ultimately
conclude that activity-dependent neurotransmitter phenotype acquisition may be cell-
autonomous (Borodinsky et al., 2004). However, later experiments with induced mosaic
expression of \textit{hKir2.1}, which suppresses calcium spiking, in developing spinal neurons did
not find it to increase the likelihood of an excitatory neurotransmitter phenotype in the
targeted cells, nor to decrease the likelihood of an inhibitory phenotype (Guemez-

These findings call into question validity of activity-dependent neurotransmitter
phenotype regulation as a cell-autonomous process. To this end, we designed experiments
to investigate whether the correlations between spike frequency and neurotransmitter
phenotype persist \textit{in vitro}, using dissociated presumptive neural tissue from various
developmental stages of \textit{Xenopus laevis}. Tissue was dissected, dissociated, and plated
before being loaded with a fluorescent calcium indicator and imaged for 2 hours. Plates
were then fixed and used in fluorescent \textit{in situ} hybridization in order to assay gene
expression. To most directly investigate the homeostatic model, \textit{vGlut} expression was used
to distinguish glutamatergic (excitatory) neurons, while \textit{GAD} was used to mark GABAergic
(inhibitory) neurons. However, in order to more thoroughly understand the changes in
calcium dynamics across the neuronal differentiation process, two additional marker gene
probes were used. \textit{NBT} served to mark all differentiated neurons, while \textit{sox2} marked
undifferentiated neural precursor cells. A particular cell’s gene expression profile was then
collated with its calcium activity pattern in order to determine if any correlations exist
between the two variables.
In addition to investigating the relationship between gene expression and calcium activity, we also investigated calcium activity profiles across developmental stages. The aforementioned neural tissue dissections were performed at three time points: Stage 14 (neural plate), Stage 18 (neural tube), and Stage 22 (early tailbud) (staged according to Nieuwkoop and Faber, 1956) (Figure 1). By comparing patterns of calcium activity between cells dissected at each stage, we were able to gain insight into the ways in which single-cell calcium dynamics change across embryonic development.

In order to investigate these research questions in a nuanced, informative way, we worked to develop novel methods of calcium data analysis based on unbiased, quantitative metrics rather than on simple manual observation (Marken et al., unpublished). In addition to allowing us to probe the relationship between calcium activity and cell phenotype in a rigorous way, this methodology promises useful application to any calcium time-series data.

Taken as a whole, this project explores single-cell calcium dynamics in a more intensive way than has been previously done, applying novel analysis techniques to the determination of activity-dependent neurotransmitter phenotype specification as a cell-autonomous or non-cell-autonomous process, as well as investigating the changes in calcium activity pattern over the course of embryonic nervous system development.
2. Materials and Methods

2.1. Animal Care

Adult *Xenopus laevis* pairs were injected with human chorionic gonadotropin (1000U/mL, Chorulon) to induce natural matings. Females received 0.6 mL of HCG, and males received 0.4mL. Embryos were collected and dejellied by gently washing with a 2% cysteine solution (pH 8.0) for 2-4 minutes. Embryos were then rinsed three times in 0.1X Mark’s Modified Ringer’s solution (MMR) composed of 100 mM NaCl, 2 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂, and 5 mM HEPES. Embryos were transferred to 100mm glass petri dishes containing 0.1X MMR with gentamycin (50μg/mL) and incubated at 14°C, 16°C, 18°C, or 22°C during development at an average density of 50-100 embryos per plate. Plates were periodically sorted to removed unfertilized, necrotic, and abnormally-developing embryos. All animal care protocols have been approved by the Institutional Animal Care and Use Committee (IACUC) at the College of William and Mary.

2.2. Embryo Dissection for Calcium Imaging

2.2.1. Dissection Setup and Solution Preparation

For each dissection date, one 60 mm Fisherbrand plate and two 50mL Falcon tubes were placed in a laminar flow hood (Labconco Purifier Class II Biosafety Cabinet). For each plate to be imaged, two 35 mm Fisherbrand plates and one 35 mm Nunclon plate were also placed inside the hood. These materials were sterilized for 20-30 minutes with ultraviolet light.
The following solutions were prepared prior to the dissection date and stored at 4°C:

2mM Ca²⁺ “Spitzer” Solution: 116 mM NaCl, 0.67 mM KCl, 2 mM CaCl₂•2H₂O, 1.31 mM anhydrous MgSO⁴, 4.6 mM Tris; adjusted to a pH of 7.8 (Xiao et al., 2010). 1 ml/100 ml Penicillin/Streptomycin (10,000 units/ml penicillin; 10,000 μg/ml streptomycin) was added.

Calcium-magnesium free medium (CMF): 116 mM NaCl, 0.67 mM KCl, 4.6 mM Tris, 0.4 mM EDTA, adjusted to a pH of 7.8 (Ferrari and Spitzer, 1999).

Solution bottles were wiped down with 70% ethanol before being moved into the hood. Ten mL 2mM “Spitzer” Solution was added to one Falcon tube. Ten mL 2mM “Spitzer” Solution per plate to be imaged was added to the second Falcon tube. For each image, 2 mL 2mM “Spitzer” Solution was added to one 35 mm Fisherbrand plate and one 35 mm Nunclon plate. 2 mL CMF medium was added to the remaining 35 mm Fisherbrand plate.

Outside of the laminar flow hood, two 100 mm Fisherbrand plates and one 60 mm Fisherbrand plate were prepared, as well as one 35 mm Fisherbrand plate per image. 0.1X MMR solution with gentamycin (50μg/mL) was added to the 100 mm and 35 mm plates. 70% ethanol was added to the 60 mm plate to sterilize forceps during the dissection process.
2.2.2. Dissection, Dissociation, and Plating

Directly prior to dissection, 0.01g Collagenase B (Roche) was added to the tube containing 10mL 2mM “Spitzer” solution. Once mixed, this solution was transferred to the empty 60 mm Fisherbrand plate. Embryos of the appropriate stage (Nieuwkoop and Faber 14, 18, or 22) were selected and transferred to a 100 mm Fisherbrand plate containing 0.1X MMR + gentamycin (“holding” plate). One at a time, an embryo was transferred to the second 100 mm Fisherbrand plate (“dissection” plate). Forceps were used to carefully remove the vitelline membrane as well as to perform an initial ‘rough’ dissection, separating the dorsal and ventral halves of the embryo. The dorsal portion was transferred to the collagenase dish, and the ventral portion was discarded. After a few minutes, the dorsal explant was returned to the dissection plate to complete the ‘fine’ dissection. All endoderm and mesoderm was removed and discarded, leaving only the presumptive neural tissue of the ectoderm. Cleanly dissected explants were transferred into a 35 mm Fisherbrand plate containing 2mM “Spitzer” Solution until four explants from the same stage were collected. Once four explants were dissected, a p1000 pipetteman was used to transfer them all to the plate containing CMF.

Explants were allowed to dissociate for 60 minutes before being transferred to the 35 mm Nunclon plate with a p100 pipette man. Before this plating, a micron grid (Nexcelom, Cellattice) was attached to the bottom of the Nunclon plate with superglue. Cells were incubated for 60 minutes to allow adhesion to the plate.

Before the dissection, additional appropriately-staged embryos were selected and transferred to the holding plate. After the dissection, remaining embryos (at least two per
image) were transferred to a 35 mm Fisherbrand dish containing 0.1X MMR + gentamycin to serve as sibling controls.

2.2.3. Fluorescent Dye Loading

After this plating step, 2μL 10% pluronic acid was added to a 5μL aliquot of Fluo-4 AM. The Nunclon plate was moved to a dark room, the contents of this aliquot were added to the plate, and the plate was covered with aluminum foil to prevent light exposure. The plate was then incubated for 60 minutes to allow the fluorescent dye to load. After this incubation, the plate was washed three times with 2mM “Spitzer” Solution to remove excess Fluo-4. Four mL 2mM “Spitzer” Solution were left in the plate during imaging.

2.3. Calcium Imaging

2.3.1. Confocal Microscopy

Imaging was performed on a Nikon TE2000 inverted confocal microscope using Nikon Elements software. Bright-field images of both cells and corresponding micron grid locations were taken at 20X magnification. The position of the image was chosen based on optimal cell density as well as the presence of a numerically-identifiable grid location within the field of view.

Samples were then illuminated with a 488nm laser to visualize Fluo-4 AM. Laser intensity was held constant across experiments at 18.32%, while HV and offset values were optimized for each experiment to ensure a dynamic range of fluorescence.
Images consisted of 901 frames taken over 2 hours (scan time of 3.93 seconds). After each image was completed, a second set of bright-field images were taken of both the cells and grid to confirm that no movement occurred during the imaging period.

2.3.2. Plate Fixation

Plates were then removed from the confocal microscope. 3mL solution was removed from each plate, and replaced with 1mL 2X MEMFA (200 mM MOPS, 2 mM EGTA, 2 mM MgSO₄, in 7.4% formaldehyde). During fixation, plates were incubated for 2 hours at room temperature or overnight at 4°C. After this period, all solution was removed and replaced with 2mL 1X PBS. Plates were stored at 4°C to await further processing. Sibling control stage was recorded at the beginning of the fixation step to confirm an appropriate experimental time frame.

2.4. Gene Expression Analysis

2.4.1. Purification and Linearization

Probes were prepared for *Xenopus* neural beta tubulin, *Xenopus* glutamate decarboxylase 1, *Xenopus* vesicular glutamate transporter 1, and *Xenopus* SRY-box 2 (Table 1). Neural beta tubulin (NBT) encodes a microtubule component distinct to mature, differentiated neurons. Glutamate decarboxylase 1 (GAD) encodes the rate-limiting enzyme for GABA synthesis, and therefore specifically marks differentiated GABAergic neurons. Vesicular glutamate transporter 1 (vGlut) is a solute carrier gene involved in glutamate reuptake, and has expression specific to differentiated glutamatergic neurons.
SRY-box 2 (sox2) is a transcription factor whose expression is distinct to undifferentiated neuronal precursors.

Plasmid constructs containing 836-1600 base pair segments of each gene of interest were transformed into bacterial cells and grown up in 150mL of Luria broth at 37°C until turbid. Plasmid DNA was then isolated using a Promega PureYield Plasmid MidiPrep kit. Appropriate restriction enzymes were used to diagnostically digest inserts to confirm sequence length and identity. Plasmid samples were also sent to the Molecular Core Lab for sequencing to confirm sequence fidelity.

Plasmid constructs were linearized using appropriate restriction enzymes (Table 2). A 100µL reaction consisting of 20µg plasmid DNA, 20U Promega restriction enzyme, and 1X appropriate Promega restriction enzyme buffer was incubated at 37°C for a minimum of 2 hours before precipitation. Samples were extracted with P/C:IAA followed by C:IAA, then stored at -80°C for at least 30 minutes after the addition of 0.1 volume NaOAc and 2 volumes cold 100% ethanol. Precipitated samples were then spun in a refrigerated microcentrifuge for 20 minutes at 16,000g at 4°C. After the supernatant was removed, the pellet was washed with 200 µL room temperature 70% ethanol and returned to the microcentrifuge to spin for 5 minutes at 16,000g at 4°C. The supernatant was removed and the pellet was air-dried for 5-10 minutes. The pellet was resuspended in 20 µL TE and DNA concentration was quantitated using a NanoDrop 1000 Spectrophotometer (Fisher). Successful linearization was also confirmed through agarose gel electrophoresis.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Full Name</th>
<th>Expression Profile</th>
<th>GenBank Accession Number</th>
<th>Cloning and Characterization in X. laevis</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBT</td>
<td>neural beta tubulin</td>
<td>all differentiated neurons</td>
<td>379220</td>
<td>Good et al., 1989</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamic acid decarboxylase</td>
<td>GABAergic (inhibitory) neurons</td>
<td>378551</td>
<td>Brox et al., 2003</td>
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<tr>
<td>vGlut</td>
<td>vesicular glutamate transporter 1</td>
<td>glutamatergic (excitatory) neurons</td>
<td>398748</td>
<td>Gleason et al., 2003</td>
</tr>
<tr>
<td>sox2</td>
<td>SRY-box 2</td>
<td>undifferentiated neural precursors</td>
<td>398000</td>
<td>Mizuseki et al, 1998</td>
</tr>
</tbody>
</table>

**Table 1:** Selected marker genes used to assay neurotransmitter differentiation status and phenotype.
2.4.2. mRNA Probe Synthesis

Antisense mRNA probes were generated from these linear DNA templates for use in fluorescent *in situ* hybridization. 4 µg linearized DNA were combined with 1.5 µL appropriate RNA polymerase (Promega) (Table 2), 15 µL 2.5 mM rNTP mix containing dig-11 UTP (Roche), 10 µL 5X transcription buffer (Promega), 0.5 µL 20U/µL RNAsin (Promega), and 5 µL 0.1M DTT. The 50 µL reaction mixture was incubated at 37°C. An additional 1.5 µL of the appropriate RNA polymerase was added after 1 hour, and the reaction was incubated for a further hour after this addition. 1 µL RQ1 DNase (Promega) was added to degrade the DNA template, and the reaction was incubated an additional 10 minutes at 37°C.

Transcribed RNA probe was then purified with the addition of 30 µL LiCl solution. The reaction was placed at -20°C for at least one hour, then pelleted by spinning in a refrigerated microcentrifuge for 25 minutes at 14,000 RPM at 4°C. After the supernatant was removed, the pellet was washed with 500 µL 70% ethanol and returned to the microcentrifuge for 5 minutes at 14,000 RPM at 4°C. Supernatant was removed, and the pellet was allowed to air-dry for 5-10 minutes. The pellet was then resuspended in 20 µL nuclease-free water. Probe yield was quantitated using a NanoDrop 1000 Spectrophotometer (Fisher).

Probe integrity was confirmed through agarose gel electrophoresis. Successful probes were diluted to 10X stock in ISH Buffer (50% formamide; 5X SSC; 1 mg/ml Torula RNA; 100 µg/ml heparin; 1X Denhardt’s solution; 0.1% Tween-20; 0.1% CHAPS; 10 mM
EDTA) and stored at -20°C. Probes were diluted to 1X in ISH Buffer before use, and each aliquot of dilute probe was used up to three times before being discarded.

### 2.4.3. Fluorescent in situ Hybridization

This fluorescent in situ hybridization protocol has been adapted from protocols developed by Anderson Lab and Vize Lab, as well as those published by Davidson and Keller (1999) and Xenbase. It has been further optimized through lab experience to account for the delicacy of plated cells. Plated cells are washed with fresh 1X PBS before being incubated in 0.1M triethanolamine with acetic anhydride for 10 minutes at room temperature. Cultures were washed in 1X SSC (saline sodium citrate) before being permeabilized with 0.02M HCl in H₂O for 10 minutes at room temperature. Cultures were again washed with 1X PBS before being prehybridized in 1mL ISH Buffer for at least 6 hours in a 60°C shaking water bath. After this incubation, ISH buffer was removed and replaced with 750μL dilute (1X) probe solution. One plate per procedure was incubated with a sense mRNA probe for use as a negative control. This hybridization step was carried out overnight (8-14 hours) in a 60°C shaking water bath.

After probe was removed and stored, cultures were briefly rinsed in 0.2X SSC before being incubated in 0.2X SSC for 1 hour at 60°C. Plates were then allowed to adjust to room temperature before additional 0.2X SSC washes. This was followed by a 15-minute wash in 1X PBT, a 1-hour wash in fresh 2% H₂O₂ (diluted in PBT, incubation performed in darkroom), and a 15-minute wash in 1X TBST (tris-buffered saline with Tween-20). Cultures were blocked in 2% BMB in MAB for at least 1 hour at room
<table>
<thead>
<tr>
<th>Construct</th>
<th>Linearization Restriction Enzyme (Antisense)</th>
<th>RNA Polymerase Enzyme (Antisense)</th>
<th>Linearization Restriction Enzyme (Sense)</th>
<th>RNA Polymerase Enzyme (Sense)</th>
<th>Fragment Length</th>
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</thead>
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<tr>
<td>NBT</td>
<td>BamHI</td>
<td>T7</td>
<td>HindIII</td>
<td>T3</td>
<td>841 bp</td>
</tr>
<tr>
<td>GAD</td>
<td>NotI</td>
<td>Sp6</td>
<td>HindIII</td>
<td>T7</td>
<td>836 bp</td>
</tr>
<tr>
<td>vGlut</td>
<td>NotI</td>
<td>T3</td>
<td>XhoI</td>
<td>T7</td>
<td>1600 bp</td>
</tr>
<tr>
<td>sox2</td>
<td>EcoRV</td>
<td>Sp6</td>
<td>HindIII</td>
<td>T7</td>
<td>961 bp</td>
</tr>
</tbody>
</table>

*Table 2: Constructs used for mRNA probe synthesis.*
temperature. Anti-digoxigenin POD-conjugated antibody (Roche) was diluted at a concentration of 1:1000 in 2% BMB in MAB, and plates were incubated overnight at 4°C in 1mL of this diluted solution.

After this incubation, plates were rinsed three times briefly in TBST, then placed on a rocking platform and washed 4 times with TBST for 15 minutes each. Plates continued to rock during two 10-minute PBT washes. 750 µL 1:25 Cy3-tyramide was then applied to each plate, during and after which the plates were extremely light sensitive and were kept in a foiled container in a dark room to prevent any exposure. After 5 minutes, 2.5 µL 0.3% H₂O₂ was added to each plate, and plates continued to rock for an additional 40 minutes. Four 15-minute TBST washes were performed, followed by a brief wash in 1X PBT. Cultures were then fixed in 1X MEMFA for 1 hour at room temperature, then stored in 2mL 1X PBS at 4°C in a foiled container to prevent light exposure before imaging.

2.4.4. Confocal Microscopy

Post-FISH images were taken on either a Zeiss LSM 510 confocal microscope using Axiovision and Zeiss LSM software or a Nikon TE2000 inverted confocal microscope using Nikon Elements software.

For plates imaged on the Zeiss LSM 510, samples are illuminated using a HeNe 543 laser set to 15% excitation. First, the negative control plate (hybridized with a sense mRNA probe) was placed under the microscope and visualized using Fast X/Y scan. Once cells are in focus, gain and offset values are adjusted. After changing “Palette” settings to Range Indicator, amplifier offset is adjusted until the negative control image appears as a
completely blue background. This step serves to determine a background fluorescence 
value against which to calibrate the probe-induced fluorescence present on experimental 
plates. These offset and gain values are recorded and maintained throughout imaging of 
any particular batch of post-FISH plates.

A similar procedure was followed for plates imaged on the Nikon TE2000, with the 
negative control plate used to set baseline values. Samples are illuminated using a TRITC 
laser (\(\lambda=595\)nm) set to 6.35% excitation. Once cells are in focus, offset values are held at 
0 while gain values are adjusted until the image appears as a completely black 
background.

The micron grid images taken of each experimental plate before the initial calcium 
imaging are pulled up for use as references; the field of view for each experimental plate is 
adjusted to perfectly match the field of view of the calcium image. Once this position has 
been located, the image is refocused to the plane of the cells, and offset and gain values 
are reset to the previously-determined values. Images are taken of both the cells and the 
corresponding micron grid location, with any fluorescence above background indicating 
expression of a particular gene within that cell.

2.5. Image Preparation

2.5.1. Binary Generation

Both calcium and FISH images are opened in Nikon Elements for further analysis 
(.lsm files must be converted to .nd2 files before this can occur). Once an image is 
opened, the “Binary” menu is opened and the “spot detection” tool selected. By selecting
“bright spots” under the appropriate fluorescence channel (FITC for calcium images, TRITC for FISH images), all cells within a particular image are circled. Diameter, circularity, and contrast settings can be adjusted to ensure that cells are appropriately captured. Although cells that are distorted or overlap significantly cannot be distinguished, >80% of cells on any given image can be accurately assigned binaries. For calcium images, the binaries must be applied to all frames of the image, and the image is played to ensure consistency of binaries across frames. Any objects that do not consistently correspond to a single cell across all frames (e.g., one binary covers multiple cells, two binaries overlap on a single cell, or binaries ‘jump’ between adjacent cells) are manually vetted and deleted if appropriate.

2.5.2. Cell Tracking and Calcium Data Generation

Because cells may shift in position between frames of a calcium image, a cell tracking program is applied to detect frame-to-frame ROI overlap and track individual cells across the entire image. This program is implemented through the Nikon Elements software by selecting View > Analysis Controls > Tracking Options, allowing 5 frames as a maximum gap between tracks and deleting any tracking with fewer than 600 frames. The “Close Gaps” feature is selected, and “Track Binaries” is then selected to initiate the tracking program. After the program has completed, the image is viewed with ROI overlay to ensure that cells are accurately tracked. Any objects that do not accurately correspond to an individual cell across all frames of the image can be manually deleted before the results are saved.
Now that each cell is identified throughout the 901 frames of the image, fluorescence data is collated for each individual cell. Within the Tracking window (automatically opened when “Track Binaries” has been performed), the user can view graphed time-series data for a particular cell or group of cells by selecting the characteristic of interest (e.g., Mean Intensity, Speed, etc.) (Figure 1). The data for all cells is then exported by selecting all objects and selecting the “Export Data to Excel” option. This spreadsheet is then saved as a CSV (Comma Separated Values) file, allowing plain-text storage of significant amounts of data.

2.5.3. Overlay Confirmation

During the numerous washing steps of the in situ hybridization, plated cells may be either shifted in their position or washed out of frame entirely. Therefore, a judgment must be made about the overall fidelity of cell identity before cell coregistration can be performed. This is done by overlaying the post-FISH image onto a still image of the cell field of view after the calcium image has been completed. These images were generated through GIMP (GNU Image Manipulation Program) v. 2.8.16, an open-source image editor available for free download (www.gimp.org/downloads/).

The brightfield cell image taken after calcium imaging was converted to JPEG format and uploaded as the first image layer, after which the post-FISH image was converted to JPEG format, uploaded as the second image layer, and adjusted to ~50% transparency. The images were shifted to align distinct patterns or clusters of cells that were visible in both images. Images originally taken as .nd2 files could be converted to
**Figure 1:** (A) is a representative image of a calcium image to which binaries have been applied. Each colored circle represents a defined binary and an individual cell. (B) graphs the composite fluorescence data for each cell over the 2-hour imaging time course (where the x-axis represents time and the y-axis represents fluorescent intensity). (C-H) graph individual calcium activity traces for two representative cells to demonstrate the diversity of signals that are seen. (C) is an example of a cell that exhibits a single spike event over the imaging period, while (D) exhibits multiple spiking events fairly regularly across the two hours. (E) demonstrates variable calcium activity across time, yet its trace is less neatly defined as a particular number of spikes or waves. (F) is an example of a cell that does not show spiking activity, yet has a much more long-form pattern of calcium influx and efflux over about 90 minutes. Both (G) and (H) do not show calcium spikes. However, while (G) is a fairly noisy signal, (H) exhibits relatively little noise. The overall downward trend in (H) is most likely an artifact of slight photobleaching across the imaging period (Bootman et al., 2013), which can also be seen in the general form of composite trace (B).
.jpg files within Nikon Elements. Images originally taken as .lsm files were converted to .jpg files using ImageJ (available for free download at imagej.nih.gov/ij/download.html).

Overlays were graded into four categories based on the fidelity of cell position between images. Schematic images for each category can be seen in Figure 2. All grades were confirmed by at least two independent observers. If the two images corresponded perfectly, the plate was assigned a score of 1. A score of 2 was assigned if at least half of the cells corresponded perfectly, but a significant amount of the remaining cells could not confidently be corresponded between the two images. A score of 3 was assigned for plates in which patterns and clusters were maintained, although different groups of cells moved in different directions during the FISH. Therefore, while only a small subset of cells could be perfectly overlaid at any one time, shifting the alignment of the FISH image in various directions allowed the majority of the cells to be confidently identified across images. A grade of 0 was assigned to plate on which little/no cells remained and/or could be identified consistently between the two images. Plates with grades of 1, 2, and 3 were considered usable, while plates with grades of 0 were considered unusable.

2.5.4. FISH Data Generation

Since binaries have already been generated to define cells on the post-FISH image, simply selecting the “Perform Measurement” function on the Nikon Elements software will generate a spreadsheet of relevant variables of interest for each cell, including X/Y position, mean intensity, and intensity variation. The “Export Data to Excel” function is selected, and the resulting file is saved as a CSV (comma separated values) file.
**Figure 2:** Post-FISH images were overlaid on brightfield images taken directly after calcium imaging to confirm that cells could be accurately traced between the two images, with scores assigned to grade the fidelity of cell positions. (A) represents a brightfield post-calcium image with no overlay. (B) represents an overlay that would be given a 1, signifying perfect or near-perfect alignment. (C) represents an overlay that would be given a 2; while a significant portion of cells are remained in perfect alignment, others cannot be coregistered. (D) represents an overlay that would be given a 3, as general cell patterns remained the same, yet different clusters of cells moved in different directions. (E) represents an overlay that would be given a 0, as no cells can be confidently corresponded between the two images due to extensive cell movement.
2.5.5 Gene Expression Scoring

Gene expression was manually scored on the basis of fluorescence intensity within each binary. When the post-FISH image is opened on Nikon Elements, selection of the “Show Binary IDs” function shows the ID number corresponding to each binary overlaid on the image. An Excel spreadsheet was generated in which the first column held binary ID numbers and the second column held a call of positive (high expression), low-level (low-expression), or negative (no expression).

Because background fluorescence was variable between plates, no universal threshold was instated across all plates. Instead, manual calls were based on holistic assessment of each plate as a whole. LUTs (Look-Up Tables) were adjusted per user preference to better visualize cells and fluorescence contrast. Expression profiles were highly variable across probes. sox2-probed plates generally had high levels of positive and low-level expression (up to 90% of cells on each plate demonstrating some expression). In contrast, NBT-, vGlut-, and GAD-probed plates tended to have few or no positive cells, a moderate amount of low-level cells, and many negative cells. This scarcity in expression was even more pronounced at earlier stages (14 and 18), a logical result of assaying for terminal neural differentiation genes at early neural plate and neural tube stages.

Because manual calls were performed by different individuals, we recognize potential concern for inter-experimenter bias and variability. To control for this, we had several plates manually scored by multiple people, and found that >95% of calls were identical across all experimenters.
2.5.6. Manual Coregistration

Because of the unpredictable differential movement of different cell clusters on a particular plate, automated correspondence techniques could not be trusted to confidently coordinate the binary ID of a particular cell on the tracked calcium image with the binary ID of that same cell on the post-FISH image. Therefore, these correspondences were determined manually, with the help of binary-ID-labeled images and the previously generated overlay files. A list of all post-FISH image binary IDs was pulled from the .csv file generated previously. This was necessary because, due to manual vetting, binary IDs were not always completely sequential; manually deleting a particular binary simply skips that ID number, rather than readjusting all IDs to result in a gapless sequence.

Corresponding calcium binary IDs were recorded for each cell. Any ambiguous correspondences (i.e., those for which the experimenter could not be confident in the fidelity of cell identity, but could make a guess) were not included. The number of cells that could be coregistered across both images varied considerably between plates, ranging from 4-200+. These discrepancies arose from numerous variable, including number of cells remaining on the post-FISH image, overlay score, the presence of distinguishable pattern or clusters of cell, and precision of the field of view captured in each image.

2.5. Data Analysis

As previously discussed, a novel analysis pipeline has been developed simultaneously with this project in order to investigate calcium dynamics in a rigorous way that does not rely on predetermination of features of interest (Marken et al., in
preparation). The following methodology will be discussed only in general outline. A complete explanation of the underlying mathematical principles, as well as a full justification for the validity of this analysis method, is being prepared for publication. A simplified schematic outline can also be found in Figure 3.

Final data preparation involved using the calcium tracking .csv file, post-FISH measurement .csv file, list of positive/low-level/negative cells, and list of cell-to-cell coregistration to collate 900 frames of calcium activity in a particular cell to the gene expression profile of that same cell. Stage of dissection, as an additional variable, was also noted for each plate. Additionally, baseline removal was applied to remove artifacts of photobleaching or Fluo-4 accumulation.

For each calcium trace, fluorescence values were binned into even quartiles (with ‘A’ representing the lower 25% of recorded fluorescence values across the time series for that particular cell and ‘D’ representing the upper 25% of recorded fluorescence values across the time series for that same cell). Each cell’s activity profile was thereby converted into an alphabetic sequence representative of its variable fluorescence. Transition matrices were generated to quantify the likelihood that that cell would make a particular transition from frame to frame (e.g., moving directly from the ‘A’ quartile to the ‘D’ quartile). The information entropy value for the matrix was then calculated.

This metric allows the reduction of a 900-frame fluorescent time-series to a single value (the information entropy of the transition matrix), allowing more straightforward statistical conclusions to be drawn. Comparisons were then made between entropy values across stages, genes, and gene expression levels.
Figure 3: This figure represents a highly simplified representation of the calcium analysis methodology applied to this data. The quartiles are demarcated as A-D, with the black text presenting the symbolic representation of the data and the table showing the likelihood of occurrence of particular transitions.
3. Results

3.1 Overview of the Project and Results

The intent of this project was to explore the relationship between calcium activity and neurotransmitter phenotype in development using an *in vitro* system and a novel method of data analysis that emphasizes entropy over narrowly-defined features. To this end, we performed both calcium imaging and gene expression assays, and then investigated whether significant differences in calcium activity profiles exist between groups of interest. We will present data on (1) correlations between calcium activity and developmental stage and (2) correlations between calcium activity and expression of various neural marker genes. In order to most thoroughly understand the second data set, we will specifically discuss (a) the differences in calcium activity between positive and negative cells, (b) the differences in calcium activity between positive and negative cells at particular developmental stages, and (c) the differences in calcium activity between cells positive for individual neural marker genes.

3.2. Calcium Activity by Developmental Stage

While our primary motivation for performing this research was to determine differences in calcium activity patterns associated with neural marker gene expression, an understanding of how calcium dynamics change across development provides a vital foundation for these questions. To this end, we collated entropy values for cells by the stage of dissection and used Cohen’s d and the two-sample Kolmogorov-Smirnov test (KS
test) to determine statistical significance of the difference between these groups. These results can be seen graphically in Figure 4, and statistical significance can be seen in Tables 3 and 4,

Average entropy of calcium activity was found to decrease over time; cells dissected from embryos at neural plate stage (stage 14) had the most entropic behavior, followed by cells dissected at neural tube stage (stage 18). Cells dissected at early tailbud stage (stage 22) showed the least entropic (most predictable) calcium activity patterns. All d-values were statistically significant, with small and moderate effect sizes. However, all KS test p-values were <0.001 (significant at <0.05), indicating that all distributions are significantly different from one another.
Figure 4: Each data point represents an individual cell plotted according to its stage (x-axis) and entropy value (y-axis).
<table>
<thead>
<tr>
<th></th>
<th>Stage 14</th>
<th>Stage 18</th>
<th>Stage 22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 14</td>
<td>--</td>
<td>0.2424</td>
<td>0.6445</td>
</tr>
<tr>
<td>Stage 18</td>
<td>-0.2424</td>
<td>--</td>
<td>0.4009</td>
</tr>
<tr>
<td>Stage 22</td>
<td>-0.6445</td>
<td>-0.4009</td>
<td>--</td>
</tr>
</tbody>
</table>

**Table 3:** Cohen’s d value for differences between cells at each developmental stage. Positive values for Cohen’s d indicate that the stage on the vertical axis was more entropic than the stage on the horizontal axis, while negative values indicate that cells positive for the gene listed along the vertical axis were less entropic. A Cohen’s d value of >|0.2| is considered small, but significant. A Cohen’s d value of >|0.4| corresponds to a medium effect size.
<table>
<thead>
<tr>
<th></th>
<th>Stage 14</th>
<th>Stage 18</th>
<th>Stage 22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 14</td>
<td>--</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Stage 18</td>
<td>&lt;0.0001</td>
<td>--</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Stage 22</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>--</td>
</tr>
</tbody>
</table>

**Table 4:** KS test values for entropy comparisons between cells at different developmental stages. A value <0.05 is considered significant.
3.3. Calcium Activity and Neural Gene Expression

3.2.1. Comparisons Between Positive and Negative Cells

One of our primary research aims was to investigate whether differential patterns of calcium activity existed between cell positive for selected neural marker genes (specifically, \textit{NBT}, \textit{vGlut}, \textit{GAD}, and \textit{sox2}). To answer this question, we assigned entropy scores to each cell with recorded gene expression (positive, low-level, or negative). Mean entropy values where then determined for cells positive and negative for each probe (low-level cells were grouped with positive cells because low numbers of positive cells inhibited statistical analysis). Cohen’s \textit{d} was applied to determine the effect size of gene expression on entropy value, and the two-sample Kolmogorov-Smirnov test (KS test) was applied to compare the probability distributions of the relevant samples. Plots of cell entropy data for each probe can be seen in Figure 5, and values of statistical significance for each comparison can be seen in Table 5.

Three of the four probes (\textit{NBT}, \textit{vGlut}, and \textit{sox2}) showed a small significant difference in mean entropy of calcium activity between cells expressing the gene of interest and cells not expressing the gene of interest when the Cohen’s \textit{d} metric was used. However, only \textit{vGlut} and \textit{sox2} were found by the KS test to be significantly different from non-expressing cells. \textit{GAD} did not show an effect large enough to be considered significant by either metric. The Cohen’s \textit{d} values for both \textit{NBT} and \textit{GAD} were negative, indicating that cells positive for these genes tend to have less entropic calcium activity than cells negative for these genes. Conversely, both \textit{vGlut} and \textit{sox2} had negative Cohen’s
d values, suggesting that cells positive for these genes behave in a less ordered manner than cells that do not express these genes.
Figure 5: Each data point represents an individual cell plotted according to its entropy value (y-axis). Data points are color-coordinated based on gene expression scoring, although positive and low-level cells were binned for statistical analysis.
Table 5: Measures of statistical significance of differences between cells positive for each probe vs. cells negative for that same probe. Positive values for Cohen’s d indicate that gene-positive cells were more entropic than gene-negative cells, while negative values indicate that gene-positive cells were less entropic. A Cohen’s d value of >|0.2| is considered small, but reportable. KS test values of <0.05 are considered significant.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Cohen’s d</th>
<th>KS Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBT</td>
<td>-0.2779</td>
<td>0.0936</td>
</tr>
<tr>
<td>GAD</td>
<td>-0.1673</td>
<td>0.0854</td>
</tr>
<tr>
<td>vGlut</td>
<td>0.2275</td>
<td>0.0162</td>
</tr>
<tr>
<td>sox2</td>
<td>0.2197</td>
<td>0.0467</td>
</tr>
</tbody>
</table>
3.2.2. Comparisons Between Positive and Negative Cells by Stage

In order to more thoroughly understand the differences in calcium dynamics between cells across the development time span, we performed the previously-described statistical tests for cells clustered by stage (14, 18, and 22). Plots of cell entropy data for each probe can be seen in Figure 6, and values of statistical significance for each comparison can be seen in Tables 6 and 7.

This analysis revealed changes in time in the relationship between cells positive for marker genes and cell negative for these marker genes over the developmental time period. Cohen’s d suggests that NBT-positive cells were significantly less entropic than NBT-negative cells at stage 14, significantly more entropic than NBT-negative cells at stage 18, and significantly less entropic than NBT-negative cells at stage 22. While this could be the result of actual changes in the activity of these cells over time, we remain wary of the fact that the low number of positive and low-level cells for this particular probe may be skewing this interpretation. Additionally, this significant is not supported by the KS test, which did not find statistically significant differences at any stage.

vGlut-positive cells showed the strongest difference from their respective negative cells, with vGlut-positive cells significantly less entropic than vGlut-negative cells at stage 14, significantly more entropic than vGlut-negative cells at stage 18, and not significantly different from vGlut-negative cells at stage 22. The results were supported by both Cohen’s d and the KS test. Again, although this effect may be the result of an authentic biological phenomenon, it could be further bolstered by data from additional vGlut-positive cells.
Across all developmental stages, neither GAD-positive cells nor sox2-positive cells were significantly different from GAD-negative and sox2-negative cells when analyzed with Cohen’s d. The KS test suggests that there may be a significant different between GAD-positive and GAD-negative cells at stage 18, as well as between sox2-positive and sox2-negative cells at stage 18. These suggestions would be more throughout investigated with an increased sample size.
Figure 6: Each data point represents an individual cell plotted according to its stage (x-axis) and entropy value (y-axis). Data points are color-coordinated based on gene expression scoring, although positive and low-level cells were binned for statistical analysis.
<table>
<thead>
<tr>
<th></th>
<th>Stage 14</th>
<th>Stage 18</th>
<th>Stage 22</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBT</td>
<td>-0.2749</td>
<td>0.3979</td>
<td>-0.3536</td>
</tr>
<tr>
<td>GAD</td>
<td>-0.0579</td>
<td>0.0172</td>
<td>0.03687</td>
</tr>
<tr>
<td>vGlut</td>
<td>-1.5783</td>
<td>1.0795</td>
<td>0.1451</td>
</tr>
<tr>
<td>sox2</td>
<td>-0.1462</td>
<td>0.0878</td>
<td>0.0436</td>
</tr>
</tbody>
</table>

**Table 6**: Cohen’s d values for differences between cells positive for each probe vs. cells negative for that same probe at each developmental time point. Positive values for Cohen’s d indicate that gene-positive cells were more entropic than gene-negative cells, while negative values indicate that gene-positive cells were less entropic. A Cohen’s d value of >|0.2| is considered small, but significant. A Cohen’s d value of >|0.4| corresponds to a medium effect size, and a Cohen’s d value of >|0.8| corresponds to a large effect size.
<table>
<thead>
<tr>
<th></th>
<th>Stage 14</th>
<th>Stage 18</th>
<th>Stage 22</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBT</td>
<td>0.1379</td>
<td>0.6773</td>
<td>0.2732</td>
</tr>
<tr>
<td>GAD</td>
<td>0.3501</td>
<td>0.8421</td>
<td>0.0174</td>
</tr>
<tr>
<td>vGlut</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.1596</td>
</tr>
<tr>
<td>sox2</td>
<td>0.3914</td>
<td>0.0445</td>
<td>0.3205</td>
</tr>
</tbody>
</table>

**Table 7:** KS test values for differences between cells positive for each probe vs. cells negative for that same probe at each developmental time point. A value of <0.05 is considered significant.
3.2.3. Comparisons Between Cells Positive for Neural Marker Genes

One approach to our core research question involved making comparisons between cells that expressed a particular scene and cells that did not. While this is certainly a meaningful way to understand differences between groups, it provides an incomplete understanding of the relationship between gene expression and calcium activity profile. A complementary analysis involves comparing cells positive for each gene with cells positive for other genes. That is to say, we investigated whether a GAD-positive cell has a pattern of calcium activity distinct from that of a vGlut-positive cell, a sox2-positive cell, and/or an NBT positive-cell. To this end, we performed the same statistical analyses as previously discussed (Cohen’s d and the KS test) between cells graded positive and low-level for each gene of interest. A plot of cell entropy data can be seen in Figure 7, and values of statistical significance for each comparison can be seen in Tables 8 and 9.

The results revealed significant differences in cells positive for specific neural marker genes, most notably GAD and vGlut. Indeed, the stronger effect size was found when comparing GAD-positive cells to vGlut-positive cells, with the former cells exhibiting less entropic patterns of calcium activity. Both GAD-positive cells and vGlut-positive cells differed in entropy from both NBT-positive cells and sox2-positive cells. However, no difference was found between the calcium dynamics of NBT-positive cells and sox2-positive cells.

Overall, these results suggest that vGlut-positive cells exhibit the most unpredictable calcium dynamics, followed by sox2-positive cells, and NBT-positive cells. GAD-positive cells were found to exhibit the most predictable, or least entropic, patterns of calcium
activity. All determinations of significance were consistent between our two statistical metrics.
Figure 7: Each data point represents an individual cell plotted according to its entropy value (y-axis). Data points are color-coordinated based on its expression of a particular marker gene with positive and low-level cells were binned for statistical analysis.
Table 8: Cohen’s d values for entropy comparisons between cells positive for expression of individual marker genes. Positive values for Cohen’s d indicate that cells positive for the gene listed along the vertical axis were more entropic than cells positive for the gene listed along the horizontal axis, while negative values indicate that cells positive for the gene listed along the vertical axis were less entropic. Values $> |0.2|$ signify a small significant effect size, values $> |0.4|$ signify a moderate effect size, and values $> |0.8|$ signify a large effect size.
<table>
<thead>
<tr>
<th></th>
<th>NBT</th>
<th>GAD</th>
<th>vGlut</th>
<th>sox2</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBT</td>
<td>--</td>
<td>0.0286</td>
<td>0.0026</td>
<td>0.3499</td>
</tr>
<tr>
<td>GAD</td>
<td>0.0286</td>
<td>--</td>
<td>&lt;0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>vGlut</td>
<td>0.0026</td>
<td>&lt;0.0001</td>
<td>--</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>sox2</td>
<td>0.3449</td>
<td>0.0001</td>
<td>&lt;0.0001</td>
<td>--</td>
</tr>
</tbody>
</table>

Table 9: KS test values for entropy comparisons between cells positive for expression of individual marker genes. A value <0.05 is considered significant.
4. Conclusions

4.1. Experimental Outcomes and Conclusions

Although several experiments have correlated intracellular calcium activity with the acquisition of a particular neurotransmitter phenotype, many contributions remain to be made to the understanding of this relationship. In particular, contradictory suggestions about whether this process is cell-autonomous have yet to be resolved (Borodinsky et al., 2004; Guemez-Gamboa et al., 2014). Additionally, the wide range of single-cell calcium activity patterns we observed during imaging experiments prompted a need to move away from traditional methods of data analysis, which rely on the imposition of a particular threshold or kinetic profile as defining a calcium spike, and then simply tally the number of spike events occurring over a particular period. Coincident with the cell culture experiments carried out in this project, members of our lab developed a novel analysis pipeline for calcium fluorescence data. This methodology, which does not rely on the definition of a particular feature of interest, has been applied to multiple distinct data sets from both our lab and others, and had proved reliable and robust as a biologically-relevant classifier.

With this technique, we approached our central research question, investigating whether cells expressing particular neuronal phenotypes also express distinct profiles of calcium activity. Experimentally, this was approached via calcium imaging of dissociated presumptive neural tissue \textit{in vivo}, followed by fluorescent \textit{in situ} hybridization as a gene expression assay. Analytically, we compared calcium activity across developmental
stages, across cells positive vs. negative for specific chosen marker genes, and across cells positive for different chosen marker genes.

Analysis of calcium activity by stage reveals that cells dissected at neural plate stage (stage 14) were most entropic, followed by cells dissected at neural tube stage (stage 18). Cells dissected at early tailbud stage (stage 22) were found to be the least entropic. Perhaps counterintuitively, this means that in a general sense, cells dissected at stage 14 showed a narrower range of calcium influx and efflux, while stage 22 plates showed the widest fluctuations in intracellular calcium. This application of entropy values to their biological relevance is rooted in the division of the original calcium fluorescence trace into equal quartiles. When the trace of a cell that exhibits a narrow range of calcium flux is divided, even the most distant quartiles (A and D) remain quite close in raw distance. This means that transitions between these distant quartiles can easily occur between frames, as the raw fluorescence increase that must occur to make this transition is nominal. When this data is converted to a matrix, the distribution of the values within the matrix becomes much more equal. A-to-D transitions may not be significantly less likely than A-to-B or A-to-C transitions, because the real difference between these thresholds may be relatively small.

In contrast, a trace representative of a cell that experiences broad, high-amplitude fluctuations in calcium concentration would be divided into quartiles spanning much broader fluorescence ranges. The range of each quartile may be so large that it becomes unlikely for a cell to exhibit such a quick and dramatic change in calcium concentration so as to complete an A-to-D transition in a single frame. Therefore, values in this matrix
tend to be largest for proximal transitions (e.g., A-to-B, C-to-D). This creates an uneven matrix with a higher entropy value.

Previous lab experiments have suggested that cells dissected at stage 18 exhibit a higher spike frequency than cells dissected at both earlier and later stages. This result is not inconsistent with our findings, as our methods of calcium activity are now sensitive to long-scale changes in calcium concentration that would not be detected by a spike-counting methodology. For example, it could be true that, while stage 18 cells exhibit more stereotypical spiking behaviors, stage 22 cells exhibit a broader range of slower-form calcium activity. The occurrence of slow rises and falls in calcium concentration would contribute to a lower entropy level; transitions like A-to-A or B-to-B would be more common, since fluorescence intensity would increase or decrease gradually over numerous frames. Indeed, this is consistent with the hypothesis that calcium waves (with slower kinetics than calcium spikes) are important regulators of neurite extension, because studies have found that the calcineurin gene proposed as responsive to this type of activity does not demonstrate robust expression until stage 22 (Lautermilch and Spitzer, 2000).

In a broad sense, this data leads us to conclude that significant differences exist between the calcium activity profiles of cells dissected at different developmental stages. However, it does not lead us to favor any particular reason for this discrepancy. For example, one hypothesis would propose that these entropy phenotypes are generalizable to all cells within a particular population. Another hypothesis holds that these results actually arise because of the confounding variable of differential gene expression. For example, it could be true that stage 14 cell populations have higher frequency of
expression of a particular gene, and that only cells expressing this gene exhibit the high-entropy phenotype. However, since this gene is expressed in such a high proportion of presumptive neural cells, it is the most significant contributor to the ultimate average entropy value.

Although our data suggests that gene expression can be correlated with calcium activity, it does not go so far as to confirm the validity of this second hypothesis. For the genes we assayed, the proportion of cells expressing a positive phenotype was so low that their nominally-different activity profiles would not skew the average values. However, a whole host of additional neural-development-related genes are differentially expressed at the studied stages, and may represent potential modulators of this observed effect.

These differences in activity may also be the result of changes in voltage-gated calcium channel expression over development. Ca\textsubscript{v}1.2, for example, is expressed at the neural plate stage, but not at detectable levels later in development. Ca\textsubscript{v}1.3 is expressed at the neural tube stage, but not earlier in development (Lewis et al., 2014). Beginning at early tailbud stages, Ca\textsubscript{v}2.3 also begins to be expressed (Lewis et al., 2009). The variable profile of calcium channel phenotype over time provides a potential mechanism for this shift in entropic activity. This hypothesis would hold that particular calcium channels (or particular combinations of coexpressed calcium channels) tend to allow calcium fluxes of a particular entropic profile, and that these profiles will then change over time with changing VGCC distribution. Previous lab work has demonstrated that Ca\textsubscript{v}2.1 morpholino-knockdown embryos show significantly fewer calcium spikes than control
embryos (Herbst, unpublished), suggesting that these VGCCs have the capability to play a significant role in modulating the profile of calcium activity.

In this way, our studies of calcium activity across developmental time points is intimately related to our investigation into correlations between calcium activity and neurotransmitter phenotype. An understanding of baseline activity profiles across time is a vital foundation for further comparative studies, as well as for any future work that seeks to test the effects of genetic or pharmacological perturbations on calcium flux. In part, these results are consistent with previous literature about both spike frequency across time and wave frequency over time. However, the use of a metric that is not limited by the definition of these features is novel, and revealed an overall decrease in calcium activity entropy over development that would not have been observed by a ‘spike-counting’ methodology.

As a final note on these stage-wise calcium comparisons, we would like to emphasize their robustness as the result of a significantly larger set of input data. Because this question did not involve querying gene expression data, all calcium data recorded in the 2-hour image was able to be statistically analyzed. Conversely, significant washing away of cells during the FISH procedure as well as the presence of cells with gradable gene expression that could not be confidently coregistered contributed to a significant diminishment of the size of our gene-expression-related data sets as compared to this one.

With this information, we move to our core research question: Is there a relationship between calcium activity profile and expression of neural marker genes? Marker genes were chosen to distinguish undifferentiated neural precursors (sox2-positive)
from differentiated neurons (NBT-positive), as well as to distinguish excitatory glutamatergic neurons (vGlut-positive) from inhibitory GABAergic neurons (GAD-positive). The performance of various statistical comparisons allowed us to understand (a) if cell expressing particular marker genes differ in calcium activity from cells that do not express these genes, (b) if this difference changes over time, and (c) if cells expressing particular marker genes have distinctly different profiles of calcium activity compared to cells expressing other marker genes.

Comparisons between cells positive for NBT, GAD, vGlut, and sox2 and same-plate cells negative for expression of these genes revealed a small effect size for all genes except GAD, which showed no significant difference. These results suggest that NBT-expressing cells tend to have less entropic calcium activity than cells that do not express this gene, while vGlut- and sox2-expressing cells tend to have more entropic calcium activity than their respective negative cells. However, the low proportion of positive cells across NBT-probed, GAD-probed, and vGlut-probed plates means that more experiments would ideally be done to increase confidence in this effect.

It should not be surprising that GAD and vGlut show relatively little expression in the stages assayed. As seen in Appendix 2, expression of both genes remains at relatively low levels early in development. In Xenopus laevis, vGlut expression does not show a fold change of 2 until stage 23, while GAD expression does not show a fold change of 2 until ~stage 27. For cells that were dissected at stage 22, sibling controls had generally reached stage ~stage 25 by the beginning of the imaging period; therefore, robust expression of these genes in cell culture is not expected.
Similarly, high expression levels of sox2 across all developmental time points are consistent with microarray-validated expression data (Yanai et al., 2004). sox2 expression is significantly higher than that of the terminal neural differentiation markers even at the blastula stage, and dramatically increases before gastrulation. Comparable expression levels are found at each of the developmental stages assay in our experiment.

Although NBT expression is characterized as increasing over gastrulation (to a fold change of ~4 by stage 14), our results showed many less NBT-positive cells than would be expected. This could be the result of our relatively low plate n; a few plate on which very few cells were maintained could result in a much less robust data set for a particular probe. Another explanation would be that our NBT-probe was weaker than probes for other marker genes, and therefore low levels of expression that were noticeable on other plates were not detectable for NBT. Although this explanation could not be proven within the confines of our current data set, additional experiments with freshly-synthesized probe (perhaps based on a different region of template DNA) could be performed.

The result of this discrepancy on our data was an artificially high threshold for expression in NBT-positive cells. Therefore, if we were to increase the sensitivity of our probe, we would expect more ‘negative’ cells to be re-evaluated as having low-level expression. Based on our data as a whole, as well as inferences that can be drawn from GAD- and vGlut-positive cells (which ostensibly would have also expressed NBT if probed), we do not expect that our results would be dramatically altered. If anything, we might expect NBT-positive cells to, on average, show an intermediate activity profile to
GAD- and vGlut-positive cells. With the small effect sizes we report, this hypothetical shift would be reasonable.

We attempted to further understand these differences in calcium activity by analyzing its changes over developmental time points. Appendices B and C may be helpful in understanding the general profiles of marker gene expression over the course of *Xenopus* development, as well as in understanding the approximate developmental stage of cultured cells (This was determined based on sibling control embryos staged at the time of experimental dissection. Although we cannot assume that dissociated cells develop along an entirely identical timeline, we believe that no significant discrepancy arose over our relatively short imaging time span).

*NBT*-positive cells seem to experience the most changes in comparison to negative cells across stages. However, the previously-discussed low levels of positive cells in this data set lead us to be wary of drawing definite conclusions from this result. Particularly, the dearth of *NBT*-positive cells successfully coregistered from Stage 18 plates may be confounding our overall interpretations.

The profiles of *GAD*-positive and *vGlut*-positive cells suffer less from this scarcity, yet we must still use caution when extrapolating from this data. Preliminary analysis seems to show that *GAD*-positive cells do not significantly differ from *GAD*-negative cells across any developmental time point, consistent with our earlier findings that no significant difference exists between *GAD*-positive and *GAD*-negative cells when stages are pooled. *vGlut*-positive cells, conversely, seem to differ dramatically from *vGlut*-negative cells in cells dissected at stages 14 and 18. *vGlut*-positive cells dissected at stage 14 show
significantly less entropic calcium activity than negative cells, while this phenomenon reverses at stage 18 (vGlut-positive cells exhibit, on average, more entropic calcium activity). This rather striking statistical significance is one of few that can be easily visually appreciated (Figure 6).

Although, again, we must be cautious in drawing general conclusions from such a small pool of data, findings in the literature support these stage-dependent discrepancies. For example, cells exposed to different concentrations of calcium channel blocker diltiazem after dissection at various developmental stages show differential vGlut expression compared to one another and compared to control embryos. Cells dissected at neural plate stage show increased vGlut expression positively correlated to increased diltiazem concentration. However, cells dissected at neural fold stage only show significant change in vGlut expression at exposure to an intermediate diltiazem concentration (10 μM), and not at exposure to higher levels (100 μM). Cells dissected at neural tube stages did not show any effect at intermediate concentrations, but the number of vGlut-positive cells increased approximately four-fold with expression to high concentration of diltiazem (Lewis et al., 2014). Taken in conjunction with our findings, we recognize the suggestion that cells do experience a stage-dependent differential relationship between vGlut expression and calcium activity.

In cells positive for sox2 expression, no significant difference was found when compared to cells negative for sox2 expression across each developmental stage. While our stage-pooled analysis found a slight overall increase in calcium activity entropy in
sox2-positive cells, this effect was too small to be detected when the data was parsed by stage.

A final avenue of analysis focused on comparisons between cells expressing different marker genes. Our previous approaches sought to investigate whether, for example, cells expressing GAD show an average calcium activity profile different from that of cells not expressing GAD. In contrast this analysis seeks to elucidate whether cells expressing NBT, GAD, vGlut, or sox2 have significantly different calcium activity profiles from one another.

Our results suggest that cells expressing these genes have characteristic calcium activity profiles that may be distinct from those of cells expressing other genes. This evidence is especially compelling when understood within the larger body of work studying the relationship between calcium activity and neurotransmitter phenotype. Previous experiments, both in our lab and in the literature, have been inconsistent in their conclusions about the presence of such a correlation. However, our results, coupled with a recognition of the discrepancies in the thresholds used in the literature to define certain forms of calcium activity, may provide additional explanation for these ambiguities.

No significant difference was found between cells expressing sox2 and cells expressing NBT across all stages. This is not particularly surprising; both sox2 and NBT are expressed in neurons and neuronal precursors regardless of neurotransmitter phenotype, and their expression has not been implicated in the acquisition of an excitatory or inhibitory fate.
However, significant differences were found between GAD-expressing cells and cells expressing vGlut, NBT, or sox2, as well as between vGlut-expressing cells and cells expressing GAD, NBT, or sox2. These results show that vGlut-positive cells are significantly more entropic than cells positive for other marker genes, GAD-positive cells are significantly less entropic than cells positive for other marker genes, and that NBT- and sox2-positive cells exhibit an intermediate entropy phenotype.

We will first address the comparisons of vGlut and GAD to NBT. These results are consistent with the underlying biology of gene expression. Although this was not explicitly tested, we can assume that all cells positive for GAD and vGlut would also be positive for NBT (that is to say, all differentiated inhibitory neurons are differentiated neurons). Therefore, we would anticipate the entropy value of NBT-positive cells to be intermediate to that of both GAD- and vGlut-positive cells. We found this prediction to be confirmed.

The most significant differences were found between the calcium profile entropy values of vGlut-positive cells and those of GAD-positive cells. The distribution of entropies for vGlut-positive cells was almost a full standard deviation higher than the distribution for GAD-positive cells (Cohen’s d=0.9034). Cells that express vGlut (i.e., excitatory neurons) have more entropic, less predictable activity patterns than cells that express GAD (i.e., inhibitory neurons).

These results are not inconsistent with the proposed homeostatic model (Spitzer et al., 2005). In a simplified sense, higher entropy values are associated with less spiking activity, while lower entropy values are associated with a higher prevalence of these highly-stereotyped occurrences. Per this interpretation, our data is consistent with the
suggestion that a high frequency of calcium spikes during development is correlated with an inhibitory neuronal fate, while a low frequency of calcium spikes specifies an excitatory phenotype (Borodinsky et al., 2004).

However, this simplification of entropy as the inverse metric of spike frequency is inappropriate. Our analysis metrics, while accounting for the type of rigorously-defined calcium spikes that predominate analysis methods in the field, also incorporate longer forms of calcium activity (such as waves), as well as ambiguous activity patterns that may inconsistently be classified as spikes based on the chosen threshold. An appreciation for the broad range of calcium activity patterns that can occur within a single cell can be found in Figure 1. This figure demonstrates the limitations of relying on the automated recognition of a single, narrowly-defined trace feature. Furthermore, as a review of the literature demonstrates, multiple types of calcium events can occur within cells at the same time, as well as sequentially. For example, gastrulating Xenopus cells may exhibit calcium spikes superimposed onto a slower increase in calcium concentration (Webb et al., 2005), and a significant proportion of cultured Xenopus neurons were found to exhibit both spikes and waves over an extended imaging period (7-12 hours) (Gu and Spitzer, 1995). Our entropy-based analysis does not favor any particular form of calcium flux over any other, and is therefore a valid metric for quantifying time-series calcium activity in a general sense.
4.2. Possible Limitations

The primary limitations of this project arise from the use of a cell culture methodology. Although this approach allows definitive coregistration of cells between calcium imaging and gene expression analysis, it artificially removes elements of cell-cell communication that are vital to development and patterning nervous system. Therefore, conclusions drawn from an *in vitro* study cannot necessarily be applied directly to actual embryonic development.

Additional limitations resulted from the low number of positive cells present in plates probed for GAD, vGlut, and NBT. This limited the statistical power of comparisons between positive cells and low-level and negative cells. Since positive and low-level cells were then grouped together during statistical analysis, differences that exist in only highly-positive cells may not be accurately revealed.

A final point of limitation was the relatively low number of cells that were able to be coregistered. The extent of cell loss over the FISH and coregistration processes can be visualized in Figure 8. Although this should not impact the comparisons made between groups (as it equally affects all probes and stages), it does represent a significant loss of data that would have provided additional statistical power to our conclusions.
Figure 8: Entropy values for all cells on plates probed for NBT. The overwhelming number of cells without correlated gene expression data (shown as white data points) is the result both of cells that were washed away during *in situ* hybridization and of cells that could not be confidently coregistered between calcium and FISH images.
4.3. Future Directions

Cell-cell interactions are crucial within the developing nervous system, and recent work has lent credence to the idea that the effect of calcium activity on neurotransmitter phenotype is, at least in part, non-cell-autonomous (Guemez-Gamboa et al., 2014). Therefore, future investigations into the effect of the activity on neural gene expression would ideally be performed in vivo.

Genetically-encoded calcium indicator GCaMP is widely used to visualize dynamic patterns of calcium activity (Nakai et al., 2001), and microinjection of capped GCaMP mRNA at the 2-cell stage has been shown to induce robust expression of the GCaMP protein in the embryo (Sible and Wroble, 2009). Co-injection with memRFP, (which localizes to the cell membrane and allows visualization of the extent of each cell) allows distinction of individual cells within and between which calcium activity occurs. Transgenic methods could also be used to generate a stable Xenopus line expressing these two constructs. Gene expression profiles of individual cells could be generated either by whole-mount in situ hybridization after fixation, or in vivo with reporter constructs. Novel methods for visualizing real-time transcription have been established in zebrafish, and may soon be optimized for use in Xenopus (Campbell et al., 2015).

While this in vivo approach offers many possibilities for understanding the cell-cell interactions that seem vital to the establishment of a balanced neurotransmitter phenotype pool, it also provides additional challenges in terms of pattern analysis. Although single-cell calcium dynamics could be analyzed via the existing pipeline, spatial analysis adds an additional level of complexity. Further investigations into calcium dynamics across
neural tissue would require significantly expanded methods of analyzing coordination of calcium activity between cells and of characterizing particular types of calcium events. Cardiac calcium dynamics have been analyzed via Spatial Permutation Entropy, a potential future approach conceptually similar to our current cell-culture methodology (Schlemmer et al., 2015).
5. References


Appendix A
Table of all experimental plates

<table>
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All analyzed plates sorted by probe (columns) and stage of dissection (row).
Dates correspond to dates of dissection and imaging, respectively.
Initials correspond to experimenter who performed the dissection and image, respectively.
Numbers were assigned sequentially to every plate dissected/imaged on a particular date.
Appendix B
Developmental stage profiles for genes of interest
All expression profiles were derived from microarray data obtained by Yanai et al., 2004.
**Appendix C**
Approximate sibling stage data for cells dissected at each stage of interest

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