UNIVERSITY OF CALIFORNIA SAN DIEGO

Mechanism for Spontaneous Calcium Activity and Spinal Cord Neurotransmitter Specification, and the Role of Calcium Activity in Dopamine Specification in the Brain and Spinal Cord

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Neurosciences

by

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2009

The Dissertation of Norma Andrea Velázquez Ulloa is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2009

Dedication

I dedicate this thesis to

My family
My friends

Quiero dedicar esta tesis a mi mamá, mi papá, mi hermana y mi abuelita. Mi familia ha sido una fuente de apoyo incondicional todos estos años. Su ética de trabajo y entusiasmo por lo que hacen han sido un ejemplo a seguir, difícil de igualar en ocasiones, pero fuente de inspiración en momentos difíciles. Su confianza en mí y palabras de aliento me han ayudado a sobrellevar y superar los baches y frustración durante estos años lejos de casa. Agradezco las conversaciones semanales y la manera en que nuestra relación familiar se ha mantenido intacta y si acaso tal vez fortalecido en la distancia. A mi abuelita, le agradezco todos los fines de semana que pasamos juntas, las historias sobre su juventud y su ejemplo de ética de trabajo y por ser una mujer luchona, con fuerte carácter. A mi papá le agradezco las conversaciones sobre la vida y la política, que me mantienen informada sobre mi país. Le agradezco su entereza, su paciencia y por ser un ejemplo de hacer las cosas bien y dar todo en el trabajo y con la familia. A mi mamá le agradezco por sus porras y canciones, y por su actitud ante la vida, por

ser un ejemplo de fuerza y perseverancia para conseguir lo que quiere, y por dar todo por la familia. A mi hermana le agradezco el ser un ejemplo de arrojo y valentía para seguir sus sueños tanto en el ámbito profesional como en el personal. Le agradezco sus ganas de vivir y personalidad tan distinta a la mía, social y fiestera, que me han enseñado a valorar más las relaciones personales y la forma de distribuir el tiempo entre trabajo y diversión, aun en momentos en que las cosas que hacer parecen rebasar la cantidad de tiempo disponible cada día! Gracias a todos ustedes por ayudarme a mantener perspectiva sobre las cosas que son importantes.

I also dedicate this thesis to my friends. I don't mention specific names because you know who you are. Some of you are here in San Diego, most of you are away. Some of you I haven't talked to in months or years, but the connection between us remains. I thank you all for the shared moments, happy and sad, for the laughs and the tears, and the hugs. I thank you for letting me be part of your life, and making me part of yours. Thank you for the thoughts, the conversations, the support, for accepting me for who I am, and for contributing to make me who I am. It can be hard to find good friends, and I feel extremely lucky to be able to count many of you in this category. Thanks for being there for me throughout all these years.

Epigraph

Si lloras por haber perdido el sol,
las lágrimas te impedirán ver las estrellas

Rabindranath Tagore

Pausa

De vez en cuando hay que hacer
una pausa

contemplarse a sí mismo
sin la fruición cotidiana

examinar el pasado
rubro por rubro
etapa por etapa
baldosa por baldosa

y no llorarse las mentiras
sino cantarse las verdades.

Mario Benedetti

El éxito es aprender a ir de fracaso en fracaso sin desesperarse.

Winston Churchill

Lo importante no es saber, sino tener el teléfono del que sabe.

Les Luthiers

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Acknowledgements

I thank Nick for his enthusiasm, and love of science, and for discussions about the big picture, philosophical questions about what constitutes cell identity, about the life of a scientist, and for being patient, understanding and supportive, through the good and the rough parts of this journey. He has also been a great teacher of the lore of manuscript and scientific writing, and has provided a model of how to be a scientist. I thank him for questioning me, and pushing me to think hard about science, and explain myself clearly to get my point across.

I also want to thank my committee members, Darwin Berg, Bill Kristan, Jeff Isaacson, and Binhai Zheng for useful discussion, and support throughout these years. I would also like to thank Paul Slesinger, who was a member of my pre-advancement committee, and provided useful insight into the signaling cascades that was very useful for the experiments and discussion of the results presented in Chapter 2.

I thank Paul, Jeff, Bill, Kathy French and Dan Feldman for being great teachers. Thanks to Jeff for introducing me and my classmates to the early electrophysiology papers. Thanks to Paul for the ion channel readings course. This class was arguably one of the most valuable courses I took in grad school, including going through the exercise of

developing a proposal and having a mock study section that gave me a type of training I had not been exposed to before and were invaluable help and preparation for minor prop. Bill and Kathy were amazing from the beginning welcoming us to the program with the unforgettable and irreplaceable experience of boot camp, a signature of the UCSD Neuroscience program that sets the ground for future friendships, and is a preview of the collaboration and interactive spirit of the Neuroscience community affiliated to the program. It is also an invaluable way to get exposure to all realms of Neuroscience in a nutshell! Thanks!

Dan Feldman, a former member of the community, was not only a great teacher, but also a great mentor and example for how to put together a class, integrating innovative teaching techniques, combined with the latest discoveries in research, and including the teaching of the scientific thinking to students. Being a TA in his Systems Neuroscience undergraduate course was an incredible experience that reinforced my love of teaching. I thank him for the guidance during the course, and for his valuable feedback about teaching and science.

I also want to acknowledge Darwin Berg, Marla Feller, and Anirvan Ghosh for their mentoring concerning postdoctoral choices. They went out of their way to talk to me, cheer me up, and give me advice on this and other aspects of being a scientist.

I also want to thank former and current members of the lab, my family away from home; I have interacted with through the years of graduate school. I want to particularly stress the help and support of Laura Borodinsky and Brent Miller during my first couple years in the lab. I want to thank my co-authors of the Root et al., 2008 publication Cory Root, Gabriela Monsalve, Elena Minakova, and Nick for the collective effort to prepare the manuscript and see it through to publication. I learned a lot about the writing process during these months. Gabriela and Elena were amazing collaborators, with hard to bit enthusiasm, and willingness to undertake even the most challenging experiments.

I want to acknowledge and thank Sharon Sann, and Linda Chang for many conversations over food, or in our office, and their support and advice on being and surviving the graduate student years, and for technical assistance in lab. The European gang (Michaël Demarque, Xavier Nicol, and Davide Dulcis) have been a source of continuous support, fun moments in lab, never-ending technical, and theoretical support, food advice, and croissant or bread and nutella providers. I thank them for introducing me to a proper espresso and croissant, and for showing me a different way to think about and carry out science. I want to thank Qian for being an example on how to enjoy life while being a graduate student, and both Qian and Lin for fun moments in our shared

office. I thank Lisa for sharing the grad school experience with me, including support during some hard times. Her strong personality and way of taking things and making them happen have been an inspiration to me. I want to thank Armando de la Torre for getting me pink hand wraps and giving me fighting tips, and rides, and providing the occasional Mexican sweet bread. I thank Paola Plazas, for her easy going personality and fun discussions over coffee in the mornings. I thank the Berg Lab for feedback on lab meetings.

Thanks to all my fellow neuroscience students in classes above and below, and to the Neurodinner community. Special thanks to the members of my class for the shared moments, conversations, and support throughout graduate school.

Additional important recognition should be made to Mentor Net, and AWIS San Diego. Through Mentor Net I got in contact with two female scientists that through email or chats over coffee, gave me the support I needed and gave me important advice on how to establish better relationships at work, and keep a balanced life at the same time.

AWIS San Diego has been a great place to develop leadership skills, and working in teams. It has also been great for my writing skills, and provided me with a network of like-minded female scientists whose experiences and advice have been invaluable to me since I joined AWIS.

Being part of this community has opened up a whole new world of options within science that I did not know existed, and the workshops and events are always delightful to attend, useful, and great opportunities for personal development.

I thank my friends here in San Diego and across the globe for their support, and keeping in touch. Special recognition here goes to, Tathali, Carla, Ana Ligia, Tanya, Rocío, Ana, Paola, Nadia, Candela, Xoxo, Aline, Jessica, Lisa, John, Jyoti, Dhakshin, Dan, Sam, Joy, Justin, and Beth, for their support during tough personal times, and for multiple shared good moments. I thank the Tocayo gang: Miguel Torres (tocayo), Alex, Laia, Stephane, Maite, and Miguel Yañez (tocayito) for volleyball and especially the post volleyball socials! I also want to thank the third Miguel gang (Guerrero), who introduced me to the world of chemists, and Choao spicy maya chocolate. Thanks to Marcos, Gaby, Andreita, Norma Angélica, David, Mariana, Victoria, Mariangela, Marion, Bárbara, Rubén, Ana and Erik, for trips, multiple gatherings, and making my last year of gradschool my most socially active and fun.

I also want to thank UCSD recreation, UCSD extension, and Villa Musica for a variety of classes offered that have kept my need to keep busy with other interests possible. Special recognition goes to my teachers and classmates in the Translation and Interpretation Certificate

program, that did wonders to my writing and presentation skills, improved my English level, and got me reacquainted with my love of language. Villa Musica was also a great find to bring back music into my life on a regular basis at an affordable price, and with the added value of becoming part of a community of individuals who share the joy of music, keeping it fun, at whatever level of skill you have!

A special acknowledgement goes to Krav Maga San Diego, and my classmates and instructors there that have introduce me to a totally different way of life outside of the world of science and academia, and provided a challenge that has shown me and made me feel I can do anything and everything! The training I have gotten in class goes well beyond the physical self-defense movements we learn in class. This class has allowed me to discover a new side of my personality I hadn't exploited before; a side of me that won't quit, that fights back, and is strong. For me the most important lessons I have learned are to be more assertive, stay focused under pressure, become more aware of my surroundings, observant, and vigilant, and unleash a fighting spirit I didn't know I possessed, that has done wonders for my self-esteem, and provided the greatest, most fun, efficient, and effective escape-valve for the stress of gradschool and life. Thanks for being tough on me, but gentle and patient too, and pushing me out of my comfort zone, especially Dana, Drew,

Benjamin, Ben and Nancy. I thank Chris Horton for infecting me with his enthusiasm for Krav Maga. I thank Tim, Mike, Ed, Jay, Darren, Ari, Mara, Ray, Sherry, Pat & Ed, Lourdes, Miguel, and George for practicing for the P1 test with me and being great partners that are helpful inside and outside of class, and for being great Krav buddies. Additional thanks to Drew for the extra help and encouragement! In connection to thesis writing, special thanks go to David Haas for being an example of dedication, and a fun and helpful thesis-writing buddy at coffee shops around town. Mike Wychick also gets extra points for being available for distracting activities that kept me sane and entertained during the thesis-writing process.

My family is more properly acknowledged in the dedication, but I can't leave them out of this part, so here once more I thank them and acknowledge them for their unconditional support throughout graduate school.

Chapter 2 is a reprint of the material as it appears in "Embryonically Expressed GABA and Glutamate Drive Electrical Activity Regulating Neurotransmitter Specification" Cory M. Root, **Norma A. Velázquez-Ulloa**, Gabriela C. Monsalve, Elena Minakova, and Nicholas C. Spitzer.

Journal of Neurosciences, 2008. Apr 30; 28(18):4777-84. It is included with the permission of all the authors on the final publication.

Chapter 3 is material currently being prepared for submission for publication. It is included with the permission of all the authors of the manuscript, Davide Dulcis, and Nicholas C Spitzer. The dissertation author was the primary author on this paper.

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-Velázquez-Ulloa N.A*, Dulcis D.*, Spitzer N.C. Heterogeneous specification of dopaminergic phenotype: transcription factors, electrical activity and neurotransmitters. In preparation.

-Velázquez-Ulloa N.A., Spitzer N.C., Dulcis D. (2008) Activity-dependent specification of dopaminergic neurons in the embryonic nervous system. International Journal of Developmental Neuroscience. 26: 887.

-Root C.R., **Velázquez-Ulloa N.A**., Monsalve G.C., Minakova E. Embryonic transmitters modulate activity regulating later neuronal differentiation. *Journal of Neuroscience*. Vol 28(18), 4777-4784.

-García-Pérez E., Vargas-Caballero M., **Velázquez-Ulloa N**, Minzoni A., De Miguel F.F., 2004. Synaptic integration in electrically coupled neurons. *Biophysics Journal*, Vol 86 (Pt 1), 646-655.

-**Velázquez-Ulloa N**.*, Blackshaw S.E.*, Szczupak L., Trueta C., García E., De Miguel F.F., 2003. Convergence of mechanosensory inputs onto modulatory serotonergic neurons in the leech. *Journal of Neurobiology*, Vol. 54(4), 604-17.

-Velázquez-Ulloa N.A., Spitzer N.C., Dulcis D. Heterogeneous specification of dopaminergic phenotype: electrical activity and transcription factors. Society for Neuroscience Meeting, Washignton D.C., USA, November 2008.

-**Velázquez-Ulloa N.A.**, Monsalve G.C., Minakova E., Root C.R., Spitzer N.C. GABA and glutamate modulate calcium spike activity and neurotransmitter expression via metabotropic receptor signaling. Society for Neuroscience Meeting, Atlanta, GA, USA. October 2006.

-**Velázquez Ulloa N.A.**, Vargas M., Coello G, Fernández de Miguel F. The role a morphology in synaptic integration in electrically coupled cells (Papel de la Morfología en la Integración Sináptica en Neuronas Acopladas Eléctricamente). XLIV Meeting of the Physiological Sciences Mexican Society, Monterrey, Nuevo León, Mexico, 2001.

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Major Field: Neurosciences

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ABSTRACT OF THE DISSERTATION

Mechanism for Spontaneous Calcium Activity and Spinal Cord Neurotransmitter Specification, and the Role of Calcium Activity in Dopamine Specification in the Brain and Spinal Cord

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Doctor of Philosophy in Neurosciences

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Professor Nicholas C. Spitzer, Chair

Professor Binhai Zheng, Co-Chair

Spontaneous electrical activity is a feature of the nervous system from early stages of development preceding synapse formation. An example of this is calcium-spike activity, which is displayed by embryonic *Xenopus laevis* spinal cord neurons, and has a role in neurotransmitter specification.

Here I present data identifying a mechanism for calcium-spike activity that depends on GABA or glutamate activation of metabotropic receptors, and their recruitment of PKA or PKC. This work attributes a role to neurotransmitters and metabotropic receptors prior to synapse formation in modulating electrical activity that in turn modulates neurotransmitter specification. These results contribute to our knowledge of the molecular mechanisms recruited during development.

In addition to electrical activity, transcription factors play a role in neurotransmitter specification. Here I present a study of the development of the dopaminergic system in *Xenopus laevis* to characterize the co-expression of dopamine with additional neurotransmitters, and with transcription factors. This led to identification of subclasses of dopaminergic neurons based on these markers. Calcium-spike activity plays a role in dopamine specification, but different dopaminergic nuclei respond to activity manipulations in a different way. Examination of the *in situ* calcium spike activity of spinal cord neurons showed that neurons with different molecular markers have distinct pattern of calcium-spike activity. These results provide the basis for further studies to establish the interplay between calcium-spike activity and transcription factors in the dopaminergic system.

1 Introduction

Development in biology is the term used to encompass the changes and processes that give rise to a mature organism. For multi-cellular organisms this involves going from a single cell to multiple cells organized in subsystems with specialized functions. Early neuronal development has three main stages: proliferation, migration, and differentiation. During proliferation cells divide to increase their numbers. Migration follows, during which cells move to different regions where they will become established. The last step is differentiation, the process by which a given neuron becomes specialized and acquires characteristics that define its function within the nervous system.

One critical aspect of neuronal differentiation is the acquisition of a specific neurotransmitter phenotype. Neurons use neurotransmitters to communicate with one another in neuronal circuits. Neurotransmitters can have excitatory, inhibitory, or modulatory functions, depending on the particular neurotransmitter and the receptors to which it binds. This choice is crucial because it defines a key neuronal function in the nervous system and it is also a component of the neuron's identity.

1.1 Neurotransmitters: types and functions

Neurotransmitters are molecules that are synthesized and released by neurons. There are three main forms of neurotransmitter release, synaptic, non-synaptic, and extrasynaptic or volumetric. The role of neurotransmitters as molecules for synaptic communication between neurons is well established. Non-synaptic release “en passage” from varicosities is the form of transmission of autonomic nerve fibers at neuro-effector junctions (Burnstock, 2008). Now it is also recognized that neurotransmitter release can occur at extrasynaptic locations, and diffuse through the extracellular space to activate receptors over longer distances and have a paracrine effect (Vargová and Syková, 2008). This form of neurotransmitter release has been documented for serotonin, for example (De-Miguel and Trueta, 2005). Neurotransmitters are also expressed before synapse formation (Lauder, 1993; Ma et al., 1992; Antal et al., 1994; Berki et al., 1995) and released in a calcium and SNARE-independent way at this early stage (Demarque et al., 2002). They have additional roles including growth regulation (Lauder, 1993), cell migration (Yacubova and Komuro, 2002; Bolteus and Bordey, 2004; Manent et al., 2005), as trophic factors (Jonsson and Hallman, 1982; Zagon and

McLaughlin, 1986; Bulloch, 1987; Hauser et al., 1987), and in proliferation (Lo Turco et al., 1995; Liu et al., 2005; McDearmid et al., 2006).

Traditionally, it has been believed that neurons express only one neurotransmitter. However, accumulating information indicates that this is not the case. Classical neurotransmitters and neuropeptides are co-expressed and co-released (see Hökfelt et al., 1984; Hökfelt, 1991 for reviews). Rapidly-acting classical transmitters and more slowly-acting neuropeptides are expressed in cells involved in background adaptation in adult *Xenopus* frogs (Ubink et al., 1998). Fast neurotransmitters are also co-expressed and co-released (Kosaka et al., 1991; Gao et al., 2001; Gonzalez-Hernandez et al., 2001; Gutierrez, 2003; Trudeau and Gutierrez, 2007 for review; Mendez et al., 2008).

Although the presence of more than one transmitter is normal in the adult nervous system, it is more prevalent at early stages of development and in the adult nervous system following changes in electrical activity, such as an epileptic crisis, and in disease (Fureman et al., 1999; Fureman et al., 2003; Gutierrez, 2003; Dal Bo et al., 2008).

1.2 External and internal factors in neural development

It is now recognized that both extrinsic and intrinsic factors are involved in the process of neuronal differentiation, and interplay between

these two types of factors is necessary for normal neuronal differentiation (Edlund and Jessell, 1999). Among the external factors cell adhesion molecules (see Jessell, 1988 for review), sonic hedgehog (Ericson et al., 1995); FGF (Liu et al., 2001); Wnts (Patapoutian and Reichardt, 2000; Salinas and Zou, 2008), and GDNF (Haase et al., 2002) have been shown to play a role in neuronal development.

The internal factors are the genetic programs in the cell that involve the activation of specific transcription factor cascades. Transcription factors are sequence-specific DNA-binding proteins that activate or repress the expression of a gene. Multiple transcription factor cascades have been identified that control different stages of neuronal development, including neurogenesis, patterning and differentiation (Tanabe and Jessell, 1996; Jurata et al., 2000; Bertrand et al., 2002; Schuurmans and Guillemot, 2002; Dasen et al., 2003; Dasen et al., 2005; Guillemot et al., 2006). This has led to the understanding of transcriptional codes that regulate neuronal fate (Jessell, 2000; Lee and Pfaff, 2001; Shirasaki and Pfaff, 2002). Transcription factors have also been identified that are important for neurotransmitter specification. For example, Tlx3 is involved in the regulation of glutamate and GABA expression in the spinal cord (Cheng et al., 2004). Ptf1a, Lbx1, Pax2, Rbpj have been implicated in the specification of inhibitory neurotransmitters including GABA and glycine

and neuropeptides such as NPY, somatostatin, enkephalin, dynorphin and galanin (Huang, 2008; Hori, 2009). Pet1, Nkx2.2, Lmx1b, Mash1, Gata2, Gata3, and Phox2b are involved in serotonergic specification (Alenina et al., 2006).

However, other factors also have important roles in development. Electrical activity regulates neurotransmitter specification as well as other aspects of differentiation (Borodinsky et al., 2004; Spitzer, 2006). Natural stimuli that generate electrical activity regulate the number of neurons that express a given transmitter (Dulcis and Spitzer, 2008).

1.3 Role of electrical activity in development with emphasis on calcium signaling

Electrical activity in neurons is generated by the flux of ions across the cell membrane. These ion fluxes can be depolarizing or hyperpolarizing depending on the charge of the ions and the direction in which they flow. The most studied form of electrical activity is that generated by the flow of sodium ions to generate an action potential. The role of this type of activity in synapse formation and neuronal survival is also well established (see Mennerick and Zorumski, 2000; Zito and Svoboda, 2002, for reviews). In addition, it is now recognized that patterned activity in the cortex and retina is important for proper

establishment of maps, and refinement of synaptic connections (Roerig and Feller, 2000).

Synchronized electrical activity that is sodium-channel dependent and driven by synaptic input has been reported in many taxa and across different regions of the developing nervous system (see Feller, 1999; Spitzer, 2006 for reviews). Examples include electrical activity in the embryonic and neonatal mouse and embryonic chick spinal cord (Hanson and Landmesser, 2003; O'Donovan et al., 2005), correlated wave activity in embryonic rodent brainstem (Momose-Sato et al., 1999), cortex and hippocampus (Ben-Ari et al., 1989; Garaschuk et al, 2000; Corlew et al., 2004), and retinal waves in rodents (Roerig and Feller, 2000; Stellwagen and Shatz, 2002; Torborg et al., 2005). This form of electrical activity is displayed and propagated across large groups of cells that fire bursts of action potentials in a highly correlated fashion with periodic rhythmicity. Calcium entry is elicited by the action potentials, generating rhythmic increases in intracellular calcium that, given its role as second messenger, could play a role in the effects exerted by this form of electrical activity by regulating gene transcription (West et al, 2001; Flavell and Greenberg, 2008). Retinal waves recruit the cAMP/PKA pathway in a calcium-dependent manner (Dunn et al., 2006). In the case of retinogeniculate

refinement, the CRE/CREB pathway has been implicated in the segregation of retinal cell ganglion axons (Pham et al., 2001).

Other forms of electrical activity are mediated by different ions and channels. Calcium-dependent electrical activity is important in proliferation, migration, and differentiation (see Spitzer, 2006 for review), an early example of which is its role in neurotransmitter choice in sympathetic neurons (Walicke et al., 1977; Walicke and Patterson, 1981). Calcium transients are now recognized as another prevalent form of activity during early stages of development of vertebrates and invertebrates. In *Xenopus laevis*, different types of calcium transients have been described, each with different functions in neuronal development. Somatic calcium spikes and waves in spinal neurons have a role in neurotransmitter maturation and neurite extension, respectively (Gu et al., 1994; Gu and Spitzer, 1995). Growth cone transients regulate axon extension and pathfinding (Gomez and Spitzer, 1999), and filipodial calcium transients mediate growth-cone turning (Gomez et al., 2001). Local calcium transients distributed throughout the lamellipodia modulate de-adhesion from the substrate (Conklin et al., 2005). The interaction between extracellular matrix molecules like laminin beta2 and calcium channels has been shown to play a role neurite outgrowth (Sann et al., 2008).

Calcium transients have also been reported in many other nervous systems, including developing zebrafish spinal cord (Ashworth and Bolsover, 2002), chick dorsal root ganglion neurons (Gomez et al., 1995), precursor cells in embryonic rat cortex (Owens and Kriegstein, 1998), mushroom body neurons in fly pupae (Jiang et al., 2005), and in moth pupal antennal-lobe neurons (Mercer and Hildebrand, 2002).

1.4 Calcium signaling in neurotransmitter specification in *Xenopus laevis*

An early form of calcium-dependent spontaneous electrical activity has been studied in spinal cord neurons of *Xenopus laevis* embryos. This calcium spike activity is important for neurotransmitter specification and is present during a specific window in development before synapses are formed and through the beginning of synapse formation (Borodinsky et al., 2004). This activity consists of transient elevations of intracellular calcium, generated by extracellular calcium entry through voltage-gated calcium channels and an intracellular component of calcium released from calcium stores (CICR) (O'Dowd et al., 1988; Holliday et al., 1991; Lockery and Spitzer, 1992; Gu and Spitzer, 1993; Gu et al., 1994; Gu and Spitzer, 1995). The frequency of these transients is low, with only ten or fewer per hour, and they are present both *in vivo* and in neurons dissociated and

cultured *in vitro*. Borodinsky et al. (2004) characterized normal spontaneous activity of neurons in the dorsal and ventral sides of the spinal cord and identified differences in the frequency of calcium transients among classes of neurons that express different neurotransmitters. They hypothesized that this calcium activity was important for neurotransmitter specification and showed this is the case by manipulating this activity and determining the effect on neurotransmitter expression. Manipulations were carried out either pharmacologically or by over-expression of mRNA encoding depolarizing or hyperpolarizing channels. In both cases the authors observed an increase in the number of neurons expressing excitatory neurotransmitters when activity was reduced, and an increase in the number of neurons expressing inhibitory neurotransmitters when activity was increased, seemingly following a homeostatic paradigm. Homeostasis has been demonstrated during later developmental periods for synaptic plasticity, when synapses are altered to compensate for chronic alterations of activity (Belousov et al., 2001; Turrigiano and Nelson, 2004).

More recent work has shown that calcium spike activity is also present in the developing diencephalon and rhombencephalon of *Xenopus laevis* (Dulcis and Spitzer, 2008; Demarque and Spitzer, personal communication). However, the mechanisms generating this spontaneous

electrical activity were still unknown, and it was not known if calcium-spike activity had a more widespread role in neurotransmitter specification.

1.5 Questions addressed in this thesis

This thesis examines the mechanisms that trigger calcium-spike activity in the spinal cord and the role of this type of activity in specification of dopamine in various nuclei of the nervous system.

Many forms of electrical activity are termed “spontaneous”. Identifying the triggers for these forms of activity is important to begin to understand the molecular mechanisms recruited during development. We investigated the role of early-expressed neurotransmitters on calcium-spike activity in developing spinal neurons in *Xenopus.* The results of this work are reported in Chapter 2.

The dopaminergic system is a useful model with which to determine the relevance of calcium-dependent neurotransmitter specification during the development of heterogeneous populations that express a common neurotransmitter: dopamine. Hints to the diversity among neurons in a midbrain dopaminergic nucleus are provided by Korotkova et al. (2004), who describe the divergent electrophysiology and different susceptibility to neurodegeneration and regulation by neuropeptides of neuronal subpopulations within the midbrain.

The dopaminergic system is complex, being composed of nuclei that are located in different regions of the nervous system, and provides an opportunity to study the development of co-expression with other neurotransmitters and transcription factors. Knowledge of the development of the dopaminergic system and the different types of dopaminergic cells could help bridge the gap between treatments aimed at replacing specific dopaminergic neurons that are affected in neurological diseases, such as Parkinson's disease, or other conditions in which the dopaminergic system malfunctions, such as drug addiction, schizophrenia, Tourette's syndrome, and bipolar disorder (Hirsch et al., 1988; Kienast and Heinz, 2006; Miklowitz and Johnson, 2006; Meisenzahl et al., 2007).

We investigated the development of the dopaminergic system in *Xenopus laevis,* identifying co-expression with other neurotransmitters and transcription factors, and testing the role of calcium-spike activity in specification of dopamine in the different nuclei of this system. The results of this work are presented in Chapter 3.

In the final chapter I synthesize the findings from my research and identify key future directions for work on these problems.

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2 Mechanism for calcium spike activity in *Xenopus laevis*

2.1 Abstract

Neurotransmitter signaling in the mature nervous system is well understood, but the functions of transmitters in the immature nervous system are less clear. Although transmitters released during embryogenesis regulate neuronal proliferation and migration, little is known about their role in regulating early neuronal differentiation. Here, we show that GABA and glutamate drive calcium-dependent embryonic electrical activity that regulates transmitter specification. The number of neurons expressing different transmitters changes when GABA or glutamate signaling is blocked chronically, either using morpholinos to knock down transmitter-synthetic enzymes or applying pharmacological receptor antagonists during a sensitive period of development. We find that calcium spikes are triggered by metabotropic GABA and glutamate receptors, which engage protein kinases A and C. The results reveal a novel role for embryonically expressed neurotransmitters.

2.2 Introduction

Assembly of the nervous system is a complex biological process that involves three distinct but overlapping stages of proliferation, migration, and differentiation. These phases of development determine much of its basic structure and function by producing the appropriate numbers of neurons, moving them into the correct positions, and endowing them with the machinery to establish proper connections and neuronal circuits underlying behavior. The generative roles of these early aspects of development have contributed to the interest in understanding the mechanisms involved.

Differentiation of the nervous system is achieved by a partnership between selective gene expression, gradients of signaling molecules, and electrical activity-driven mechanisms. Dependence on electrical activity used to be viewed as reliance on sodium-dependent action potentials with susceptibility to tetrodotoxin, which can appear late in development. It is now recognized to include other voltage-dependent channels, ligand-activated channels, and transient receptor potential (TRP) channels discovered more recently. In addition, many structural and functional components used in the mature nervous system have been discovered to have different functions during its construction. Mechanoreceptors and

transmitter transporters acting in reverse generate electrical signals that can play roles in neuronal development. Thus, activity is now recognized to exist in multiple forms, many of which are present early in embryogenesis (Spitzer, 2006).

Neurotransmitters are expressed at early stages of development, long before synapse formation (Lauder et al., 1986; Ma et al., 1992; Antal et al., 1994; Berki et al., 1995), and their release can stimulate elevations of intracellular calcium (Blanton et al., 1990; Flint et al., 1999). They regulate proliferation in rat embryonic neocortical progenitor cells and subventricular zone neuroblasts, and zebrafish spinal interneurons (LoTurco et al., 1995; Liu et al., 2005; McDearmid et al., 2006). Embryonic transmitters also regulate the migration of cerebellar granule cells and neuronal precursors in the mouse postnatal subventricular zone (Komuro and Rakic, 1996; Yacubova and Komuro, 2002; Bolteus and Bordey, 2004). However, their roles in nervous system differentiation beyond proliferation and migration have been elusive (Spitzer, 2006). We, therefore, tested the hypothesis that embryonic transmitter signaling triggers machinery initiating activity-dependent differentiation.

We find that GABA and glutamate (Glu) are promiscuously expressed in the neural plate of developing *Xenopus* embryos.

Suppressing synthesis of GABA or blocking GABA or glutamate receptors (GABARs or GluRs) causes an increase in the number of neurons expressing excitatory transmitters and a decrease in the number of neurons expressing inhibitory transmitters, which appear to be independent of cell proliferation or cell death. GABA and glutamate stimulate generation of calcium spikes that are an early form of excitability in these neurons, via metabotropic GABAB receptors and group III metabotropic GluRs (mGlu-RIIIs) that activate protein kinases A (PKA) and C (PKC). The role of this early electrical activity in transmitter specification is restricted to a brief period during which synapse formation takes place and swimming behavior begins. We propose a model in which the release of neurotransmitters at early stages of development has a function distinct from that associated with synaptic transmission, driving what was previously termed spontaneous activity that leads to gene expression necessary for appropriate transmitter specification. Maintaining the incidence of calcium spiking enables frequency-dependent control of the specification of different transmitters (Borodinsky et al., 2004).

2.3 Materials and methods

Immunocytochemistry. Embryos and larvae were fixed in 4% paraformaldehyde, with or without 0.025 to 0.1% glutaraldehyde, in PBS

at pH 7.4, for 30min to 2 h at 4°C, incubated in sucrose for 2.5 h, and embedded in OCT. Cryostat sections 10 μm in thickness were made starting 400 μm posterior to the back of the eyes over a region of 400 μm. Sections were incubated in a blocking solution of 2% goat serum,1% fish gelatin, or 2% goat serum/2% BSA for 0.5 h at 20°C, followed by overnight or 3 d incubation with primary antibodies to glycine (Gly), GABA, choline acetyltransferase (ChAT), lim-3 (Millipore Bioscience Research Reagents, Temecula, CA), vesicular glutamate transporter 1 (VGluT1; Millipore Bioscience Research Reagents or Sigma-Aldrich, St. Louis, MO), glutamate, or human natural killer-1 (HNK-1) (Sigma-Aldrich) at 4°C and 2 h incubation with fluorescently tagged secondary antibodies at 20°C. Triple stains were incubated at 4°C for 3 d. Sections were mounted in Vectashield mounting medium with or without 4',6'-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) to track cell numbers. Labeling was examined on an Axioskop with 40X air or water immersion objectives and the appropriate excitation and emission filters for Alexa 350, Alexa 488, Alexa 594, and DAPI.

Morpholinos. Transcription-blocking morpholinos (MOs; GeneTools, Philomath, OR) were designed to the start sequences of *X. laevis glutamic acid decarboxylase 65* (*GAD65*) and *GAD67* genes. The *GAD65*

sequence was identified by PCR using primers from the *X. tropicalis* genome and *GAD67 X. laevis* sequence was taken from GenBank (accession number U38225). Respective morpholino sequences were AAAACCCCGAGCCAGGAGACGCCAT and GCAGGGTGTTCTCTTTCCTAAGCAT. Saline solution (7–10 nl) containing 300 pg/nl of each morpholino and 30 ng/nl fluorescent dextran tracer (10,000 molecular weight) were injected into both blastomeres at the two-cell stage. The control morpholino was CCTCTTACCTCAGTTACAATTTATA, a standard sequence from GeneTools that did not recognize any sequence in GenBank.

Pharmacology. Stock concentrations of drugs were 50 or 25 mM (*RS*)-α-methylserine-*O*-phosphate (MSOP), 50 mM (*S*)-α-methyl-4-carboxyphenylglycine (MCPG), 5 mM D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5), 100 mM phaclofen (Tocris Bioscience, Ellisville, MO), 1mM (-)-bicuculline methobromide, 100 μM GVIa ω-conotoxin, 100 μM flunarizine, 100 μg/ml tetrodotoxin, 10 mM veratridine, 1 μM PMA, 1 M tetraethylammonium (TEA; Sigma-Aldrich), 1 μM N6-benzoyladenosine-3',5'-cyclic monophosphate sodium salt (N6-benzoyl-cAMP; Axxora, San Diego, CA), 2 μM calcicludine, and 1 μM KT5720[(9*S*,10*S*,12*R*)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-

oxo-9,12-epoxy-1*H*-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4l][1,6]benzo-diazocine-10-carboxylic acid hexyl ester; Calbiochem, San Diego, CA],dissolved in 2 mM calcium saline. 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7 sulfonamide (NBQX; 50 mM; Tocris Bioscience) and 10 μM bisindolylmaleimide (Sigma-Aldrich) were dissolved in DMSO. These stock solutions were diluted in saline 100X when acutely applied to preparations for calcium imaging and 10X when loading agarose beads. Fluorescently tagged ω-conotoxin was purchased from Hans-Günther Knaus (Innsbruck Medical University, Innsbruck, Austria). To alter spike activity *in vivo*, an 80 μm agarose bead (Bio-Rad, Hercules, CA) loaded with drugs or bovine serum albumin (control) was inserted between the neural tube and myotomes ~400 μm behind the eye primordium at 20 h of development (stage 18) and immunostaining was performed 2 d later (Borodinsky et al., 2004). Sections of bead implanted embryos were collected anterior, through, and posterior to the bead.

Imaging. Neural tubes of stages 25–28 *X. laevis* embryos were dissected to expose the dorsal or ventral surface and loaded for 45–60 min with 5μM Fluo-4 AM/0.01% pluronic acid/1% DMSO in 2 mM calcium saline. Images were acquired with Bio-Rad laser confocal systems at 0.2 Hz for periods of 30 min or 1 h. The baseline incidence of calcium spiking

was determined for the dorsal and ventral neural tube; values lying outside the normal distribution (incidence >2 SD from the mean; ventral, 21 ± 7%; dorsal, 15 ± 7%) were excluded from further consideration. Antagonists were then applied directly to the bathing solution and images were acquired for another 30 min. In pharmacological rescue experiments, kinase agonists were applied 15 min after receptor antagonists. Movies were analyzed with Image J and NIH Image.

Statistics. Significance was assessed with Student's two-tailed *t* test and values were considered different when $p < 0.05$.

2.4 Results

2.4.1 Neurotransmitters are promiscuously expressed at early stages

To evaluate the extent of early expression of neurotransmitters in the spinal cord, we examined four transmitters during *Xenopus* embryonic development. At neural plate and early neural tube stages (stages 15 and 20), GABA and glutamate expression assayed by immunoreactivity (IR) to GABA (GABA-IR), glutamate (Glu-IR), and the vesicular glutamate transporter (VGluT1-IR) are observed in a large number of cells and often coexpressed (Fig. 2.1*A*,*E*). To investigate cell-type expression of these

transmitters, we determined whether the presence of GABA and glutamate is consonant with that observed at later stages of development. Fully differentiated motoneurons (MNs) and Rohon-Beard sensory neurons (RBs) are identified by expression of molecular markers lim-3 and HNK-1 and are cholinergic and glutamatergic, respectively. Lim-3-IR is first detected at stage 14 (Taira et al., 1993) and HNK-1-IR is first detected at stage 20 (Nordlander, 1993). The numbers of neurons expressing these markers does not change significantly from the neural tube stage to the 3 d larva (stages 20–41; HNK-1-IR, 14 _ 2 to 10 _ 1 neurons per 100 _m of spinal cord; lim-3-IR, 21 _ 1 to 26 _ 2; *p*_0.05). Fifty percent of HNK-1- and lim-3-expressing neurons are both GABA and Glu immunoreactive at early stages (Fig. 2.1*B*–*D*). Glu-IR becomes restricted to RBs and GABA-IR to GABAergic (lim-3 and HNK-1 negative) interneurons by 3 d of development.

Because the developmental appearance of neurotransmitters in *Xenopus* is quite rapid, occurring within hours, the time resolution of our experiments does not reveal whether the appearance of GABA precedes the appearance of glutamate (Ben-Ari et al., 2007; Deng et al., 2007). However, both transmitters are expressed at the neural plate stage, preceding axon outgrowth and synapse formation. This question could be answered by live imaging of fluorescent reporter transgenes at a higher

sampling rate, combined with immunostaining of transmitters, transmitter receptors, and molecular markers for different classes of interneurons.

Gly- and ChAT-immunoreactive neurons are first detected later in tailbud stage embryos (stages 25 and 30) (Roberts et al., 1988; Lopez et al., 2002), at which time they are appropriately expressed in glycinergic interneurons (lim-3 and HNK-1 negative) and lim-3-immunoreactive MNs (Fig. 2.1*C*,*D*,*F*). Neither neurogenesis nor cell death can account for differences in the number of immunoreactive neurons per section between stages (Hensey and Gautier, 1998; Schlosser et al., 2002). Thus, the initial expression of GABA and glutamate, but not ChAT and glycine, is promiscuous (Fig. 2.1*E*). The normal later pattern of transmitters in the spinal cord involves respecification of GABA and glutamate expression and de novo specification of acetylcholine and glycine expression (Fig. 2.1*F*).

2.4.2 GAD knockdown alters neurotransmitter expression

The early transient expression of GABA and glutamate led us to test whether these neurotransmitters play a role in subsequent transmitter specification in embryonic spinal neurons. We first injected MOs to knock down expression of GAD, the GABA synthetic enzyme. The effectiveness of MOs for *xGAD67* and *xGAD65* injected into both blastomeres at the

two-cell stage was confirmed by reduction in the incidence of GABA-IR. Residual expression of GABA may be caused by differential knockdown of GAD translation resulting from inconsistent distribution of the morpholinos at subsequent cell divisions. When GAD is knocked down, the incidence of both GABA- and Gly-immunoreactive neurons is decreased in 3 d larvae. In contrast, the incidence of Glu- and ChAT immunoreactive neurons is increased (Fig. 2.2*A*,*C*). DAPI staining of nuclei indicates that the number of cells in the spinal cord does not differ between *GAD* MO, control MO, and control embryos both at the early tailbud stage and at 3 d larvae, suggesting that the changes in transmitter expression are not caused by cell proliferation or death (Fig. 2.8).

2.4.3 GABA and glutamate receptor antagonists alter neurotransmitter expression

To test further the role of early transmitter signaling in subsequent transmitter expression, we implanted pharmacologically loaded agarose beads adjacent to the neural tube to block transmitter receptors chronically. This approach allowed us to restrict the perturbation to specific stages of development and to assay the role of early glutamate signaling as well as signaling by GABA. Beads were loaded with a combination of GABA receptor (GABAR) ionotropic (bicuculline) and

metabotropic (phaclofen) antagonists, or a combination of GluR ionotropic (D-AP5, NBQX) and metabotropic (MSOP, group III, and MCPG, group I and II) antagonists. Beads were implanted at the time of neural tube closure and larvae were fixed at 3 d of age to examine transmitter expression. When beads are implanted with GABAR antagonists, the incidence of Glu- and ChAT-IR is increased and the incidence of GABA- and Gly-IR is decreased, mirroring the effect of GAD knockdown on transmitter specification. Strikingly, when beads are implanted with GluR antagonists, the incidence of Glu- and ChAT-IR is again increased and the incidence of GABA- and Gly-IR is decreased (Fig. 2.2*B*,*C*). DAPI staining shows that the number of cells in the spinal cord does not change (Fig. 2.8) and implanting control beads loaded with bovine serum albumin has no effect on transmitter expression.

2.4.4 GABA and glutamate regulate the incidence of calcium spiking

To determine whether calcium spikes are driven by GABA and glutamate, we imaged spike activity on the dorsal and ventral surfaces of the neural tube at tailbud stages (stages 25–28) when activity is highest (Borodinsky et al., 2004), both in *GAD* MO-injected embryos as well as before and after acute application of transmitter receptor antagonists. In *GAD* MO-injected embryos, the incidence of calcium spike activity is

reduced on both the dorsal and ventral surfaces (Fig. 2.3*A*,*B*) and frequency is reduced only ventrally (Fig. 2.9*A*). Injections of control MO have no effect on calcium spike incidence or frequency. Applying combinations of antagonists for GABA receptors (bicuculline and phaclofen) or glutamate receptors (D-AP5, NBQX, MSOP, and MCPG), we observed decreases in the incidence, but not frequency of calcium spikes, on the dorsal and ventral neural tube (Fig. 2.3*A*,*C* and Fig. 2.9*B*).

2.4.5 GABA and glutamate act primarily via GABAB and mGluRIII receptors

To assess the contributions of specific ionotropic and metabotropic receptors, we tested individual GABAR and GluR antagonists. On the dorsal surface, the incidence of calcium spikes is reduced in the presence of MSOP, phaclofen, and bicuculline and NBQX, implicating mGluRIII, GABABR, GABAAR and AMPA receptor (AMPAR). Ventrally, only MSOP and phaclofen reduce spike incidence, implicating mGluRIII and GABABR (Fig. 2.3*D* and Fig. 2.9*C*). Thus, GABAergic and glutamatergic modulation of calcium spikes is mediated largely by metabotropic receptors. Spikes are triggered by calcium-dependent action potentials that are terminated by activation of potassium channels. Accordingly, these results are consistent with a more depolarized resting potential that reduces the

distance to threshold, enhancement of voltage-gated calcium channel activity, or postinhibition rebound excitation after potassium channel activation (Takeshita et al., 1996; Kim et al., 1997; Luthi et al., 1997; Linn and Gafka, 1999; Shen and Slaughter, 1999; Mathie, 2007). GABAAR and AMPAR may contribute to spike incidence on the dorsal but not ventral surface because RBs are the only ones depolarized by GABA throughout development (Bixby and Spitzer, 1982) and dorsal interneurons express calcium-permeable AMPAR (Rohrbough and Spitzer, 1999).

The previous demonstration of activity-dependent transmitter specification by the frequency of calcium spiking (Borodinsky et al., 2004) was said to be homeostatic based on consideration of the effects of transmitters on ionotropic receptor activation. In contrast, the results reported here reveal that activation of metabotropic receptors regulates the incidence of calcium spiking that enables generation of different frequencies of spiking. Thus, embryonic GABA and glutamate appear to drive the activity that causes activity-dependent homeostatic transmitter specification.

Because GABA and glutamate generate calcium spikes mainly through activation of metabotropic receptors, we tested whether blockade of either GABABR or mGluRIII alone caused changes in transmitter

expression similar to those observed with antagonist combinations (compare Fig. 2.2). Beads were loaded with phaclofen or MSOP and implanted at the time of neural tube closure; larvae were fixed at 3 d of age to examine transmitter expression. Indeed, when beads were implanted with either drug by itself, changes in neurotransmitter expression were similar to those after application of GABAR or GluR antagonist cocktails (Fig. 2.4). Blockade of either phaclofen or MSOP increases the number of neurons expressing excitatory transmitters and decreases the number of neurons expressing inhibitory transmitters, consistent with a homeostatic response to suppressed calcium spiking.

2.4.6 GABAB and mGluRIII activate PKA and PKC

To identify steps in signal transduction downstream of GABABR and mGluRIII, we imaged calcium spikes in the presence of antagonists of PKA or PKC, because these kinases are targets of GABABR and mGluRIII (Olianas and Onali, 1999; Shen and Slaughter, 1999; Catsicas and Mobbs, 2001; Evans et al., 2001; Couve et al., 2002; Martin et al., 2004) and modulate the properties of calcium and potassium channels (Taylor et al., 2000; Park et al., 2003; Zhang et al., 2003; Hoogland and Saggau, 2004; Mathie, 2007). PKC and PKA could inhibit tandem pore domain potassium channels that set the membrane potential or engage

low voltage- and high-voltage-activated calcium currents and potassium currents involved in generating calcium spikes in these neurons (Gu and Spitzer, 1993). Blocking PKA with KT5720 decreases calcium spiking (Gorbunova and Spitzer,2002); spike incidence is reduced on the dorsal surface, with no effect on spike generation on the ventral surface. Blocking PKC with bisindolylmaleimide reduces spike incidence both dorsally and ventrally (Fig. 2.5). No changes in spike frequency are detected (Fig. 2.9*D*). To determine whether the kinases are downstream of GABAB and mGluRIII, we tested whether stimulation with agonists of these kinases bypasses the effect of receptor antagonists and rescues calcium spiking. Indeed, decreases in spike incidence in the presence of either MSOP or phaclofen are prevented by N6-benzoyl-cAMP to activate PKA on the dorsal surface of the neural tube and by PMA to stimulate PKC on either the dorsal or ventral surface of the neural tube (Fig. 2.5). Although we do not know whether the actions of kinase antagonists and agonists are cell autonomous, these results suggest that PKA and PKC mediate the effects of activation of GABABR and mGluRIII on calcium spiking.

2.4.7 Neurotransmitter specification occurs during a restricted period

We tested the presence of a sensitive period for GABA- and Glu-mediated transmitter specification by implanting beads loaded with GABAR and GluR antagonists to suppress calcium spikes at different stages of development. Altering GABAergic and glutamatergic signaling leads to an increase in the numbers of neurons expressing glutamate and a decrease in the number of neurons expressing GABA during an interval of ~15 h (Fig. 2.6). To correlate the sensitive period for GABA and glutamate regulation of transmitter expression with that of calcium spikes, we then determined the sensitive period for calcium spike dependent regulation of transmitter expression. We suppressed or enhanced spike activity by local delivery of voltage gated channel antagonists or veratridine, a sodium channel agonist (Borodinsky et al., 2004), and identified a similar interval for activity-dependent regulation of transmitter expression (Fig. 2.6 and Fig. 2.10). The stages during which calcium spikes are generated spontaneously are extended by application of TEA to block voltage-gated potassium channels, but local delivery of TEA plus veratridine does not increase the duration of the sensitive period (Fig. 2.10*C*–*E*). These results imply that GABA and glutamate regulate

transmitter expression via calcium spikes during a restricted period, but that calcium spikes are not sufficient to do so on their own and require a specific developmental context (Fig. 2.7).

2.5 Discussion

The widespread embryonic expression of GABA and glutamate suggested that these neurotransmitters could have significant functions (Lauder et al., 1986; Ma et al., 1992; Antal et al., 1994; Berki et al., 1995). Our findings demonstrate that GABA and glutamate signaling drive the generation of calcium spikes that in turn specify transmitter expression. These actions are mediated largely by metabotropic receptors that activate several kinases, as indicated by the actions of pharmacological antagonists suppressing spike generation in imaging experiments. Interestingly, this signaling regulates the incidence, but not the frequency, of calcium spikes. GABA and glutamate appear to open an activity gate that allows downstream mechanisms to generate the cell-type-specific frequencies of spikes reported previously (Borodinsky et al., 2004). The incidence of calcium spiking is permissive for spike frequency-dependent transmitter specification. Thus, when the incidence is reduced, spike frequency is zero in a population of neurons, leading to an increase in the

incidence of expression of excitatory transmitters and a decrease in the incidence of expression of inhibitory transmitters.

We hypothesize that the activation of PKA and PKC leads to phosphorylation of ion channels that changes the amplitude of their current or shifts their conductance–voltage relationship. Likely candidates include tandem pore domain potassium channels (e.g., TASK [two-pore domain weak inwardly rectifying K^+ channel (TWIK)-related acid-sensitive K^+ channel] or TREK [TWIK-related K^+ channel]) that regulate the resting potential, setting the distance from threshold and thereby the incidence of firing. The low-voltage-activated calcium channels and one or more classes of voltage-activated potassium channels are also candidates. The results of changes in channel properties could be to increase the excitability of the neurons and, thus, the incidence of calcium spikes. The frequency of firing would then depend on the constellations of ion channels expressed in different classes of neurons.

Blockade of GABA or glutamate receptors only suppresses spikes in half of the neurons on the dorsal and ventral surfaces of the neural tube, indicating that some cells generate spikes in response to GABA and glutamate whereas others do not. Interestingly, the effect on neurotransmitter expression of blocking spikes partially with *GAD* MOs or

with GABA receptor or glutamate receptor antagonists is similar to the result of suppressing spikes completely, suggesting that GABA and glutamate responsive neurons are the plastic population capable of altered transmitter expression. These findings raise two questions: first, what drives spike activity in the population of neurons that is not affected by GABA or glutamate receptor antagonists? Amine and peptide neurotransmitters are also expressed at early stages of development (Lauder et al., 1981; Hu et al., 2001; McLean and Fetcho, 2004) and are candidates to evaluate. Second, what is the function of calcium spike activity in these neurons if it does not regulate the expression of GABA, glutamate, glycine or acetylcholine? On the one hand, spike activity may regulate expression of peptide and amine transmitters, following the paradigm we report here. Alternatively, spike activity may regulate other aspects of neuronal differentiation, such as axon extension or ion channel expression.

Our results show that metabotropic receptors activate PKA and PKC signaling pathways in the embryonic *Xenopus* spinal cord, which stimulate generation of calcium spikes. These results stand in contrast with observations in the mature nervous system, reporting activation of potassium currents and suppression of calcium currents by GABAB and mGluR receptors (Isaacson, 1998; Nicoll, 2004; Giustizieri et al., 2005).

One possibility is that the G-proteins are coupled to excitatory machinery at early stages of development and to inhibitory components in the mature nervous system. Evolution is likely to have been opportunistic in mixing and matching receptors with ion channels for specific contexts in particular classes of neurons. Consistent with this view, dorsal neurons also make use of GABAA and AMPA receptors as well as PKA signaling to generate spikes, whereas ventral neurons do not. These findings indicate that different populations of neurons have distinct repertoires of signaling pathways responsible for producing activity.

We identify a sensitive period *in vivo* during which transmitter expression is altered by modulating activity. Perturbation of activity at later times is ineffective, identifying its importance during this time window. Whether changes in transmitter specification achieved in this way can be reversed is still unknown; thus, we do not know if it is a critical period as well. This period is different from most of the others described in development of the nervous system (Hensch, 2004; Knudsen, 2004) in that it precedes synapse formation. Paracrine stimulation by GABA and glutamate presumably joins with intracellular signaling by kinases, transcription factors, and other components to enable transmitter plasticity; changes in the expression of this machinery close the window of opportunity. It will be useful to analyze activity-dependent gene expression

during this sensitive period. An understanding of the molecular mechanisms involved in activity-dependent transmitter specification may have therapeutic implications for treating disorders of transmitter metabolism in the mature nervous system. Spontaneous electrical activity is a transient feature of many developing nervous systems (Spitzer, 2006) but its functions have been obscure in many cases. Our results suggest a model in which neurotransmitters synthesized and released early in the development of the nervous system trigger electrical activity that drives neuronal differentiation. This activity likely engages a cell-type-specific repertoire of signaling and effector molecules. Such an arrangement may be expected to enable environmental stimuli that alter spontaneous activity to influence early stages of neuronal development. The combination would provide a level of plasticity promoting appropriate and robust context-dependent maturation of the nervous system. It will be interesting to identify the environmental stimuli capable of altering transmitter specification before synaptogenesis.

2.6 Acknowledgements

Chapter 2 is a reprint of the material as it appears in “Embryonically Expressed GABA and Glutamate Drive Electrical Activity Regulating Neurotransmitter Specification” Cory M. Root, **Norma A. Velázquez-Ulloa**, Gabriela C. Monsalve, Elena Minakova, and Nicholas C. Spitzer. Journal of Neurosciences, 2008. Apr 30; 28(18):4777-84. It is included with the permission of all the authors on the final publication.

2.7 Figures

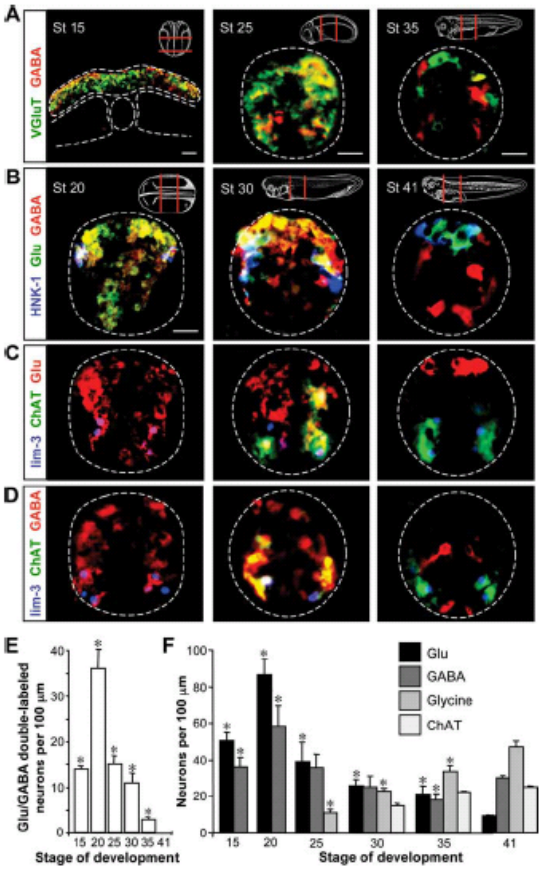

Fig. 2.1. GABA and Glu are promiscuously expressed before synapse formation. ***A***, Immunostaining for GABA and VGluT1 demonstrates expression within neural tissue at the neural plate [stage (St) 15], early tailbud (St 25), and late tailbud (St 35) stages. Insets indicate regions from which sections were taken. White dashed lines identify the margins of the neural plate, notochord, somites, and neural tube. ***B***, Triple immunostaining reveals GABA and Glu in HNK-1-immunoreactive Rohon-Beard neurons at the early neural tube (St 20) and tailbud (St 30) stages that will become glutamatergic at 3 d of development (St 41). ***C***, Triple staining for lim-3 with ChAT and glutamate shows transient expression of glutamate in motoneurons that later become cholinergic. ***D***, Triple staining for lim-3 with ChAT and GABA reveals transient expression of GABA in motoneurons. ***E***, Time course of the changes in glutamate/GABA coexpression during the development of the embryonic spinal cord. ***F***, Quantification of transmitter expression dynamics for Glu, GABA, glycine, and acetylcholine (assayed as ChAT immunoreactivity) per 100 μm of spinal cord at different stages of development. Scale bars: ***A***, ***B***, 20 μm. $n \geq 3$ embryos for ***A–F***. ***E***, ***F***, Values are mean ± SEM; asterisks indicate a significant difference from stage 41.

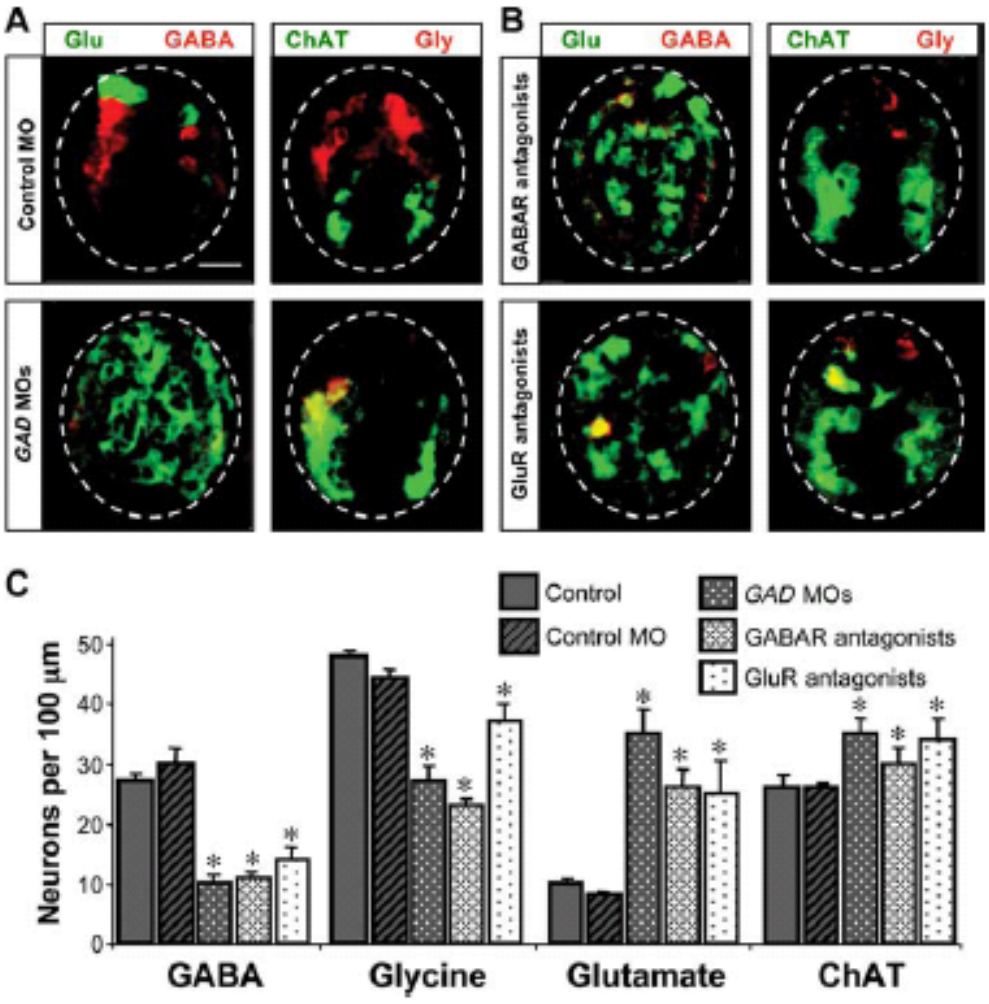

Fig. 2.2. Blocking GABAergic or glutamatergic signaling changes neurotransmitter expression. ***A***, Immunostaining for Glu/ GABA and ChAT/Gly in 3 d larvae (stage 41) developed from embryos injected with control morpholino (control MO) or *GAD* morpholinos (*GAD* MOs) at the two-cell stage. Scale bar, 20 μm. ***B***, Immunostaining is as in ***A*** of larvae from embryos implanted with beads containing antagonists of GABAR (bicuculline and phaclofen) or GluR (D-AP5, NBQX, MSOP, and MCPG). ***C***, Number of neurons expressing different transmitters at 3 d of development after suppression of GABAergic or glutamatergic signaling. $n \geq 5$ embryos for each condition. Values are mean ± SEM; asterisks indicate a significant difference from control.

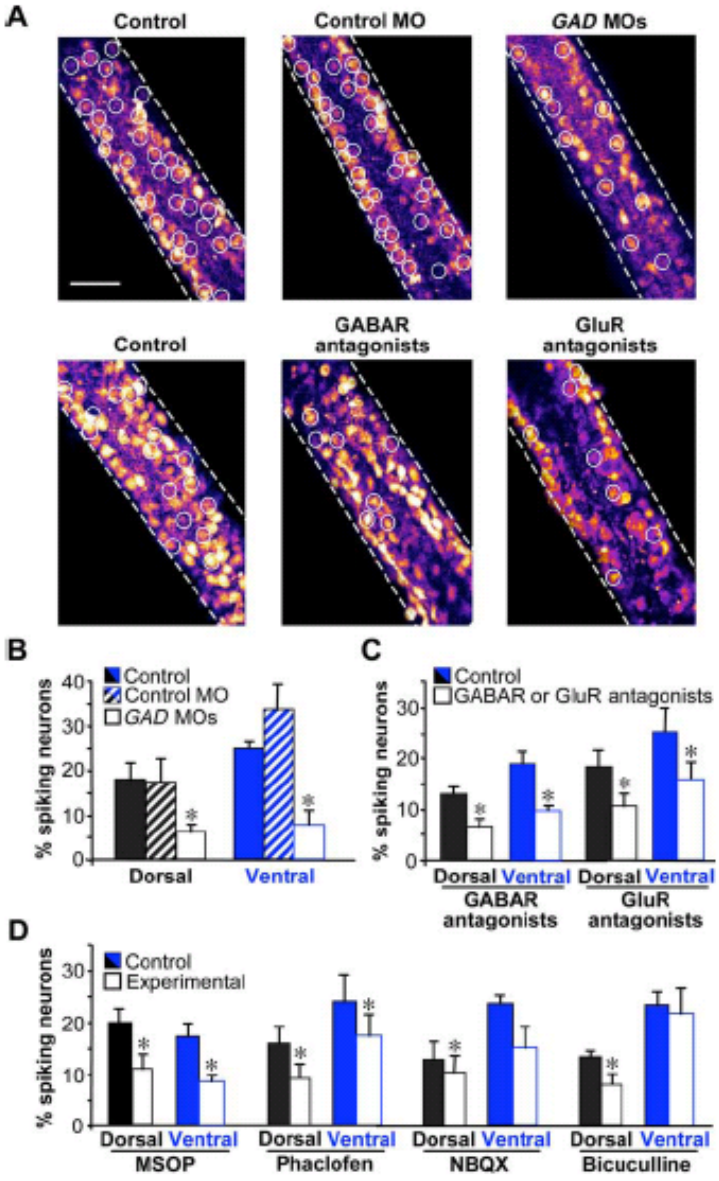

Fig. 2.3. Blocking GABAergic or glutamatergic signaling decreases the incidence of calcium spike activity. ***A***, Calcium spike incidence (white circles) on the ventral surface of the neural tube of control, control MO, and *GAD65* plus *GAD67* MO (*GAD* MO)-injected embryos or in the presence of pooled GABAR or GluR antagonists. Scale bar, 50 μm. ***B***, ***C***, Spike incidence is reduced dorsally and ventrally in *GAD65*/*67* MO-injected embryos and by pooled GABAR or GluR antagonists. ***D***, Calcium spike incidence in the presence of single receptor antagonists. $n \geq 5$ stage 25–28 embryos for each condition. Values are mean ± SEM; asterisks indicate a significant difference from control.

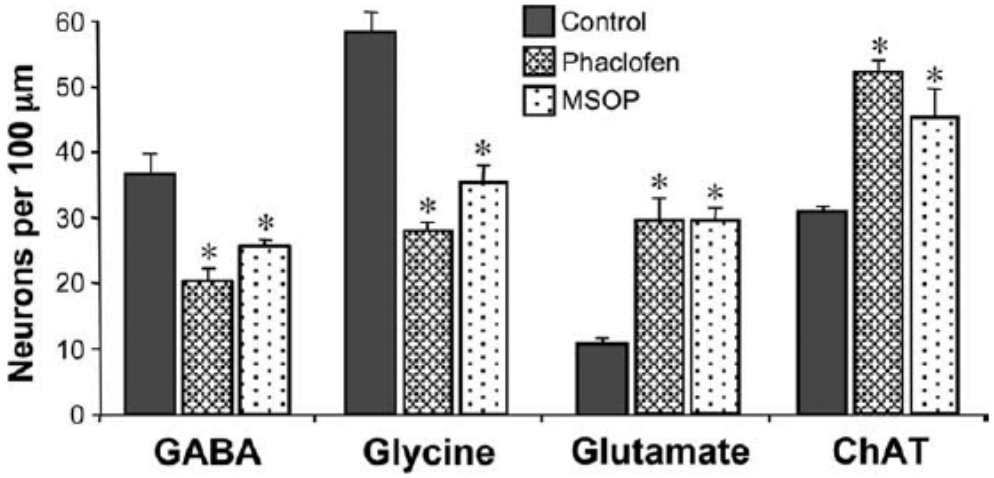

Fig. 2.4. Blocking GABAB or mGluRIII receptors changes neurotransmitter expression. Number of neurons expressing different transmitters at 3 d of development after suppression of GABAB or mGluRIII signaling. Immunostaining was performed on 3 d larvae (stage 41) developed from embryos implanted with beads containing antagonists of GABABR (phaclofen) or mGluRIII (MSOP). Control embryos were interleaved in parallel. $n \geq 4$ embryos for each condition. Values are mean ± SEM; asterisks indicate a significant difference from control.

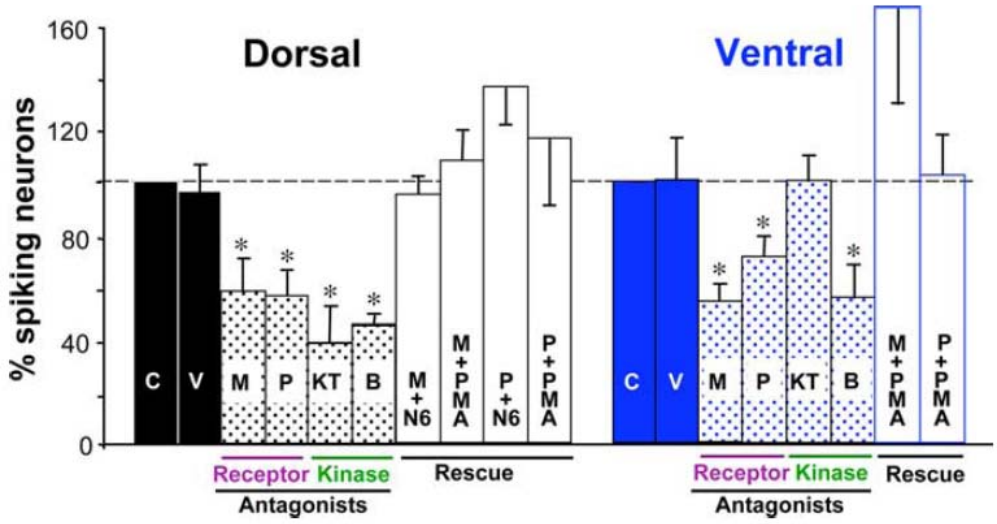

Fig. 2.5. PKA or PKC antagonists mimic the effect of metabotropic receptor antagonists on spike incidence, and the effect of receptor antagonists is reversed by kinase agonists. Values are normalized to controls for each treatment. C, Control; V, vehicle control; M, MSOP (mGluRIII antagonist); P, phaclofen (GABABR antagonist); KT, KT5720 (PKA antagonist); B, bisindolylmaleimide (PKC antagonist); N6, N6-benzoyl-cAMP (PKA agonist); PMA, phorbol myristic acid (PKC agonist). Embryos at stages 25–28 are shown. *n*≥5 embryos for each condition. Values are mean ± SEM; asterisks indicate a significant difference from control. Calcium spike activity showed no significant difference when reagent vehicle was applied alone.

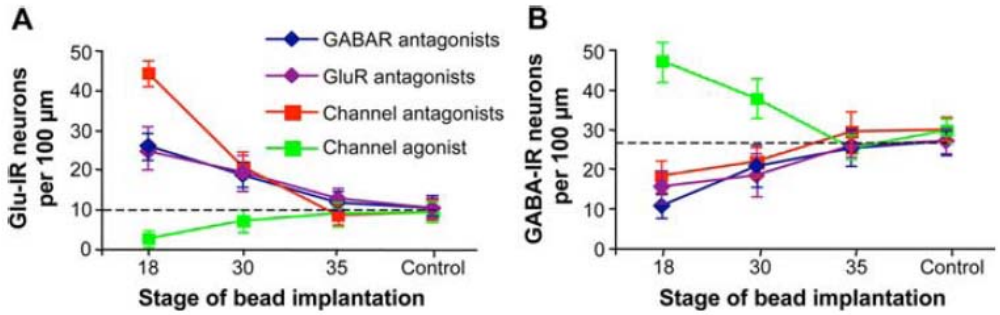

Fig. 2.6. GABA- and glutamate-driven activity-dependent transmitter expression is restricted to a sensitive period. Beads containing agents to block GluR or GABAR(as in Fig. 2.2) or to suppress or enhance voltage-gated channel activity (Borodinsky et al., 2004) were implanted at various developmental times and larvae were fixed and transmitter expression scored at 3 d of development [stage (St) 41]. Similar results were obtained after fixation of embryos at a constant interval of 2 d after bead implantation (data not shown). ***A***, GluR and GABAR antagonists increase the number of Glu-immunoreactive neurons during a period of ~15 h from St 18 to St 30; suppressing or enhancing voltage-gated channel activity increases and decreases the numbers of Glu-immunoreactive neurons during the same period. ***B***, GluR and GABAR antagonists decrease the number of GABA immunoreactive neurons during this period; suppressing or enhancing voltage-gated channel activity decreases and increases the numbers of GABA-immunoreactive neurons. Dashed lines indicate control values. Transmitter expression after bead implantation at St 18 or 30 is significantly different from control. Changes in transmitter expression after bead implants at St 30 or 35 are significantly smaller than those obtained when beads are implanted at St 18, except for GluR beads at St 30. $n \geq 5$ embryos for each condition. Values are mean ± SEM and are connected by lines according to the type of bead implanted.

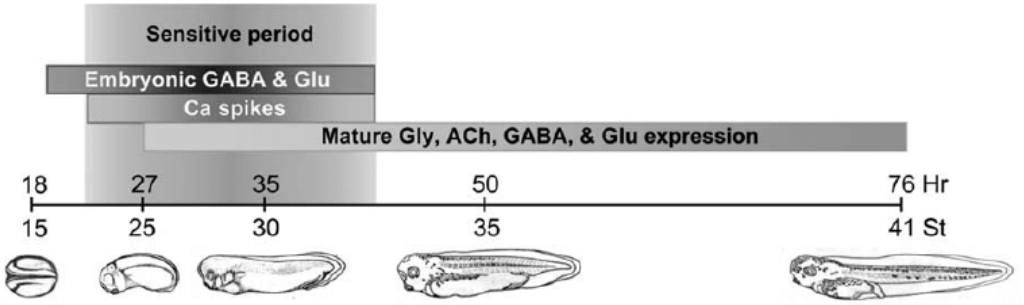

Fig. 2.7. Model for GABA/glutamate-activated Ca spike-mediated transmitter specification. GABA and glutamate are promiscuously expressed early in development. During a sensitive period, these transmitters activate receptors to trigger Ca spikes that specify transmitter expression at later stages.

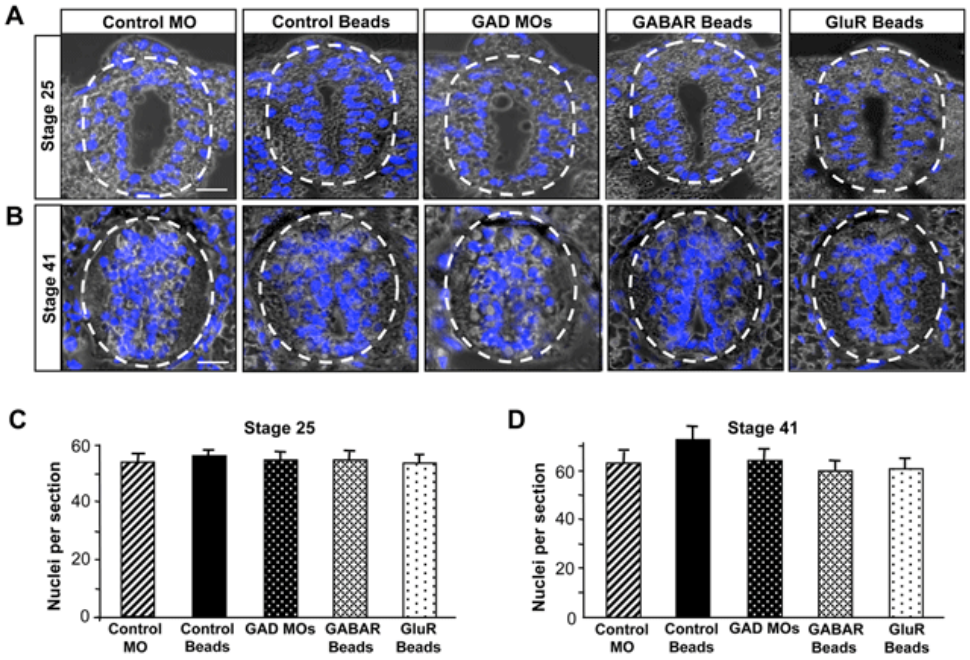

Fig. 2.8. The number of neurons per section does not change in morpholino injected or bead-implanted embryos. ***A***, ***B***, DAPI-stained nuclei (blue) from early tailbud stage (st. 25) and 3-day larvae (st. 41) are overlaid on phase contrast images. ***C***, ***D***, The number of nuclei per section from experimental embryos is not significantly different from those of controls at stage 25 or stage 41. N>9 sections from 3 or more embryos at each stage. C, D, values are mean ± SEM. Scale bars are 20 μm.

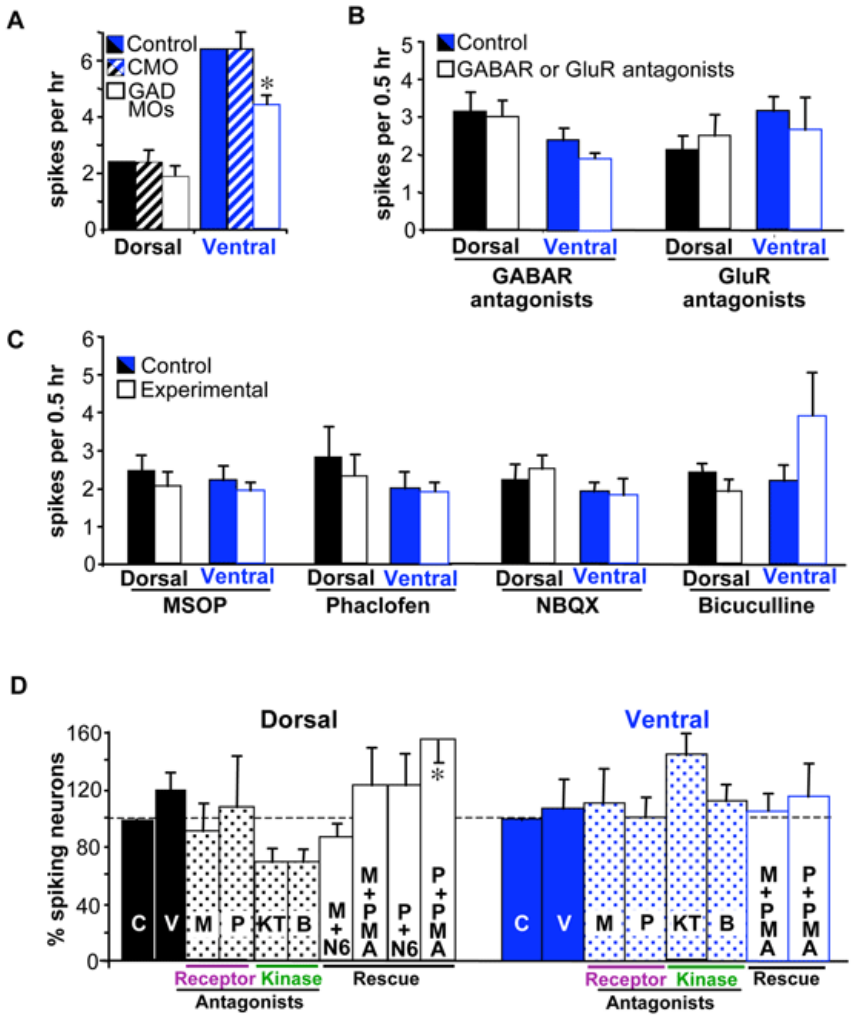

Fig. 2.9. The frequency of calcium spikes when GABAergic and glutamatergic signaling is blocked. ***A***, GAD knockdown decreases the frequency of calcium spikes on the ventral surface but not the dorsal surface of the neural tube. Neural tubes were imaged for 1 hr. ***B***, GABAR or GluR antagonists have no effect on spike frequency on both ventral and dorsal surface of the neural tube. Neural tubes were imaged for 30 min before and after addition of drugs. ***C***, Spike frequencies following treatment with individual receptor antagonists. ***D***, PKA and PKC antagonists and agonists have no effect on spike frequency, with the exception of PMA plus phaclofen. Values are normalized to controls for each treatment. C, control; V, vehicle control; M, MSOP; P, phaclofen; KT, KT5720; B, bisindolmaleimide; N6, N6-benzyl-cAMP; PMA, phorbol myristic acid. C, D, values are mean ± SEM for N>5 embryos imaged for each condition.

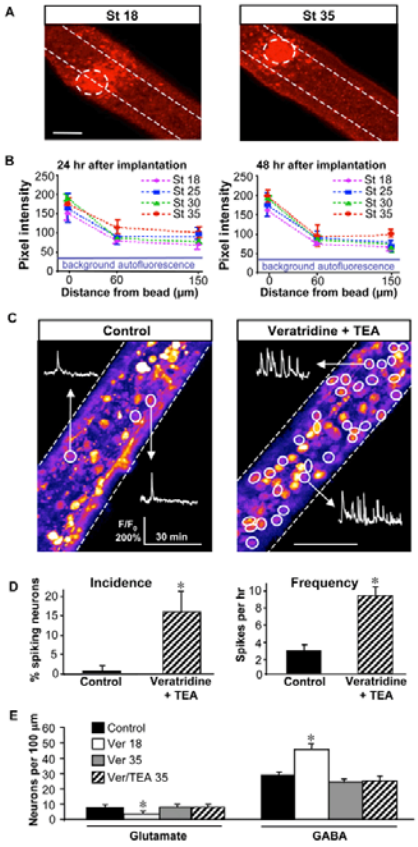

Fig. 2.10. Controls for experiments investigating the sensitive period for GABAergic and glutamatergic regulation of transmitter expression. ***A***, Dorsal coronal confocal projection images through embryos implanted with beads containing fluorescent ω-conotoxin. Beads were implanted at stages 18 and 35 and images were taken 24 hr later. Dashed lines mark 2 the margins of the neural tube and dashed circles identify dorsally positioned beads. ***B***, Fluorescence versus distance from bead at different stages of implantation; pixel intensity is not significantly different between embryos implanted with beads early rather than late in development, indicating that drug delivery is consistent across developmental stages. N>3 for A, B. ***C***, Calcium imaging of the dorsal neural tube in a stage 33 control embryo and embryo treated with TEA and veratridine. Circled cells generated spikes during the 30 min imaging session. ***D***, Addition of TEA and veratridine increased both the incidence and frequency of calcium spikes. N>4 embryos imaged for each condition. ***E***, Transmitter expression following TEA/veratridine bead implantation at stage 18 or 35. Beads implanted at stage 18 but not stage 35 produce significant changes in Glu-IR and GABA-IR neurons, demonstrating that addition of TEA to veratridine beads does not extend the sensitive period. N>5 embryos for each condition. Scale bars in A, C are 100 μm. B, D, E, values are mean±SEM. Asterisks indicate significant difference from control.

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3. Heterogeneous specification of dopaminergic phenotype: transcription factors, electrical activity and neurotransmitters

3.1 Abstract

Neurotransmitter specification is regulated by intrinsic factors such as transcription factors, by extrinsic factors, and by calcium-dependent electrical activity. We have investigated the development of dopaminergic neurons in the *Xenopus* brain and spinal cord and identified subpopulations of cells within this system. We discovered that different subpopulations co-express characteristic configurations of transcription factors along with different co-transmitters. In addition, we found that the number of dopaminergic neurons in different nuclei respond differently to manipulations of early electrical activity. Because defects in dopamine metabolism play a role in a number of neurological disorders, this knowledge is expected to help bridge the developmental complexity of the dopaminergic system with current clinical approaches.

3.2 Introduction

Calcium spike activity regulates developmental specification of classical neurotransmitters in neurons of the spinal cord (Borodinsky et al., 2004) and dopamine in neurons of the ventral suprachiasmatic nucleus

(VSC) of the hypothalamus (Dulcis and Spitzer, 2008). The dopaminergic (DA) system includes nuclei that can be distinguished by their unique localization in the brain, temporal differentiation and expression of neurotransmitter phenotype, network connectivity, and function (Smeets and Gonzalez, 2000). The DA system in *Xenopus* consists of the olfactory bulb (OB), ventral suprachiasmatic nucleus (VSC), dorsal suprachiasmatic nuclei (DLSC), posterior tuberculum nuclei (PT), and DA spinal neurons (González et al., 1993; 1994; Heathcote and Chen, 1994). We have extended these studies to understand the developmental regulation of neurotransmitter and transcription factor co-expression.

Dopamine is co-expressed with markers of other neurotransmitters including VGlut2 in rat and mouse (Dal Bo et al., 2004; Mendez et al., 2008) and GAD in rat (Hedou et al., 2000). In adult *Xenopus,* specific neuronal subpopulations within a DA nucleus co-express GABA and/or NPY (de Rijk et al., 1992; Tuinhof et al., 1994). In addition, heterogeneity is observed in the transcription factors expressed by DA neurons. The transcription factor Pax6 has been associated with the development of diencephalic and olfactory bulb DA neurons in mice (Mastick and Andrews, 2001; Kohwi et al., 2005), zebrafish (Wullimann and Rink, 2001), and *Xenopus* (Moreno et al., 2008). Transcription factors Lim1 and Lim2 are expressed in DA neurons in the suprachiasmatic region (Moreno

et al., 2004). In addition, the transcription factor Nurr1 has been linked to development of mesencephalic DA neurons (Zetterström et al., 1996; Hermanson et al., 2003; Jankovic et al., 2005). Our results show that developing DA neurons in different nuclei are heterogeneous and express specific combinations of transcription factors and neurotransmitters.

Since expression of dopamine depends on calcium spike activity in the VSC (Dulcis and Spitzer, 2008), we investigated whether calcium activity has a general role in transmitter specification in neurons of the DA system. Given its heterogeneity, we hypothesized that activity manipulations will have different effects in different nuclei. We found different responses to calcium activity manipulations suggesting that activity has distinct roles in neurotransmitter specification in different DA nuclei.

3.3 Materials and methods

Animals. Xenopus laevis embryos were generated by *in vitro* fertilization and reared at 20-22°C. Animals were staged according to Nieuwkoop and Faber (1967) for stages 27, 28, 29/30, 31, 35, 37/38, 40, 42 and 45, as described. All protocols were approved by the UCSD Animal Subjects Committee.

Immunocytochemistry. Larvae were fixed in 4% paraformaldehyde and 0.025% glutaraldehyde phosphate-buffered saline (PBS) at pH 7.4 for

1 or 2 hr at 4°C. After fixation, larvae were rinsed twice in 1x calcium-free phosphate buffered saline (1xCMF-PBS) and either cryoprotected in 30% sucrose in 1X CMF-PBS and embedded in OCT for cryostat sections or rinsed and incubated in PBS-0.5% Triton (PBT) for wholemounts. Cryostat sections 10 μm in thickness were taken following an anterior to posterior progression through the whole brain or through 600 to 800 μm of spinal cord, 300-400 μm after the eyes. For wholemounts, the brain and spinal cord of the larvae were dissected for staining after permeabilization in PBT. Sections or dissected wholemounts were permeabilized in PBT for 1 hr, and then incubated in a 1% fish gelatin in 1X CMF-PBS blocking solution for 1hr at 20-22°C. One or two night incubation at 4°C with primary antibodies to tyrosine hydroxylase (TH; Imgenex), GABA (Chemicon), neuropeptide Y (NPY; Immunostar Inc.), Pax6 (Covance), Nurr1 (Santa Cruz Biotechnology), Lim3 (Millipore Bioscience Research Reagents), Lim1,2 or Isl1,2 (DSHB) was followed by incubation with fluorescently tagged secondary antibodies for 1 hr 30 min at 20-22°C. Sections were mounted with either Vectashield mounting medium with DAPI, or Fluoromount (Southern Biotech); wholemounts were cleared in successive washes of 30%, 50%, and 80% glycerol and mounted in 80% glycerol. Immunoreactivity of sections with a single staining was examined

with a 20X water immersion objective on a Zeiss Axioscope, using a Xenon arc lamp attenuated by neutral density filters. Immunoreactivity of wholemounts and of double and triple stainings on sections was examined on a Leica confocal system; multiple focal planes were acquired to confirm co-localization and to generate through-series projections. For both microscopes, appropriate filter combinations for excitation and emission were used for Alexa 488, Alexa 594, Alexa 647, DAPI, FITC, or Cy3 fluorophores. Images were acquired and analyzed with either Axiovision or the Leica Application Suite software packages. TH, the rate limiting enzyme of dopamine synthesis, was used as a marker for dopamine throughout the paper (Gonzalez et al., 1994).

Ion channel misexpression. DNA constructs for the human Kir2.1 and the rat $Na_v2a\alpha\beta$ channels were gifts from E. Marban and W. Catterall. These constructs were subcloned, transcribed and expressed in all cells by injecting mRNA into both blastomeres at the two-cell stage along with the fluorescent tracer, Cascade Blue (Invitrogen), to identify successfully injected embryos (Borodinsky, et al. 2004; Dulcis & Spitzer, 2008).

Calcium imaging followed by immunostaining. To image dopaminergic neurons in the spinal cord, the embryo was pinned ventral side up with 0.10-mm-diameter stainless-steel Austerlitz Minuten pins (Fine Science Tools) to the side of a well carved in the middle of a Sylgard

coated dish. These embryos were incubated in 1 or 2% collagenase in 2 mM calcium saline to facilitate removal of tissue covering the ventral surface of the spinal cord, using forceps and tungsten needles. Once exposed, spinal cords were rinsed in 2mM calcium saline to which Fluo-4 was added to a final concentration of 5 μM Fluo4-AM/ 0.01% Pluronic Acid F-127 DMSO for a 1 hr incubation at room temperature, and washed in 2 mM calcium saline before imaging. Calcium imaging was carried out on a field of view covering the rostral to mid spinal cord regions, spanning a region of 1500 μm length of spinal cord. The time-lapse imaging protocol used lasted 30 min, acquiring an image every 5 seconds on a BioRAD MRC-600 confocal microscope with a 20X water immersion objective. Imaging was carried out on spinal cords from embryos at stages 27, 28, or 29/30. Movies were reviewed by eye immediately after imaging. Spinal cords that showed calcium transients were first fixed with EDAC (Sigma-Aldrich) (40 mg/1 ml of 0.1X CMF-PBS) for 1 hr at room temperature to fix the Fluo-4 (Invitrogen) (Dallwig and Deitmer, 2002), and then fixed for another hr in 4% paraformaldehyde + 0.025% glutaraldehyde at 4°C. Three 1 hr washes in 1X CMF-PBS were followed by permeabilization overnight in PBT. Spinal cords were then incubated in blocking solution (1% fish gelatin in 1X CMF/PBS) for 12-24 hr, followed by a three night primary incubation with antibodies to TH and GABA and secondary

incubation for 2 hr with appropriate secondary antibodies. Stained spinal cords were imaged on a Leica confocal system to detect TH and GABA immunostaining and fixed Fluo4, taking z-stacks covering ~40-50 μm depth. These stacks were analyzed to identify neurons immunopositive for TH, GABA or TH/GABA using the Leica Application Suite software; projection of the Fluo-4 channel was used to match the calcium imaging movie to the staining. Calcium activity was analyzed in neurons doubly marked in this way, by digitizing time-lapse movies on Image J with the "Measure Stacks" plug-in by Bob Dougherty (Optinav, Inc). A region of interest was drawn around an identified neuron, pixel intensity was measured across all frames of the movie, and intensity values were exported to Excel (Microsoft). Calcium spikes were identified by eye on digitized traces as increases in fluorescence > 2x the baseline with a single frame rise time. We analyzed spike incidence (number of spiking neurons as a percent of the total number of neurons of that staining class) and frequency (number of spikes/30 min).

Statistical analysis. Statistically significant differences in the number of TH immunoreactive neurons during development, the percent co-expression of TH with other neurotransmitters, and the effects of activity manipulations were determined by the Kruskal-Wallis test followed

by Conover's pairwise comparisons. Statistically significant differences in the percent co-expression of TH with GABA and a transcription factor were assessed by the Mann-Whitney U-test. All statistical analyses were performed on the BrightStat free online software (Stricker, 2008). Statistical significance was set at $p<0.05$.

3.4 Results

3.4.1 Qualitative and quantitative characterization of development of the dopaminergic system in *Xenopus laevis* larvae

The number of DA neurons increases throughout development of *Xenopus laevis.* In addition, differentiation of the DA phenotype follows a caudal to rostral progression with a distinct temporal and spatial pattern for different nuclei (Gonzalez et al., 1994). Wholemount preparations of brain and spinal cord were used to establish the time course of appearance of different DA nuclei as well as changes in relative position and shape of each nucleus throughout development (Fig. 3.1*A,B*). Tyrosine hydroxylase (TH), the rate limiting enzyme of dopamine synthesis, was detected in the brain at stage 35 in the posterior tuberculum (PT), which is the most caudal of the brain dopaminergic nuclei, and in the DLSC as well, both of which are composed of just a few neurons at this stage. By stage 37 a slightly larger PT and DLSC are visible. At stage 40, TH is also expressed in the ventral suprachiasmatic

nucleus (VSC) and there is an incipient olfactory bulb (OB) composed of only a few cells. A cluster of TH-positive cells is detected in the OB, the most rostral of the DA nuclei, at stage 42. The number of TH-immunoreactive (TH-IR) neuronal profiles was quantified in 10 μm transverse sections through the brain (Fig. 3.1*C,D*). All DA nuclei keep growing as development progresses. Quantification of TH-IR cells across five developmental stages is summarized for each nucleus in Table 3.1.

The three-dimensional shape and position of each nucleus also change with development. The PT nucleus moves ventrally from stage 35 to 45, while the DLSCs occupy a dorsolateral position at stage 40, but develop a ventromedial branch by stage 42 (Fig. 3.1*C*, arrowheads).

Table 3.1. Number of TH-IR neurons in DA brain nuclei during development.

	OB			**DLSC**			**VSC**			**PT**		
stage	#TH	SEM	n	#TH	SEM	n	#TH	SEM	n	#TH	SEM	n
35	NYD	-------	---	16	3	7	NYD	-------	---	19	2	7
37	NYD	-------	---	35	2	17	NYD	-------	---	31	2	21
40	3	1	5	140	9	18	11	1	18	73	4	20
42	17	2	11	186	10	15	36	2	21	117	4	24
45	38	4	9	237	27	8	66	8	9	180	11	11

NYD. Not yet developed.

TH-IR neurons appear at stage 28 in the spinal cord (Heathcote and Chen, 1994). By stage 35, these cells are located on the ventral side

of the spinal cord in two distinct longitudinal rows that undergo extensive remodeling through stage 45 (Fig. 3.2*A,B*) and beyond (Heathcote and Chen, 1994). At stage 40, growth cones start developing rostrally (Fig. 3.2*B* arrowheads), and by stage 42 axons extend anterolaterally along the longitudinal ventral axis (Fig. 3.2*B*, arrows). Imaging TH-IR profiles in wholemounts of dissected spinal cords indicates that dopaminergic cells increase in number (Fig. 3.2*D*), decrease in size (Fig. 3.2*E*), and migrate towards the midline (Fig. 3.2*F*) during development (Table 3.2).

Table 3.2. Characterization of DA spinal cord neurons during development.

Spinal Cord									
		#cells/100 μm		**max length**		**max width**		**distance to midline**	
Stage	n	number	SEM	μm	SEM	μm	SEM	μm	SEM
35	4	4	0.4	18.7	0.7	12.3	1.1	10.9	0.3
40	6	5	0.3	16.2	0.7	10.4	0.4	9	0.6
42	6	6	0.5	14.9	0.3	9.9	0.3	7.9	0.5
45	3	7	1	12.4	0.6	8.1	0.3	5.9	0.5

3.4.2 Subclasses of dopaminergic neurons: TH co-expression with other neurotransmitters

To investigate the level of heterogeneity among neurons of DA nuclei during development, we immunocolocalized TH with GABA or NPY

that have been reported to be co-expressed in the adult frog (de Rijk et al., 1992; Ubink R. et al., 1998) or with VGlut2, that has been reported to co-localize with TH in rat and mice (Kawano et al., 2006; Mendez JA et al., 2008). We found no co-localization of TH with VGlut2 (data not shown) but observed co-localization of TH with GABA, GABA and NPY, or NPY in different regions of the DA system.

Co-expression of TH and GABA was found in all four DA nuclei throughout development (Fig. 3.3). Notably, most DA cells in the OB express GABA (Fig. 3.3*A*). In the DLSC, most DA cells co-express GABA at early stages and these cells are located in bilateral dorsolateral clusters. During development more TH cells that do not express GABA are added to this nucleus around the main dorsolateral cluster (Fig. 3.3*B*). The percentage of brain TH cells that co-express GABA per nucleus at developmental stages 35, 37, 40, 42, and 45 is indicated in Table 3.3. Other combinations of NT co-expression such as TH and NPY, TH/NPY/GABA were also observed in the VSC and PT (Fig. 3.3), but were absent in the DLSC, and OB. The VSC at stage 40 consists of a core region located medially that is mostly TH-IR with some cells that co-express GABA. By stage 42 a new portion of this nucleus is apparent around the core: the annulus of the VSC. Annular DA cells express TH, NPY, and GABA (37±7% at stage 42, and 27± 8% at stage 45), while the

core is still composed of cells that express mostly TH alone or TH/GABA (Table 3.3, VSC). By stage 45, 17±4% of TH cells in the annulus co-express NPY (Fig. 3.3*C*). The PT is composed mostly of DA cells that express only TH, but there is a small population of about 10% of neurons (Table 3.3, PT) that corresponds to cells in the caudal and most ventral portions of this nucleus that co-express GABA throughout development. By stage 45, 7±2% of TH cells in PT also express NPY, and 6±2% of TH cells also express both NPY and GABA (Fig. 3.3*D*).

Table 3.3 Percentage of brain TH neurons co-expressing GABA during development.

	Stage 35		**Stage 37**		**Stage 40**		**Stage 42**		**Stage 45**	
Nuclei	TH+ GABA-	TH+ GABA+	TH+ GABA-	TH+ GABA+	TH+ GABA-	TH+ GABA+	TH+ GABA-	TH+ GABA+	TH+ GABA-	TH+ GABA+
OB	NYD	N.D.	N.D.	N.D.	N.D.	N.D.	2	98	18	82
SEM	NYD	N.D.	N.D.	N.D.	N.D.	N.D.	2	2	7	7
DLSC	0	100	8	96	13	87	28	72	42	58
SEM	0	0	6	1	4	4	2	2	3	3
VSC	N.D.	N.D.	N.D.	N.D.	87	10	49	19	32	23
SEM	N.D.	N.D.	N.D.	N.D.	6	4	8	6	9	6
PT	91	9	95	5	90	11	92	8	75	12
SEM	1	3	1	3	1	2	1	1	1	1

NYD Not yet developed.

In the spinal cord there is no co-expression of TH with NPY but widespread co-expression with GABA throughout development from stage

29 to 45 (Fig. 3.2*G,H*). The percentage of spinal cord TH cells that co-express GABA across six developmental stages is indicated in Table 3.4.

Table 3.4. Percentage of spinal cord TH neurons co-expressing GABA during development.

Stage	**29**		**31**		**35**		**40**		**42**		**45**	
	%	SEM	%	SEM	%	SEM	%	SEM	%	SEM	%	SEM
TH+ GABA-	0	0	0	0	2	1	1	1	2	1	9	4
TH+ GABA+	100	0	100	0	98	1	99	1	98	1	91	4

3.4.3 Subclasses of dopaminergic neurons: TH co-expression with transcription factors

The Pax6 and Nurr1 transcription factors have been implicated in DA differentiation (Mastik and Andrews, 2001; Zetterstrom et al., 1996, Ang, 2006). In addition, transcription factors Lim1 and 2 are expressed in DA neurons in the suprachiasmatic region (Moreno et al., 2004). We tested co-expression of TH with Lim1,2, Pax6, and Nurr1 at stage 37 and stage 42 to further evaluate heterogeneity among the different DA subclasses and determine whether transcription factor expression is correlated with different types of DA neurons identified by their

neurotransmitter expression patterns. Our data indicate that Lim1,2 is expressed in all TH neurons in DLSC, VSC, and PT at every stage tested. However, the TH neurons in the OB do not express Lim1,2 at stages 42, or 45. The other transcription factors are not as widely distributed throughout the DA system. The diencephalon shows extensive expression of Pax6 and Lim1,2, which co-localize in the DLSC in wholemount preparations. The OB and DLSC show co-localization with Pax6 but not Nurr1, while VSC and PT show co-localization with Nurr1 but not with Pax6.

Based on the co-localization of either Pax6 or Nurr1 and GABA, several subclasses of DA neurons can be identified within each nucleus. In the OB there is only one class of DA neuron, which co-expresses TH, GABA and Pax6 (Table 3.5, Fig. 3.4*A*, Fig. 3.5*OB*). The DLSC can be subdivided in three subclasses. The main subclass is predominant at stage 37 and also the largest at stage 42 and consists of cells stained for TH, GABA and Pax6. The medial class is composed of cells positive for TH and GABA and negative for Pax6, located closer to the midline and for the most part ventral compared to the main subclass. The outer class consists of cells that express only TH, and these cells are located in the perimeter of the DLSC, lateral, medial, or ventral to the other classes (Table 3.5, Fig. 3.4*B*, Fig. 3.5*DLSC*).

The VSC consists of two regions at stage 42: the core, consisting of cells located in a cluster around the midline, and the annulus, composed of cells surrounding the core. The core region is Lim1,2 positive and both Pax6 and Nurr1 negative, but can be subdivided in two subclasses according to their neurotransmitter staining pattern for TH alone or for TH/GABA. DA annular neurons express TH, GABA, Lim1,2, and Nurr1 and a few cells co-express only TH, Lim1,2 and Nurr1 (Table 3.5, Fig. 3.4*C*, Fig. 3.5*VSC*).

The PT can be subdivided in three regions: rostral, medial, and caudal. Cells in the rostral and caudal regions express TH. There are a few cells in the most rostral and caudal regions that express TH and GABA. The medial region of this nucleus at stage 42 includes cells that express TH and Nurr1 (Table 3.5, Fig. 3.4*D*, Fig. 3.5*PT*).

No transcription factors have been associated with DA specification in the spinal cord. We tested co-localization of TH with the transcription factors linked to dopamine specification in the brain, but found no co-localization of TH with Pax6, Nurr1, or Lim 1,2 in the spinal cord. We then turned to transcription factors that have been associated with neuronal differentiation of the ventral spinal cord: Lim3, and Isl 1,2 (Appel et al., 1995), but found no co-localization with these either.

Table 3.5: Subpopulations of neurons within dopaminergic nuclei

nucleus	subclass	St.37	St.42	Lim1,2	Pax6	Nurr1	GABA	NPY
OB			✓	✓	✓		✓	
DLSC	main	✓	✓	✓	✓		✓	
	medial		✓	✓			✓	
	outer		✓	✓				
VSC	core		✓	✓				
			✓	✓			✓	
	annulus		✓	✓		✓	✓	✓
PT	rostral	✓	✓	✓				
	medial		✓	✓		✓		
	caudal		✓	✓				

These subclasses should not be taken as absolute. Smaller fractions of TH cell populations, and sometimes individual cells, were occasionally found to express combinations of transcription factors and neurotransmitters that do not fit into the spatial subdivisions presented above. In summary, we can further subdivide the TH-IR neurons of each nucleus by their pattern of expression of transcription factors Lim1,2, Pax6, or Nurr1. In addition, each DA nucleus can be distinguished by the combination of neurotransmitters expressed there. This heterogeneity in

the DA system led us to ask if different DA nuclei have similar or different responses to manipulations in calcium spike activity that have been shown to regulate the number of DA neurons in the VSC (Dulcis & Spitzer, 2008).

3.4.4 Specification of the dopaminergic phenotype depends on calcium spike activity

Misexpression of Kir or Nav channels decreases or increases calcium spike activity, respectively (Borodinsky et al., 2004; Dulcis & Spitzer 2008). To test the activity-dependence of the DA phenotype we injected transcripts encoding either hKir2.1 or $rNa_v2a\alpha\beta$ along with Cascade Blue as tracer in both blastomeres at the two-cell stage of development (Fig. 3.6*A*). The results of activity manipulations were quantified at stage 42, when all DA nuclei are detected in control animals.

In the spinal cord, activity caused an increase in the number of TH-IR neurons in $rNa_v2a\alpha\beta$ mRNA injected embryos and a decrease in hKir2.1 mRNA injected embryos, compared to controls (Fig. 3.6*B,C*). In the brain, we also found an increase in the number of TH-IR profiles following increased spike activity and a decrease following a decrease is spike activity, compared to controls. However, when we analyzed the data for individual brain nuclei we found that the level of the response varies among nuclei (Fig. 3.6*D-G*). Specifically, hyperactivity caused an increase

in the number of TH-IR neurons of OB, DLSC, and VSC, respectively. The same nuclei responded to hypoactivity with a decrease in TH-IR neurons. In contrast, the number of TH-IR profiles in the PT decreased with hypoactivity, but hyperactivity yielded numbers that were not significantly different from control (Fig. 3.6*G*).

3.4.5 Subclasses of dopaminergic neurons display distinct patterns of calcium spike activity

Different responses of DA nuclei to manipulations of calcium raise the question of whether the DA nuclei have different patterns of calcium spike activity, and whether DA neurons that express specific molecular markers have characteristic patterns of calcium spike activity. To address this question we examined endogenous calcium activity, focusing our analysis on DA neurons of the spinal cord at stages 27, 28, and 29/30. These three stages encompass a period during which GABAergic midline neurons acquire the additional DA phenotype.

The proportion of GABA+/TH- and GABA+/TH+ cells within the GABA+ population on the ventral surface of the spinal cord reverses during development, as the GABA+/TH- population decreases and the GABA+/TH+ population increases (Figure 3.7A). In addition, there is an increase in calcium spike frequency in the GABA+/TH+ population at

stage 28, which is the developmental time point at which the proportion of GABA+/TH- cells becomes smaller than the GABA+/TH+ population. The spike frequency of the GABA+/TH+ cells remains constant during this period (Figure 3.7B). These results are consistent with the hypothesis that neurons with different molecular markers have different patterns of calcium spike activity.

3.5 Discussion

Development of the nervous system is a complex process that requires the interaction of extrinsic and intrinsic cues. Specific intrinsic cues are part of what determines neuronal fate, and include constellations of molecular markers that allow distinction of different neuronal cell-types. Expression of a given neurotransmitter is an easy way to sort neurons into different classes, e.g. glutamatergic, GABAergic, and dopaminergic. However, a picture of multiple transmitter co-expression in specific subsets of neuronal populations is emerging. It is important to recognize this added complexity in the study of the development of neuronal populations previously thought to be homogeneous for their expression of particular neurotransmitters. The DA system exemplifies a highly heterogeneous population of neurons, all of which share the expression of

the neurotransmitter dopamine yet diverge in their expression of additional molecular markers.

Dopamine expression appears in a distinct temporal and spatial pattern, following a caudal to rostral progression. We characterized the DA system during development in terms of expression of transcription factors and expression of additional neurotransmitters. We find that the developing DA neurons are heterogeneous, with individual nuclei composed of subpopulations that display specific combinations of GABA, NPY, Lim1,2, Pax6, and Nurr1. These patterns of expression are developmentally regulated, with GABA co-expression being more prominent at early stages of development in the spinal cord, VSC, DLSC and OB and decreasing at later stages, while GABA co-expression increases in PT throughout development. In parallel, new classes of neurons appear in the VSC and PT that express all three neurotransmitters (TH/GABA/NPY) or the TH/NPY combination that becomes more prominent during development.

Each subpopulation can also be roughly identified by its anatomical location within each nucleus and the stage of development at which this subpopulation appears. Thus, the VSC has core neurons that are either TH+/Lim1,2+/GABA- or TH+/Lim1,2+/GABA+, and the proportion of these two populations in the core of the VSC remains roughly constant over

development. However, at stage 42 a new subpopulation of annular VSC DA neurons emerges that expresses different molecular markers: TH+/Lim1,2+/GABA+/NPY+/Nurr1+. In addition, NPY and Nurr1, which are expressed at earlier stages in the area surrounding the core VSC, are never expressed in the core region of the VSC. It appears, based on their different anatomical position, that these are indeed different DA subpopulations within the VSC and not members of the same neuronal population at different stages of development.

Early calcium spike activity is an additional component that is required for differentiation of spinal neurons (Borodinsky et al., 2004). Calcium spike activity is also involved in the regulation of dopamine specification in the VSC (Dulcis and Spitzer, 2008). Given the heterogeneity of the DA system, we tested whether calcium-spike activity is involved in dopamine specification in other regions of this system. Suppressing calcium-spike activity increased and enhancing activity decreased the numbers of DA neurons. However, we observed different responses to calcium activity manipulations for specific DA nuclei.

The present work demonstrates a widespread role for calcium spike –activity-dependent neurotransmitter specification across the nervous system. This includes a role in brain nuclei located in the telencephalon, diencephalon, and mesencephalon, and neurons in the spinal cord, all of

which share the expression of dopamine as a transmitter. Our results also show that neurons that express dopamine can respond differently to activity manipulations. Different roles for electrical activity in neurotransmitter specification probably depend on the internal molecular context of different DA subpopulations.

We tested the hypothesis that developing DA spinal cord neurons with different molecular markers display different patterns of calcium spike activity. Examination of single cells identified by their expression of GABA+/TH- or GABA+/TH+ revealed that each population has different frequencies of spiking across development. One of them exhibits a peak in spiking frequency at the time that GABA cells are acquiring TH+. It is tempting to consider the possibility that the increase in frequency in the GABA+/TH- population is required for the acquisition of TH+ phenotype in the GABAergic cells of the ventral midline of the spinal cord.

The map of molecular marker expression we have developed sets the stage for experiments to study patterns of activity in additional subpopulations of DA neurons at particular times during development *in vivo*. Understanding how transcription factors and electrical activity participate in differentiation of neuronal progenitors into mature dopaminergic neurons could help to improve clinical treatments such as stem cell transplantation. Specific patterns of calcium activity may be

important in regulating expression of unique combinations of neurotransmitters and transcription factors that subclasses of dopaminergic neurons display *in vivo*.

3.6 Acknowledgements

Chapter 3 is material currently being prepared for submission for publication. It is included with the permission of all the authors of the manuscript, Davide Dulcis, and Nicholas C. Spitzer. The dissertation author was the primary author on this paper.

3.7 Figures

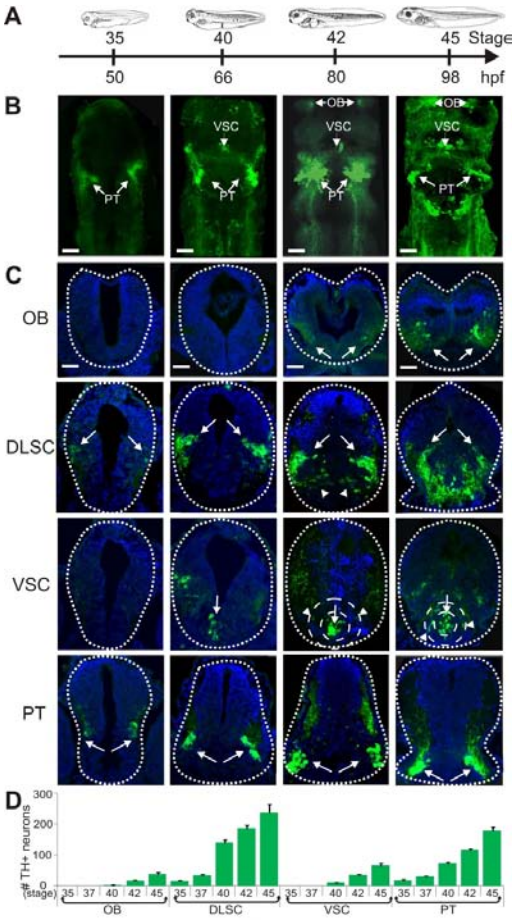

Fig. 3.1. Development of brain dopaminergic nuclei. A. Developmental timeline showing stages and corresponding hr post fertilization (hpf) at which tadpoles were fixed for the images shown below. **B.** Wholemount projections of brains stained for TH show caudal to rostral appearance of dopaminergic nuclei. Images are ventral side up, rostral end up. The PT nucleus is visible from stage 35; by stage 40 the VSC can be detected; by stage 42 the OB is also present. **C.** Cross sections through the brain were used for quantification of TH-IR cells. Images are representative for each nucleus at the corresponding stage; TH staining is shown in green and nuclei were stained with DAPI in blue. Arrows point at TH-IR cells in each section. The first row shows the olfactory bulb (OB), the second row shows the dorsolateral suprachiasmatic nucleus (DLSC). Arrowheads in this row point at a medial branch of the DLSC that becomes more prominent during development. The third row depicts the ventral suprachiasmatic nucleus (VSC). The arrows point at the core and the arrowheads at TH-IR cells in the annular region of the VSC, present from stage 42. The fourth row shows the PT nuclei. **D.** Quantification of the number of TH-IR neurons per nucleus during development. Scale bars in A and B are 50 µm. Values are mean ± SEM for N>5 embryos per stage.

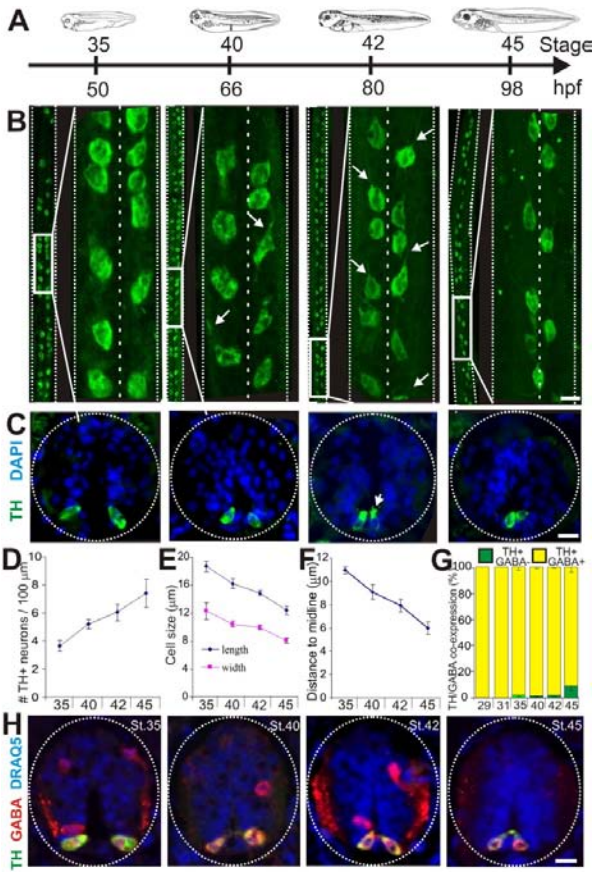

Fig. 3.2. Development of spinal cord dopaminergic neurons. A. Developmental timeline showing stages and corresponding hr post fertilization (hpf) at which tadpoles were fixed for the images shown below. **B.** Wholemount projections of spinal cord sections stained for TH immunoreactivity. At each stage, large sections of spinal cord are shown on the left side and an inset expansion is shown on the right. Spinal cord projections are shown ventral side up and rostral end up. Arrows on the stage 40 and 42 panels point at axons extending rostrally. The number of neurons per segment of spinal cord, the size of the cell bodies and the distance to midline change with development. **C.** Cross sections through the spinal cord at different stages of development stained for TH-IR and DAPI. Dopaminergic spinal neurons are located on the ventral side of the spinal cord. The short arrow on the stage 42 panel points at cilia extending into the central canal. **D.** Quantification of the number of neurons per 100 µm of spinal cord at different stages of development. **E.** Length and width measurements of dopaminergic spinal neurons**. F.** Quantification of the distance to the midline. **G.** Percent co-expression of TH and GABA over development. **H.** Cross sections through the spinal cord stained for TH and GABA, with nuclei counterstained with DRAQ5, show co-expression of these transmitters in the ventrally located dopaminergic cells. Ventral side is down. Some dorsal GABAergic interneurons are evident. Scale bars B, C, and H are 15 µm. Values are mean ± SEM for N>3 embryos per stage.

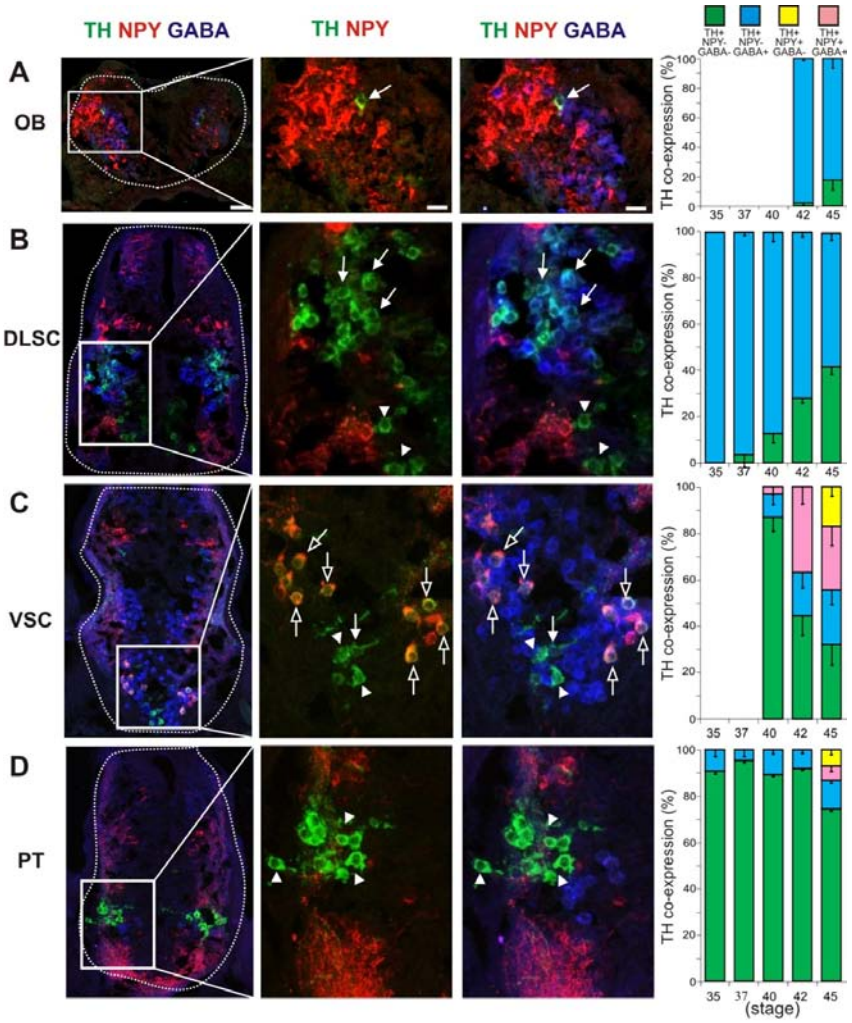

Fig. 3.3. Co-expression of TH with additional neurotransmitters during development of brain dopaminergic nuclei. Images are from stage 42 tadpoles. GABA, NPY, or GABA/NPY are co-expressed in distinct proportions in different nuclei during development. **A.** In the OB most cells co-express TH and GABA (arrows) but not NPY. **B.** In the DLSC TH is not co-expressed with NPY but is widely co-expressed with GABA (arrows). As this nucleus grows and new cells are added, some do not express GABA and the proportion of solely TH-IR cells (arrowheads) increases over time. **C.** The VSC at stage 40 consists mostly of the TH-IR core region (arrowheads) with some cells co-expressing GABA (arrow). By stage 42 there are TH-IR neurons in the annular region that are immunoreactive for TH/GABA/NPY (open arrows.) **D.** The PT is composed of a majority of TH-IR (arrows) neurons throughout development, with a small percentage of cells that co-express GABA. Scale bars in A apply to all figures in each column and are 40 µm for the first column and 20 µm for the second and third columns. Values are mean ± SEM for N>3 embryos per stage.

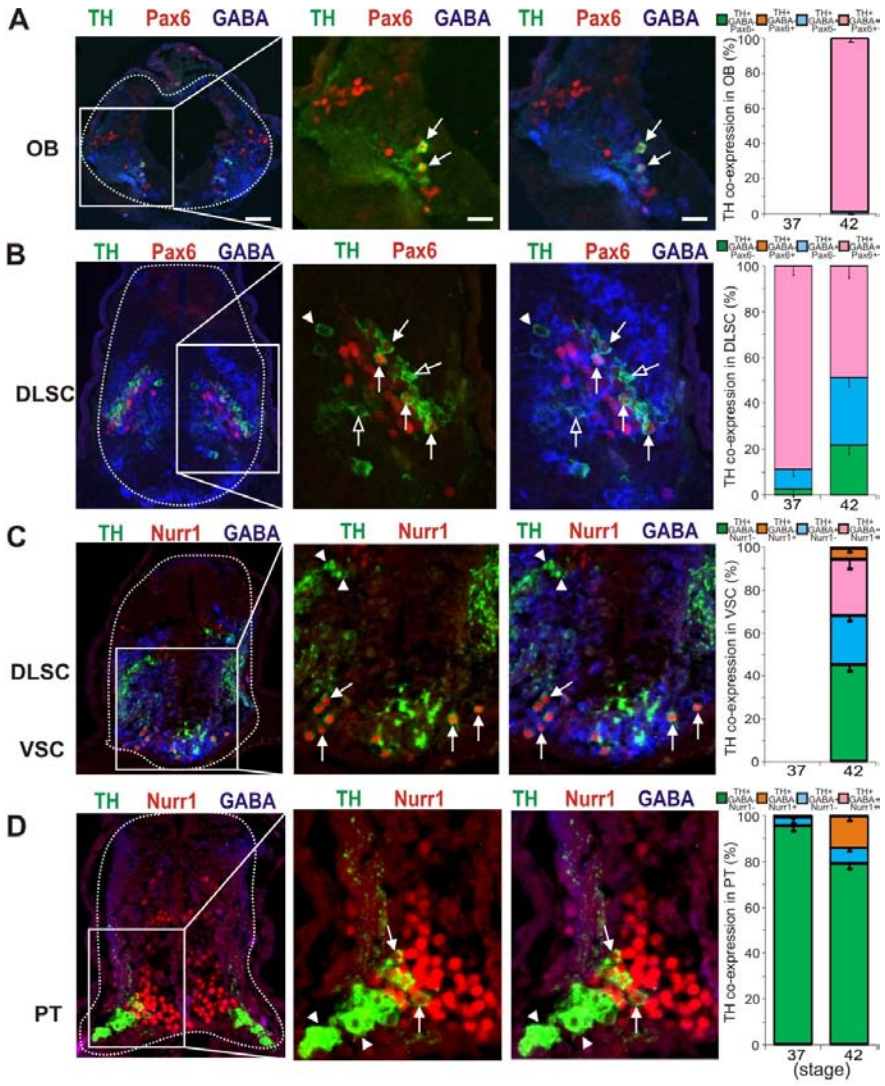

Fig. 3.4. Co-expression of TH with transcription factors during development of brain dopaminergic nuclei. Images are from stage 42 tadpoles. TH is co-expressed with Pax6, or Nurr1 in distinct proportions in different nuclei during development. **A.** In the OB neurons co-express TH, GABA, and Pax6 (arrows). **B.** In the DLSC, TH is co-expressed with GABA and Pax6 in the main DLSC cluster (arrows) at stage 37. By stage 42 new cells are added medial to the main cluster that co-express TH and GABA (open arrows), as well as cells that express only TH (arrowheads) in the periphery of the DLSC. **C.** The VSC TH-IR cells of the annular region co-express GABA and Nurr1 (arrows). **D.** The PT co-expresses Nurr1 (arrows) in a small medial region of the nucleus that develops by stage 42. Scale bars in A apply to all figures in each column and are 40 μm for the first column and 20 μm for the second and third columns. Values are mean ± SEM for N>4 embryos per stage.

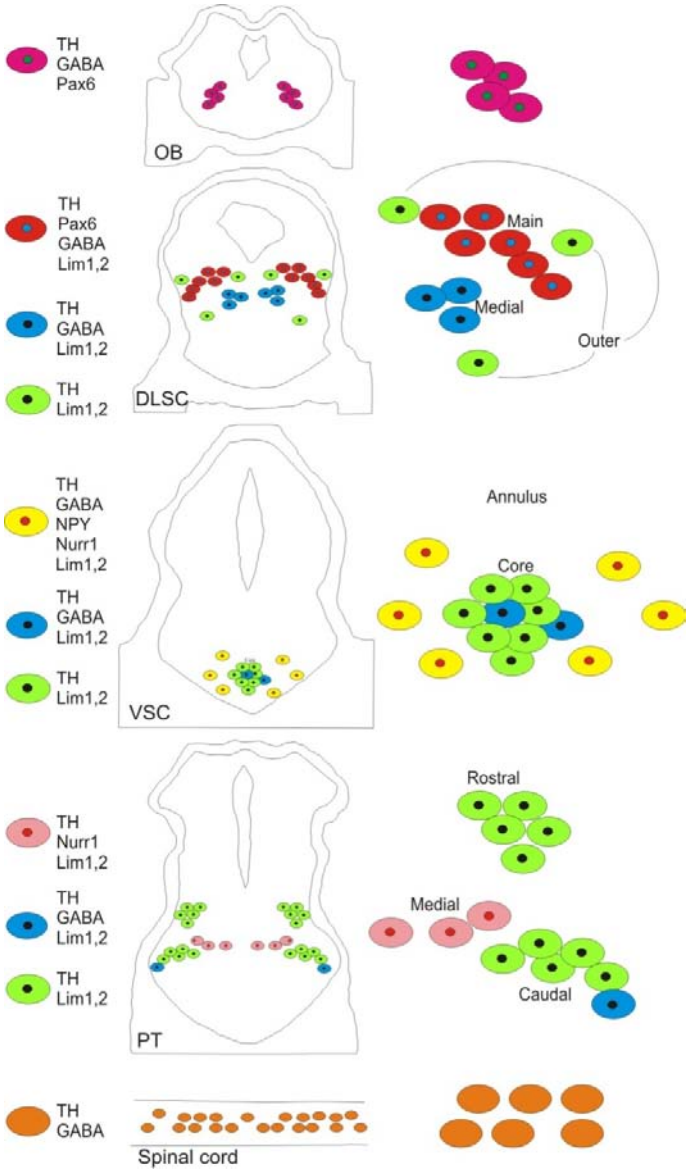

Fig. 3.5. Scheme of subclasses of dopaminergic neurons according to their pattern of co-expression with neurotransmitters and transcription factors. Each dopaminergic nucleus is composed of subclasses of dopaminergic neurons that can be identified by their co-expression of transcription factors (Lim1,2, Pax6 or Nurr1) and/or other neurotransmitters (GABA or NPY). Panels at right show expanded views of regions at left. The OB has a single class of dopaminergic cells. The DLSC can be divided into three subclasses: main, medial, and outer. The VSC can be subdivided into three subclasses: two in the core region, and one in the annulus. The PT can be divided into three subclasses: rostral, medial, and caudal. The spinal cord dopaminergic neurons form a single class.

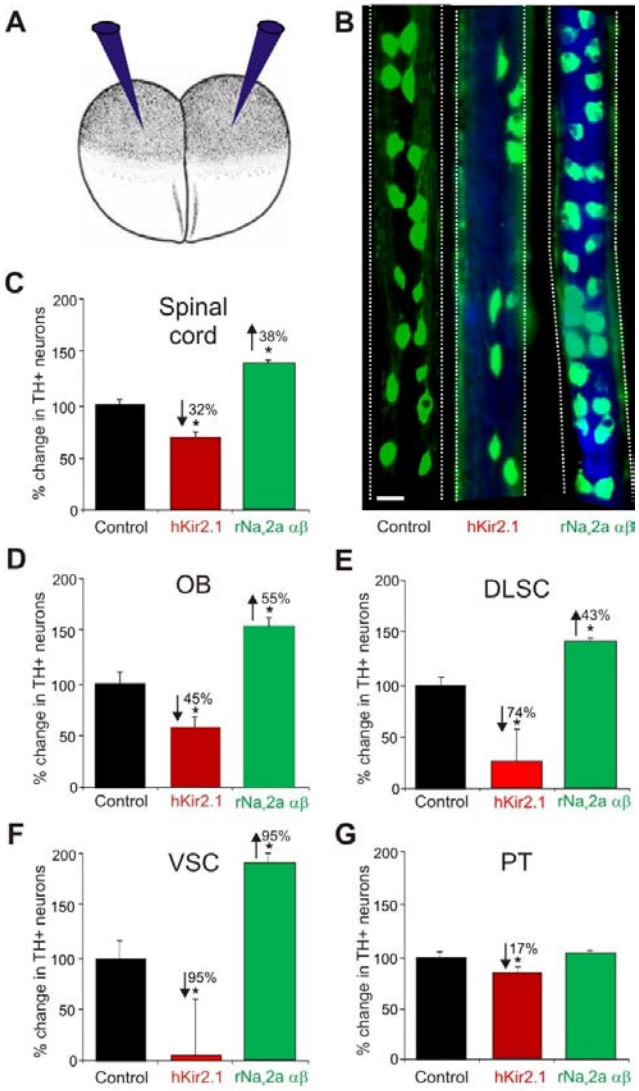

Figure 6

Fig. 3.6. Dopaminergic specification is activity-dependent. A. Embryos at the two cell stage were injected bilaterally with either hKir2.1 or $rNa_v2a\alpha\beta$ along with the tracer Cascade Blue, to decrease or increase calcium spike activity, respectively (Borodinsky et al, 2004; Dulcis & Spitzer, 2008.) **B.** Spinal cord wholemounts from stage 42 tadpoles stained for TH immunoreactivity (in green) show the effect of channel misexpression. The tracer (in blue) can be seen in the back of spinal cords from activity-manipulated tadpoles. **C-G.** Percent change in TH-IR neurons in activity-manipulated embryos compared to control in the spinal cord (**C**), OB (**D**), DLSC (**E**), VSC (**F**), and PT (**G**). Numerical percent change is indicated in cases of significant difference, marked by asterisks. Scale bar in **B** is 15 μm. Values are mean ± SEM for N>4 embryos per stage.

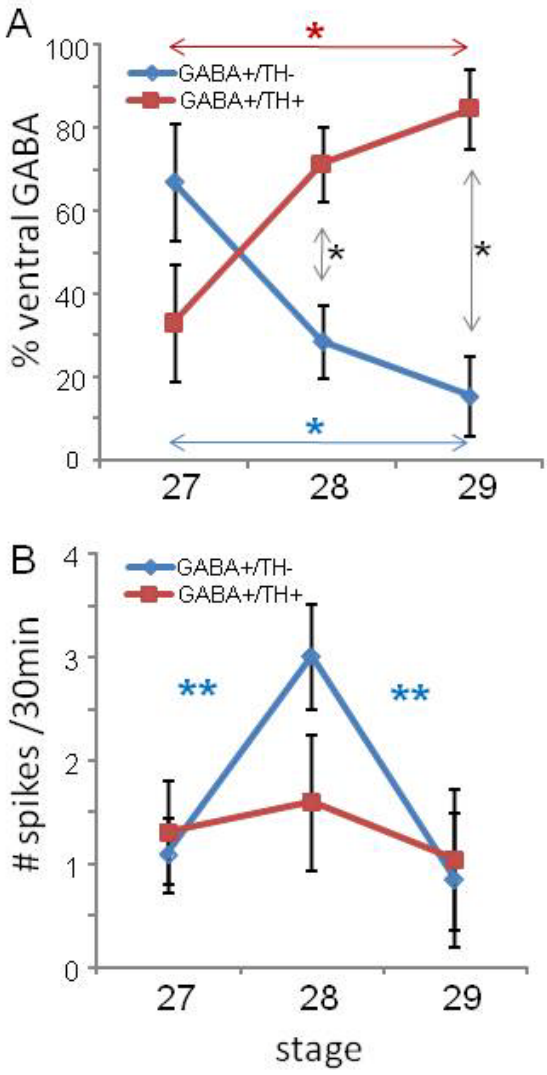

Fig. 3.7. Calcium spike activity patterns of spinal cord ventral GABA+ cells as they acquire dopamine as an additional neurotransmitter. A. The proportion of cells within the GABA immunoreactive population of the ventral spinal cord that are GABA+/TH- and GABA+/TH+ changes during development, with the first group decreasing and the second increasing between stages 27, 28 and 29/30. **B.** Spinal cord neurons with distinct molecular markers exhibit different patterns of calcium spike activity: GABA+/TH+ neurons display a low and constant frequency of calcium spikes at stages 27, 28, and 29, while the GABA+/TH- population shows an increase in frequency at stage 28 that decreases again by stage 29. Values are mean ± SEM for N>3 embryos per stage. Asterisks indicate significant differences across developmental stages (colored asterisks) or between the GABA+/TH- and GABA+/TH+ populations (black asterisks).

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4. Discussion

4.1 Summary of Chapter 2

It is now clear that neurotransmitters are expressed early in development. However, the functions of neurotransmitters and their receptors expressed prior to synapse formation are still being worked out.

In Chapter 2 I have presented evidence for early expression of GABA and glutamate in embryonic spinal cord neurons in *Xenopus laevis*. These transmitters provide a mechanism for triggering calcium-spikes that involves recruitment of metabotropic receptors mGluRIII and $GABA_B$ and kinases PKA and PKC, which have a role in calcium-spike activity-dependent neurotransmitter specification during a sensitive period in development.

This work adds to the reports of early expression of neurotransmitters, and identifies a new role for GABA and glutamate in neuronal differentiation. In addition, we elucidated the mechanism that triggers an early form of electrical activity that makes use of metabotropic receptors. Metabotropic receptors have been implicated in regulating neuronal excitability in the mature nervous system that in turn modulates synaptic transmission (Luthi et al, 1997). The results presented here identify a new role for metabotropic receptors in modulating neuronal excitability much earlier and prior to synapse formation. This role of

metabotropic receptors in modulating neuronal excitability early in development may involve the same mechanism that is used later for modulation of synaptic activity.

4.2 Future directions for Chapter 2

In Chapter 2 we identify involvement of GABA and glutamate in triggering calcium-spike activity. However, there are many questions about calcium-spike activity-dependent neurotransmitter specification that are still unanswered.

One first step would be to identify the source of these neurotransmitters. In Chapter 2 we show that GABA and glutamate are promiscuously expressed in the developing spinal cord. However, a detailed analysis of which specific cell-types express and release GABA and glutamate was not carried out due to the lack of cell-specific markers for spinal interneurons. Studying the patterns of expression of mGluRIII and $GABA_B$ in embryonic spinal cord neurons would provide additional clues about which cell-types are affected by these neurotransmitters.

A second question is, upstream of GABA and glutamate, which stimuli produce their release at this early time, before synapse formation? One possibility is that this release is stochastic. A different possibility is that it is controlled by external stimuli, for example by environmental

stimuli that the embryo is exposed to during development. External control of the internal regulation of neuronal development would allow great flexibility and provide the opportunity to adjust the development of the nervous system in a context-dependent manner. Identifying natural stimuli with the ability to modify calcium-spike activity prior to synapse formation would be exciting. Candidate stimuli include environmental factors such as oxygen level, or internal factors such as hormones. For example, oxytocin was shown to control the emergence of correlated activity in the hippocampus at birth (Crépel et al., 2007) Temperature is another stimulus that could be hypothesized to play a role in development of ectothermic animals whose development occurs in direct contact with the environment, as is the case for amphibians. We tested this hypothesis and found no evidence for a role of temperature in calcium spike activity-dependent neurotransmitter specification. The results of this work are presented in the Appendix.

A third question is what is the mechanism of release for these neurotransmitters? A likely form of release that may be involved is the paracrine release reported by Demarque et al. (2002) in the hippocampus. Involvement of these mechanisms in generating neuronal calcium spikes in the *Xenopus* spinal cord could be tested when we learn how to block paracrine release of these transmitters *in vivo*. One possibility is that these

neurotransmitters are released and act on autoreceptors, in a cell-autonomous way. Another possibility is that GABA or glutamate come from neighboring cells. Experiments in which the calcium-spike activity of individual cells is modified, while leaving the rest undisturbed *in vivo*, are underway in our laboratory (Xu and Spitzer, SfN abstract, 2009).

We show that GABA and glutamate recruit mGluRIII and $GABA_B$ receptors that activate PKA and PKC. However, it is still unclear how these kinases trigger calcium spikes. A fourth question is what are the ionic channels recruited downstream of these kinases. The ionic currents and channels that generate calcium spikes in embryonic *Xenopus* neurons have been described (O'Dowd et al., 1988; Lockery and Spitzer, 1992). The current that initiates calcium-spikes is a low-voltage-activated calcium current through T-type calcium channels (Gu and Spitzer, 1993). There are two principal ways in which to control calcium-spike production, one is by regulation of the resting potential of the neurons (Mathie, 2007), and the second is by changing the properties of T-type calcium channels (Park et al., 2003; Kim et al, 2006). These two possibilities could be distinguished by carrying out electrophysiological recordings of the currents underlying calcium spikes during blockade or activation of PKA, or PKC by mGluRIII or GABAB agonists or antagonists.

The present analysis did not attempt to distinguish the mechanisms for triggering calcium activity in specific and identified classes of developing spinal cord neurons due to the lack of neuron-specific markers that unequivocally allow identification of a given neuronal type. Future efforts would benefit from finding such markers to distinguish which cascades are recruited by which cell-types, and to determine whether the mechanism described here is a common feature of all neuronal classes, or class-specific. Such an analysis also would allow study of the ionic currents and signaling cascades recruited in each class of neurons and lead to an understanding of the mechanisms that allow different cell-types to produce spikes at different frequencies.

In our manipulations about 50% of neurons were unaffected by blockade of metabotropic receptors or kinases. It is possible that complete blockade would have been achieved if we had combined blockade of both GABA and glutamate. However, it may also be the case that 50% of neurons are affected by either GABA or glutamate, and there is another 50% whose calcium-spikes are triggered by a different mechanism. This issue could be better explored in identified neuronal classes, in which it would be possible to distinguish whether the actions of GABA and glutamate are redundant, or if they have effects on different neuronal populations. If indeed both neurotransmitters acted on the same neurons,

then we could determine whether their effects are independent, additive, or are synergistic in a non-linear way.

It is satisfying to know that GABA and glutamate are modulators of calcium-spike activity during a sensitive period of development. However, these experiments did not address the mechanism that determines the onset and termination of the sensitive period. Recent work shows that downregulation of the β3 subunit of the Na+/K+ ATPase coincides with the onset of calcium spike activity in spinal cord neurons of *Xenopus tropicalis*. Moreover, knockdown of this subunit results in more depolarized membrane potentials and inhibition of the appearance of calcium spikes (Chang and Spitzer, 2009). It would be interesting to know if this mechanism is also responsible for the onset of calcium spike production in *Xenopus laevis*, setting the membrane potential of spinal cord neurons at a level that is permissive for GABA and glutamate triggering of calcium spikes.

Further experiments on the role of calcium-spike activity in neurotransmitter specification include determining whether calcium spike activity has a role in multiple regions of the nervous system, and in heterogeneous neuronal populations. These questions were addressed in Chapter 3, and summary and future direction sections follow below.

4.3 Summary of Chapter 3

The processes that lead to neurotransmitter specification involve interaction of extrinsic factors, intrinsic factors, and electrical activity. Identification of factors and specific patterns of activity that result in the specification of a given neurotransmitter is still a work in progress. In the case of dopamine specification, work has centered mostly on midbrain dopaminergic neurons. Extracellular molecules and transcription factors have been identified that contribute to the differentiation of these neurons (Zetterstrom et al., 1996; Ang, 2006; Smidt and Burbach, 2007). A role for calcium-spike activity in specification of dopaminergic neurons in the VSC has been demonstrated (Dulcis and Spitzer, 2008). However, a widespread role for calcium-spike activity in dopamine specification in other nuclei, including midbrain dopaminergic neurons, has been lacking. In addition, a comprehensive study of the development of the dopaminergic system in terms of identification of additional molecular markers had not been attempted.

The work presented in Chapter 3 is a first step to begin to understand this complexity. We found that neurons that express a common neurotransmitter, dopamine, are a heterogeneous neuronal population that can be divided not only into nuclei segregated according to their anatomical position and time of development, but can in addition be

subdivided by patterns of expression of additional markers, either neurotransmitters GABA or NPY, and/or transcription factors Lim1,2, Pax6, or Nurr1. In addition, manipulations of calcium-spike activity have different effects on different dopaminergic nuclei, suggesting that the heterogeneity within the dopaminergic system renders specific subpopulations of dopaminergic neurons more or less susceptible to activity manipulations. These results set the stage for elucidation of the relationship between patterns of calcium-spike activity and transcription factors in establishing expression of single or multiple neurotransmitters in specific neuronal populations.

4.4 Future directions for Chapter 3

We have shown that calcium-spike activity regulates dopamine specification across the nervous system and that the dopaminergic system is heterogeneous, with subpopulations that display unique combinations of neurotransmitters and transcription factors. These results provide the foundation for identification of patterns of activity in identified subpopulations of cells expressing particular transcription factors at specific times during development *in vivo*. We now aim to understand the interplay between different patterns of calcium spike activity and transcription factors in driving the dopaminergic phenotype.

Whether the molecular signature of each subpopulation modulates the sensitivity to patterns of calcium-spike activity that drive dopamine specification has become an intriguing hypothesis. A first step to begin to elucidate the correlation between calcium-spike activity, transcription factors, and neurotransmitter expression would be to determine the patterns of calcium spike activity during development of neurons identified at the single cell level in a specific subpopulation of dopaminergic cells. This objective is facilitated by the molecular markers we have used to distinguish specific neuronal subpopulations as they acquire dopamine as a unique or as an additional neurotransmitter. Aspects of calcium-spike activity that may be important to take into account are spike incidence, spike frequency, whether or not spikes occur in bursts, and if there are bursts, the burst duration, and interspike and interburst intervals.

These calcium imaging experiments would address the following questions. What are the patterns of calcium spike activity in TH precursors and in neurons that have acquired TH during development? What are the patterns of activity of neurons that share the expression of the same neurotransmitter, but are identified by expression of different transcription factors? What are the patterns of activity of neurons that share the same neurotransmitter and markers, but are at different developmental stages? What is the pattern of activity of different types of TH precursor neurons?

The results of experiments addressing these questions would allow us to establish correlations between Ca spike patterns, state of differentiation (transcription factor and neurotransmitter expression) and stage of development. One may hypothesize that Ca spikes precede the appearance of neurotransmitter (TH) expression and that the patterns of Ca spikes are different for neurons that express different transcription factors. These experiments will set the stage for perturbation experiments in which the causal role of Ca spikes is tested by experimentally altering the patterns in which they are produced. Having transgenic lines in which individual dopaminergic cells marked by expression of fluorescent reporters can be followed during development to see which additional markers they express will allow characterization of the complex differentiation of individual, identified neurons.

Of clinical relevance, it would be important to learn which specific patterns of calcium activity regulate expression of unique combinations of neurotransmitters and transcription factors that dopaminergic neuronal subclasses display *in vivo*. This knowledge is expected to help bridge the current clinical approaches with the complexity of the development of the dopaminergic system. The vision is that knowledge of what extrinsic factors, intrinsic factors, and patterns of electrical activity are required for differentiation of neuronal precursors into mature dopaminergic neurons

can be used to design more specific and targeted treatments and clinical approaches based on replacement of diseased dopaminergic neurons, such as stem cell transplantation. There are many protocols used to differentiate cells into dopaminergic cells (Lee et al., 2000; Kawasaki et al., 2000; Kim et al., 2007), and they work; based on expression of dopamine in these cells.

However, the work presented in Chapter 3 highlights the complexity within the dopaminergic system. It would be interesting to know if stem cells subjected to different, currently used protocols for dopaminergic differentiation express additional markers for specific subtypes of dopaminergic neurons. One could argue that the best differentiation protocol will be the one that recapitulates normal development of the specific subtype of dopaminergic neuron that clinical approaches are aiming to replace, and not just a generic one that may be lacking additional molecular markers that are crucial for the appropriate integration and function of a given subclass of dopaminergic neuron.

4.5 Remaining questions about calcium-spike activity-dependent neurotransmitter specification

4.5.1 Generality of these findings

The results presented in this thesis, in combination with the work from other groups, show that activity-dependent specification of neurotransmitter phenotype is a mechanism that applies to the spinal cord as well as the brain, and is not specific to a particular neurotransmitter. Calcium-dependent neurotransmitter specification has been shown for GABA, glutamate, acetylcholine, and glycine (Borodinsky et al, 2004, Chapter 2), dopamine (Dulcis and Spitzer, 2008, Chapter 3), and serotonin (Demarque and Spitzer, personal communication) in *Xenopus laevis*, and for GABA and glutamate specification in the spinal cord of *Xenopus tropicalis* (Marek et al., submitted). Calcium transients have been reported in many other developing nervous systems (Spitzer, 2006 for review), but their role in neurotransmitter specification remains to be explored.

4.5.2 Cracking the frequency code

The mean frequency for calcium-spike activity for four main classes of neurons in the embryonic spinal cord of *Xenopus laevis* has been determined (Borodinsky et al., 2004). However, these mean frequencies

do not reflect the patterns of spikes within the imaging period, which are not evenly distributed over time and occur at irregular intervals. We still do not know what aspect of calcium spike patterns is important to determine expression of one neurotransmitter over another. The number of spikes within a certain period of time may be the relevant parameter; alternatively it may be the spacing of the spikes that is important. These two scenarios could be distinguished by imposing specific calcium-spike patterns, of evenly or unevenly distributed spikes, on individual cells *in vivo.* These experiments could determine whether this causes one cell type to express the neurotransmitter of second cell type when the pattern from the second cell type is imposed. This type of experiment could be carried out with transgenic reporter lines for specific neurotransmitters in which channelrhodopsin is expressed in particular classes of neurons and blue light used to elicit calcium spikes with different spacing.

Another way to look at the frequency code is in terms of calcium concentrations, given that calcium can regulate gene expression (West et al, 2001). Dolmetsch et al. (1997) showed that the amplitude and duration of calcium signals induces differential activation of transcription factors. Different calcium concentrations could be achieved by having calcium spikes occurring more or less frequently and either spaced far apart or close together. Calcium spikes may recruit specific transcription factors,

including terminal selector genes (Hobart, 2008) that would result in calcium-dependent transcription of neurotransmitter specific genes. The code would be based in thresholds/patterns of intracellular calcium concentration that would be coupled to activation or repression of different transcription factors leading to specific neurotransmitter expression.

At later stages of development activity is driven by synaptic connections, and even later in development the trigger for activity can come from sensory stimuli. These forms of activity also elicit changes in calcium concentration. A calcium code could be an underlying mechanism for later forms of electrical activity (Moody and Bosma, 2005).

4.5.3 Interplay between environmental factors, extrinsic molecules, electrical activity, and intrinsic genetic programs.

Electrical activity prior to synapse formation has a widespread and central role in many aspects of development (Spitzer, 2006). Starting with reports of early forms of electrical activity, the field has moved forward to identify the mechanisms that trigger electrical activity at the molecular level and determine the functions of this activity in normal development.

A combinatorial code of extrinsic and intrinsic factors controls neuronal differentiation (Edlund and Jessell, 1999). Disruption of these codes leads to ectopic neurotransmitter expression (Tanabe et al., 1998;

Cheng et al., 2003). Calcium–spike activity also regulates neuronal differentiation, and alterations of this activity result in changes in neurotransmitter expression (Borodinsky et al, 2004; Dulcis et al., 2008; Chapter 2 and Chapter 3 of this thesis).

However, more work needs to be done to elucidate the nature of the interplay of these factors and their relative contributions to normal neuronal differentiation and neurotransmitter specification. One first step is to continue identifying transcription factors expressed in cells in which calcium-spike activity neurotransmitter specification has been described. Recent work shows that serotonin expression in neurons of the raphe is also calcium-spike activity-dependent, and the transcription factor Lmxb1 acts downstream of this activity (Demarque and Spitzer, personal communication). In the *Xenopus tropicalis* spinal cord transcription factor Tlx3 is downstream of calcium-spike activity and regulates the choice between GABA or glutamate phenotypes in an activity-dependent manner via transcription factor phosphorylation (Marek et al., submitted).

A second step is to identify the signaling pathways and genetic cascades recruited by electrical activity, both calcium-dependent, and calcium-independent, and determine how these pathways interact with extrinsic factors that regulate patterning and early neuronal development.

Different patterns of activity would likely engage cell-type-specific effectors. Environmental stimuli could in turn alter electrical activity and influence nervous system development in a context-dependent manner that may increase the organism's chances of survival. It will be interesting to identify the environmental stimuli capable of altering transmitter specification before synaptogenesis.

Integrative studies from gene regulation to function at the cell, circuit, and whole organism level are needed to begin to understand the interplay between these factors and how they result in normal nervous system development.

4.6 Conclusions

This thesis focuses on one form of early calcium-based electrical activity and its role in neurotransmitter specification in the spinal cord and brain. There are, however, additional roles for calcium during neuronal development, as an ion that both contributes to changes in transmembrane voltage and acts as a second messenger. It will be useful to analyze calcium-spike activity-dependent gene expression during this sensitive period to identify additional roles calcium-spike activity may have during development.

Electrical activity is well positioned to be a gateway between the outside world and the intracellular one. It is possible to imagine a scenario in which changes in the environment leads to changes in electrical activity, and consequently in neuronal development.

Establishing the role of electrical activity as a central regulator of neuronal development is likely to lead to rethinking the causes of developmental disorders. The contributions of electrical activity appear to have been overlooked in many cases. In order to make advances in understanding the causes of these disorders and designing effective treatments it is necessary to better understand the functions of early electrical activity and the mechanisms by which they act.

The field of regeneration may also benefit from incorporating a role of electrical activity in regeneration processes that re-capitulate development.

Stem cell research is probably the field that could take most advantage of the findings about specific roles for electrical activity, extrinsic, and intrinsic factors in determining neuronal fate. This knowledge should allow the development of protocols that lead to stem cell differentiation into the appropriate cell type in a more specific way, thus increasing the chances of re-integrating neurons into the appropriate circuits they are intended to replace or repair.

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5. Appendix : Role of temperature in modulating calcium-activity dependent neurotransmitter specification and test of the function of altered neurotransmitter expression: research proposal and preliminary results.

5.1 Research proposal

Spontaneous neuronal calcium (Ca) spike activity in the *Xenopus laevis* spinal cord regulates neurotransmitter (NT) expression homeostatically during a sensitive period of embryonic development (Borodinsky et al., 2004). Several questions arise in this context:

-Does activity-dependent homeostatic specification of NT expression occur in nature?

-What is the function of changing NT expression, and are these changes functionally homeostatic?

The ability to modify a physiological, morphological, or behavioral trait in response to changes in an environmental variable, such as temperature, has been proposed to be beneficial (Wilson and Franklin, 2002). Amphibians are ectotherms, and as such they are exposed to seasonal changes of temperature during development. Frogs of the *X. laevis* species grow and reproduce at ambient temperatures that range

from 2 to 35 °C (Measey, 2004). *Xenopus laevis* is a highly adaptable species that has been able to successfully colonize novel environments (Measey, 2004). The ability of *X. laevis* to adapt may be due to an ability to utilize environmental stimuli, such as temperature or light, as triggers to modify its development to meet the demands of the new environment. We are interested in identifying natural stimuli that could modulate Ca spike activity and neurotransmitter expression. These stimuli, if they exist, have to reach the nervous system without propagation of action potentials along axons and activation of chemical synapses, since axon extension and synapse formation have not yet taken place. We hypothesize that temperature could be such a stimulus. Preliminary data of Roberto Tinoco and Michaël Demarque in our lab suggested that temperature regulates neurotransmitter expression of embryonic *X. laevis* brain and spinal cord neurons. In the spinal cord, Roberto observed an increase in the number of neurons expressing glycine and a decrease in the number of neurons expressing acetylcholine with increasing temperature at which animals were raised. These opposite effects on excitatory and inhibitory neurotransmitter expression with temperature resemble the previously described Ca activity-dependent changes of neurotransmitter expression in embryonic *X. laevis* spinal cord neurons (Borodinsky et al., 2004). There are more neurons expressing excitatory neurotransmitters in

embryos with suppressed Ca activity, but more neurons expressing inhibitory neurotransmitters in embryos with enhanced Ca activity. I tested the hypothesis that temperature regulates Ca activity-dependent neurotransmitter expression, carrying out experiments to determine the effect of temperature on Ca spike activity and neurotransmitter expression (Specific Aim I).

Ca activity-dependent changes in neurotransmitter specification seem to be homeostatic, as if trying to maintain a stable level of activity. The changes in neurotransmitter expression may have an effect on the activity of early synaptic networks. One such network is the circuit that controls swimming at 2 days of development in the hatchling embryo. *Xenopus laevis* embryos grow in aquatic environments and larvae must develop efficient escape behaviors to avoid predators. However, the locomotion performance of ectotherms is highly dependent on temperature, reaching an optimum at intermediate temperatures and decreasing above and below this optimum (see Bennett, 1990; Johnston and Temple, 2002, for review). Some ectotherms, including *X. laevis,* can compensate for the rate-limiting factors of temperature by thermal acclimation of their locomotor performance (Wilson et al, 2000; see Johnston and Temple, 2002 for review). The mechanism behind thermal acclimation of locomotor performance varies across species. However,

most studies have been done in adult animals and have focused on skeletal muscle performance (see Johnston and Temple, 2002 for review).

Most relevant here are studies of developmental plasticity in response to temperature that have been carried out at the larval stage (Wilson et al, 2000; Watkins, 2000; Watkins and Vraspir, 2006), but these have also focused on the contribution of muscle, when they have addressed mechanism. Studies directly investigating the effect of temperature on the nervous system during development are lacking. *Xenopus laevis* embryos provide an excellent system with which to study the effect of temperature on development of the nervous system and specifically of the locomotor circuit. The swimming circuit of *X. laevis* embryos has been extensively studied and characterized (see Roberts et al, 1998 for review) and *X. laevis* larvae show thermal plasticity of the burst swimming behavior, exhibiting a different burst swimming behavior depending on the temperature during larval development (Wilson et al., 2000). Larvae grown at low temperatures have a faster burst swimming behavior in cold water than embryos grown in water at warmer temperatures; on the other hand, larvae grown at warm temperatures swim faster in water at warmer temperature (Wilson et al, 2000). There was no attempt to investigate the mechanism behind these phenotypes, nor was it determined whether temperature during embryonic stages

affects swimming behavior in this study. In summary, it is known that *X. laevis* has the capacity to acclimate to temperature by modifying its locomotor performance in a way that allows the animals to achieve a normal swimming pattern in different environmental conditions.

I propose developmental changes in the nervous system as a major mechanism contributing to thermal plasticity of locomotor performance in *X. laevis*. In particular, I hypothesize that temperature triggers Ca activity-dependent changes in neurotransmitter specification that compensate for deficiencies in locomotor performance at lower and higher temperatures. This hypothesis supposes that a higher level of excitation in the swimming circuit is needed to compensate for locomotion deficits at low temperatures and that an increase in the level of inhibition is needed at higher temperatures to achieve a normal swimming pattern. I propose to test the effect of temperature-dependent changes of neurotransmitter expression on the swimming behavior and on fictive swimming of hatchling *X. laevis* embryos (Specific Aim II).

Specific Aim I: Determine the effect of temperature, a natural environmental stimulus, on Ca spike activity and neurotransmitter specification in embryonic *Xenopus laevis* spinal cord neurons.

Specific Aim II: Determine the consequences of altering neurotransmitter specification due to modulation of Ca activity by temperature on early synaptic networks, and specifically the neuronal circuit that controls swimming of *Xenopus laevis* embryos.

Specific Aim I: Determine the effect of temperature, a natural environmental stimulus, on Ca spike activity and neurotransmitter specification in embryonic *Xenopus laevis* spinal cord neurons.

Experimental Aim 1.1: Determine the effect of temperature on neurotransmitter specification.

Hypothesis: Temperature homeostatically modulates neurotransmitter expression of *X. laevis* embryonic spinal cord neurons. Growing embryos at lower temperature will increase the number of neurons expressing excitatory neurotransmitters and decrease the number of neurons expressing inhibitory neurotransmitters. In contrast, growing embryos at higher temperature will result in more neurons expressing inhibitory neurotransmitters and fewer neurons expressing excitatory neurotransmitters.

Rationale: Preliminary results of the effect of temperature on neurotransmitter expression assessed at 3 days of development showed an increase in the number of cholinergic neurons at low temperature,

accompanied by a decrease in the number of glycinergic neurons. These experiments needed to be repeated and extended to include GABA and glutamate to know if temperature-dependent changes in transmitter expression parallel the Ca activity-dependent changes reported by Borodinsky et al (2004). We are interested in the consequences of changing neurotransmitter expression in early synaptic networks (Specific Aim II), and in particular the synaptic circuit that controls swimming at 2 days of development, an age at which swimming has been described (see Roberts et al., 1998 for review). Thus, I focused my study of temperature-dependent changes in neurotransmitter specification at this stage.

Experiment 1.1: I grew embryos at 16, 22 and 28°C and determined neuronal transmitter phenotype by scoring immunoreactivity to glutamate, acetylcholine, glycine and GABA on 10 μm cryostat sections of spinal cord from embryos fixed at 2 days of development.

Experimental Aim 1.2: Determine the effect of temperature on Ca spike activity.

Hypothesis: Temperature will modulate Ca spike incidence and frequency. Low temperature will produce a sustained decrease in Ca spikes, whereas high temperature will produce a sustained increase in Ca spikes.

Rationale: Preliminary data suggest that temperature can alter neurotransmitter expression in a homeostatic manner. Growing embryos at low temperature increases the number of neurons expressing acetylcholine; growing them at high temperatures results in an increase of neurons expressing glycine. We know that enhancement or suppression of Ca spike activity in *Xenopus laevis* spinal cord neurons regulates neurotransmitter expression (Borodinsky et al., 2004). Thus, the mechanism behind temperature-related changes in transmitter expression may be modulation of Ca spike activity by temperature, which in turn specifies neurotransmitter expression.

Experiment 1.2a: I propose to grow embryos at 16, 22 and 28°C, and monitor Ca spike activity of spinal cord neurons *in vivo* by Ca imaging at 16, 22 and 28°C, controlled by a Peltier-effect device, in age-matched embryos. I plan to compare the incidence (number of neurons that exhibit Ca activity) and frequency (number of Ca spikes per unit of time produced by spiking neurons) of Ca spikes.

Experiment 1.2b: I propose to interfere with Ca spike activity to prevent temperature-dependent changes in NT expression to test the link between these phenomena.

Specific Aim II: Determine the consequences of altering neurotransmitter specification by modulation of Ca activity by temperature, and specifically the effect on the neuronal circuit that controls swimming of *Xenopus laevis* larvae.

Experimental Aim 2.1: Determine the effect of altering neurotransmitter specification on the swimming behavior of X. laevis larvae.

Hypothesis: *Xenopus laevis* embryos acclimate their swimming behavior to the temperature in which they are reared. Temperature-regulated, Ca activity-dependent changes in neurotransmitter expression will be associated with thermal acclimation of locomotor performance. The level of Ca spike activity during a sensitive period is a readout of the environmental conditions in which the embryo is growing, and will trigger changes in neurotransmitter expression that will ensure a functional synaptic network. Embryos grown at low temperature, which I propose will result in low Ca spike activity, will have more neurons expressing excitatory neurotransmitters and will develop stronger excitatory synaptic connections. This will make these embryos more efficient swimmers in water at lower temperatures compared to embryos grown at warmer temperatures. Tadpoles grown in higher temperature, which I propose will result in high Ca activity, will have more neurons expressing inhibitory

neurotransmitters and will develop stronger inhibitory connections. This will make these embryos more efficient swimmers in water at higher temperatures than embryos grown at lower temperatures. Embryos grown at different temperatures will perform similarly at medium temperatures.

Rationale: Frogs lay eggs in water, where they are subject to predation. Success in surviving may depend on rapid development of a behavior, such as swimming, which provides a better chance of escaping predators (Watkins, 1996). Embryos with the ability to detect changes in the environment that alter Ca spike activity and modify neurotransmitter expression in a way that will allow them to optimize their swimming behavior for particular environmental conditions may have an advantage for survival.

Experiment 2.1: I plan to alter neurotransmitter expression by growing embryos at 16, 22 and 28°C, and let the embryos develop for 2 days, to an age at which swimming has been previously described (see Roberts et al., 1998 for review). Using high speed cinematography, I anticipate filming the burst swimming behavior of embryos grown at 16, 22 and 28°C in a temperature-controlled bath at 10, 16, 22, 28 and 34°C and will compare maximum swimming speed, the time to reach maximum speed, distance traveled after 200 ms, and swimming cycle period (Kahn et al,

1982; Wilson et al, 2000*)*. These embryos will then be fixed and processed to evaluate temperature-induced changes in neurotransmitter expression by immunocytochemistry.

Experimental Aim 2.2: Determine the effect of altering neurotransmitter specification on the swimming circuit of hatchling embryos.

Hypothesis: Temperature-regulated, Ca-dependent changes in neurotransmitter expression are functional. Synaptic input to single motoneurons and ventral root activity will provide readouts of changes in the balance between excitation and inhibition that may result from differences in neurotransmitters expressed by swimming circuit neurons. I hypothesize that growing embryos at lower temperatures will result in larger excitatory inputs to individual motoneurons and higher swimming frequency. Growing embryos at higher temperatures will result in larger inhibitory inputs to motoneurons and lower swimming frequency.

Rationale: Ventral root activity and motoneuron activity provide readouts of the activity in the swimming circuit. If changes in neurotransmitter expression occur in the swimming circuit, we may be able to see these changes reflected in these readouts. At the individual motoneuron level, changes in the balance of excitation and inhibition can be detected based

on changes on the motoneuron's electrical activity during a swimming episode, which has a tonic excitatory component, phasic excitatory component, and a mid-cycle inhibitory component (Soffe and Roberts, 1982). Raising embryos at different temperatures may change other features of the nervous system in addition to transmitter specification. Changes in excitability, outgrowth of axons and dendrites, and synapse formation could also contribute to changes in swimming behavior. However, recordings of fictive swimming and motoneuron postsynaptic currents (PSCs) should allow me to determine the extent of changes in the output of the nervous system. The appearance of new classes of PCSs would support the role of up-regulated transmitter systems. Restitution of the normal fictive swimming by application of specific receptor antagonists would argue for the specific contribution of these up-regulated transmitters.

Experiment 2.2a: I propose to alter neurotransmitter expression by growing embryos at 16, 22 and 28°C from stage 18/19 to stage 29, and then let the embryos grow for a further 2 days. I will then record fictive swimming extracellularly from anterior and posterior ventral roots of the spinal cord with suction electrodes. Temperature of the perfusate will be set and controlled by a Peltier effect device to 16, 22 or 28°C. I will

measure swimming cycle period, rostro-caudal phase lag, and burst duration (Kahn & Roberts, 1982; Sillar et al, 1991). I will use a streamlined assay for changes in neurotransmitter specification, examining expression of one excitatory and one inhibitory neurotransmitter by immunocytochemistry.

Experiment 2.2b: I plan to perform whole-cell recordings from individual motoneurons at 16, 22 or 28C. Temperature of the perfusate will be set and controlled by a Peltier effect device. I will use pharmacology to identify up-regulated neurotransmitter systems contributing to PSCs recorded from motoneurons of embryos grown at 16, 22 and 28°C. I will again use a streamlined assay for changes in neurotransmitter specification, examining expression of one excitatory and one inhibitory neurotransmitter by immunocytochemistry.

5.2 Preliminary results for Experimental Aim 1.1: Determining the effect of temperature on neurotransmitter specification.

Borodinsky et al. (2004) assayed Ca-dependent neurotransmitter specification at stage 41. My original proposal to study the role of temperature in this form of neurotransmitter specification was to carry out experiments at stage 37; the experiments to address the function of changes in neurotransmitter expression on the swimming circuit of

Xenopus laevis would be easiest to interpret at that stage, at which most work on the swimming circuit has been carried out.

A preliminary set of experiments was carried out at this stage, and also at stage 40, with inconclusive results. To exclude the possibility that these results were due to the temperature manipulation not having enough time to act to achieve changes in neurotransmitter specification, and to have a positive control for the numbers of neurons expected to express GABA, glycine, glutamate or acetylcholine at a stage that has been previously studied, I switched the experimental design to assaying the effect of temperature on neurotransmitter expression at stage 41. Those experiments are reported here.

Embryos were generated by *in vitro* fertilization and 50 embryos at the 4-cell stage were sorted into 3 different opaque plastic containers, one for each temperature manipulation, to let them mature in the dark. The ratio of Ringer's solution to embryo was kept the same across temperature manipulations (10 ml per embryo) and adjusted as sick and dead embryos were sorted over the course of the experiment. Only experiment rounds where there was <20% dead/deformed animals at each sorting point were used. These containers were kept at room temperature until the embryos reached stage 18/19 of development. The temperature manipulation was

restricted to cover the period of development during which Ca spike activity occurs, stage 20 to 28 (Borodinsky et al, 2004). At that point, the containers were placed at the experimental temperatures of 16, 22 or 28^{o}C. The 16^{o}C condition was achieved by placing the dish with embryos in a temperature-controlled room kept at 15/16^{o}C. The 22^{o}C condition was achieved by placing the plastic container in a water bath heated to 21/22^{o}C that was kept in the 15/16^{o}C temperature-controlled room. The 28/29^{o}C condition was achieved by placing the plastic container in a hybridization oven set at 28^{o}C. A record of temperature variations was taken by a thermometer probe placed inside each plastic container, which recorded the minimum and maximum temperature over the course of the temperature manipulation. Only experimental rounds with temperature variations of less than or equal to ±1^{o}C were analyzed. At stage 29, the plastic containers were transferred back to room temperature and animals grown to stage 41. To ensure normal development of the swimming circuit in temperature-manipulated embryos, we qualitatively assayed this behavior by probing 10 tadpoles chosen at random to test early swimming behavior at stage 29/30 and 35/36. Embryos or tadpoles were considered normal if they exhibited slow lateral undulating movements at stage 29/30 in response to a poke on the side with forceps, and by stage 35/36 normal swimming was taken as faster, lateral, undulating moments in response to

a poke of forceps on the tail that allowed the tadpole to move away from the forceps. At stage 41 all tadpoles with normal anatomy were fixed at 4^{o}C in either 4% PFA + 0.025% glutaraldehyde for glycine, GABA or ChAT staining for 1 hr, or in 4% PFA for 2 hr for VGlut1 staining. Fixed tadpoles were cryoprotected in 30% sucrose overnight, and then embedded in OCT and frozen. One slide of 10 μm-thick cryostat sections per tadpole was collected. Cross-sections through the spinal cord were taken spanning a region of ~500 μm starting 400 μm posterior to the eyes, to encompass the anterior spinal cord (Borodinksy et al., 2004). 15-25 consecutive sections were scored, starting where the myotomes meet dorsally. Neurotransmitter staining was carried out keeping the combinations of primary and secondary antibodies constant for all rounds of staining. Photographs of stained sections and counts of stained neurons were performed blind. Nuclei were counter-stained with DAPI to determine whether there was extensive cell birth or death. No significant difference in DAPI counts was found across temperature manipulations.

Three experiments with embryos from different clutches were carried out following the experimental design above. A total of 6 larvae per temperature manipulation for glycine staining, and 7 larvae per temperature manipulation for GABA, glutamate and ChAT, were counted.

No significant differences in neurotransmitter expression in response to the temperature manipulation were detected.

To test whether changes occurred preferentially in posterior or anterior regions of the spinal cord the data were re-analyzed by dividing and reclassifying the original counts, considering only sections with DAPI >70 for anterior regions, and DAPI < 70 for posterior regions. This analysis also yielded no significant differences in neurotransmitter expression in response to temperature manipulation. One round of staining was analyzed to look for changes in the distribution of cells in response to temperature manipulations, but no consistent changes were observed.

Inconsistent counting is not a likely explanation of these negative results because the counts for the 22^{o}C condition are similar to those expected for stage 41, based on data from previous experiments and experimenters.

Subsequent experimental aims

The absence of changes in neurotransmitter expression following development of *Xenopus* larvae at different temperatures led to cancellation of work on subsequent specific aims.

5.3 Future directions

The negative results reported here may have been due to insufficiency of the temperature manipulation protocol. The temperature range used in these experiments was 16ºC to 28ºC, for a restricted period during development (stage 19 to 29). The temperature range could be expanded in future temperature manipulations, either to more extreme temperatures or to encompass a longer period of development.

It is also possible that temperature is not a stimulus with the capacity to recruit and modulate Ca spike activity-dependent changes in neurotransmitter expression. It would be interesting to try similar experiments testing different environmental stimuli, such as oxygen availability. Hypoxia has been shown to alter development, and recent work has begun to elucidate transcription factors activated by a decrease in oxygen and how oxygen can mediate gene expression (Simon and Keith, 2008; Weidemann and Johnson, 2008).

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