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An *in vivo* screen to identify candidate neurogenic genes in the developing *Xenopus* visual system

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ABSTRACT

Neurogenesis in the brain of *Xenopus laevis* continues throughout larval stages of development. We developed a 2-tier screen to identify candidate genes controlling neurogenesis in *Xenopus* optic tectum *in vivo*. First, microarray and NanoString analyses were used to identify candidate genes that were differentially expressed in Sox2-expressing neural progenitor cells or their neuronal progeny. Then an *in vivo*, time-lapse imaging-based screen was used to test whether morpholinos against 34 candidate genes altered neural progenitor cell proliferation or neuronal differentiation over 3 days in the optic tectum of intact *Xenopus* tadpoles. We co-electroporated antisense morpholino oligonucleotides against each of the candidate genes with a plasmid that drives GFP expression in Sox2-expressing neural progenitor cells and quantified the effects of morpholinos on neurogenesis. Of the 34 morpholinos tested, 24 altered neural progenitor cell proliferation or neuronal differentiation. The candidates which were tagged as differentially expressed and validated by the *in vivo* imaging screen include: *actn1, arl9, eif3a, elk4, ephb1, fmr1-a, fxr1-1, fbxw7, fgf2, gstp1, hat1, hspa5, lsm6, mecp2, mmp9,* and *prkaca.* Several of these candidates, including *fgf2* and *elk4*, have known or proposed neurogenic functions, thereby validating our strategy to identify candidates. Genes with no previously demonstrated neurogenic functions, *gstp1*, *hspa5* and *lsm6*, were identified from the morpholino experiments, suggesting that our screen successfully revealed unknown candidates. Genes that are associated with human disease, such as such as *mecp2* and *fmr1-a*, were identified by our screen, providing the groundwork for using *Xenopus* as an experimental system to probe conserved disease mechanisms. Together the data identify candidate neurogenic regulatory genes and demonstrate that *Xenopus* is an effective experimental animal to identify and characterize genes that regulate neural progenitor cell proliferation and differentiation *in vivo*.

Introduction

The control of cell proliferation and differentiation is essential for proper development of the central nervous system (CNS). At early stages of CNS development, neural stem cells divide symmetrically to expand the neural stem cell pool (Götz and Huttner, 2005; Hardwick and Philpott, 2014). Neural stem cells change fate and undergo asymmetric regenerative divisions to generate both neural stem cells and neurons, which then organize into nascent circuits. Further cell fate changes occur when neural stem cells become quiescent or exit the cell cycle and differentiate into either neurons or astrocytes (Encinas et al., 2006). These cell fate decisions are essential events that control the patterning of the developing brain and ultimately affect brain function (Geschwind and Rakic, 2013; Kriegstein et al., 2006). Recent work has demonstrated that neurogenic cell fate decisions are influenced by the local environment and neural circuit activity (Alvarez-Buylla et al., 2008; Bestman et al., 2012; Conover and Notti, 2008; Encinas et al., 2006; Giachino and Taylor, 2009; Holmes, 2009; Sharma and Cline, 2010; Vergano-Vera et al., 2009), suggesting that an *in vivo* screen may reveal novel candidate neurogenic regulators.

The *Xenopus laevis* tadpole is ideally suited to screen for candidate neurogenic genes. Cell proliferation continues throughout the development of the nervous system in *Xenopus*. In the visual system, for example, new neurons are generated in the optic tectum throughout larval development and integrate into the developing retinotectal circuit. Because the tadpole is transparent at early stages of development, *in vivo* time-lapse confocal imaging of

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GFP-expressing progenitor cells in the *Xenopus* brain allows direct observations of the fates of the proliferating cell population (Bestman et al., 2012). We developed an *in vivo* screen to identify candidate genes affecting cell proliferation or differentiation in *Xenopus* tectum. First, we used cDNA microarrays and NanoString analysis to identify transcripts that are differentially expressed between neural progenitor cells (NPCs) and their progeny. Next, a subset of gene candidates was evaluated in a secondary screen: after morpholinos were electroporated to knockdown candidates, differences in proliferation or differentiation were determined by *in vivo* time-lapse imaging of NPCs and their neuronal progeny. These analyses identified a diverse range of candidate neurogenic genes that modulate proliferation and neuronal differentiation in the brain, thus implicating a variety of regulatory pathways affecting neurogenesis. Mechanisms controlling cell proliferation and differentiation are highly conserved across evolution (Chapouton et al., 2007; Cheung et al., 2007; Kriegstein et al., 2006; Molnar, 2011; Pevny and Nicolis, 2010; Pierfelice et al., 2011) and are fundamental for the evolution of brain structures (Charvet and Striedter, 2011; Finlay et al., 1998). Therefore, identification of regulatory mechanisms affecting neurogenesis in the *Xenopus* CNS will likely provide insights into neural stem cell fate decisions during the development of the CNS and during adult neurogenesis. Furthermore, a deeper understanding of the underlying mechanisms controlling the balance between cell proliferation and differentiation may also direct the discovery of potential therapeutics for brain injury, developmental disorders, and interventions to replace cells lost by injury and neurodegenerative diseases.

Results

A screen for differentially expressed transcripts from neural progenitor cells and differentiated neurons

The goal of our study was to identify and evaluate candidate neurogenic genes based on a 2-tiered screen in which microarray and NanoString analyses were used to identify transcripts that might regulate cell proliferation and differentiation in the brain, followed by an *in vivo*, time-lapse imaging-based screen to test selected candidate genes. We focused our attention on the tadpole optic tectum, where we had established experimental strategies to enrich for actively dividing NPCs, differentiated neurons or quiescent progenitors based on the normal time course of optic tectal cell development and the effects of visual experience (Bestman et al., 2012; Sharma and Cline, 2010). We labeled NPCs and their progeny with a construct that drives GFP reporter expression in Sox2-expressing cells, called pSox2-bd::GFP (Bestman et al., 2012) and isolated GFP-labeled cells that are enriched for active or quiescent NPCs or differentiated neurons (Fig. 1). Our previous work showed that 1 day after transfecting the optic tectum of stage 46 animals with pSox2-bd::GFP, the majority of the GFP-expressing cells are mitotically active NPCs and by three days after transfection, most GFP-expressing cells have differentiated into neurons (Bestman et al., 2012). Pulse-chase labeling tectal progenitors with CldU also demonstrated that the majority of NPCs differentiate into neurons over a two-day period (Sharma and Cline, 2010). Furthermore, rates of cell proliferation in the optic tectum decrease significantly over the five day period between stages 46 and 48 (Sharma and Cline, 2010), suggesting that Sox2-expressing progenitors are relatively quiescent at stage 48/49. We therefore collected GFP-expressing cells at different times during normal rearing to enrich the following cell populations: active NPCs (aNPCs) isolated from animals one day after electroporation at stage 46; Mature Neurons isolated from tadpoles 5 days after electroporation at stage 46; and quiescent progenitors (qNPCs) isolated from the stage 49 tadpoles 1 day after electroporation. Our previous work also showed that rearing stage 46 tadpoles in the dark for 24 h increased the proportion of actively dividing progenitor cells whereas enhanced visual experience drove cells toward neuronal differentiation (Bestman et al., 2012; Sharma and Cline, 2010). Therefore, we manipulated visual experience to produce 2 cell groups enriched for Immature Neurons (isolated from animals that were electroporated at stage 46 and exposed to visual stimulation for the next 24 h) and actively dividing NPCs (isolated from tadpoles that were electroporated at stage 46 and visually deprived for the following 24 h), called $aNPC_{vd}$ (Fig. 1).

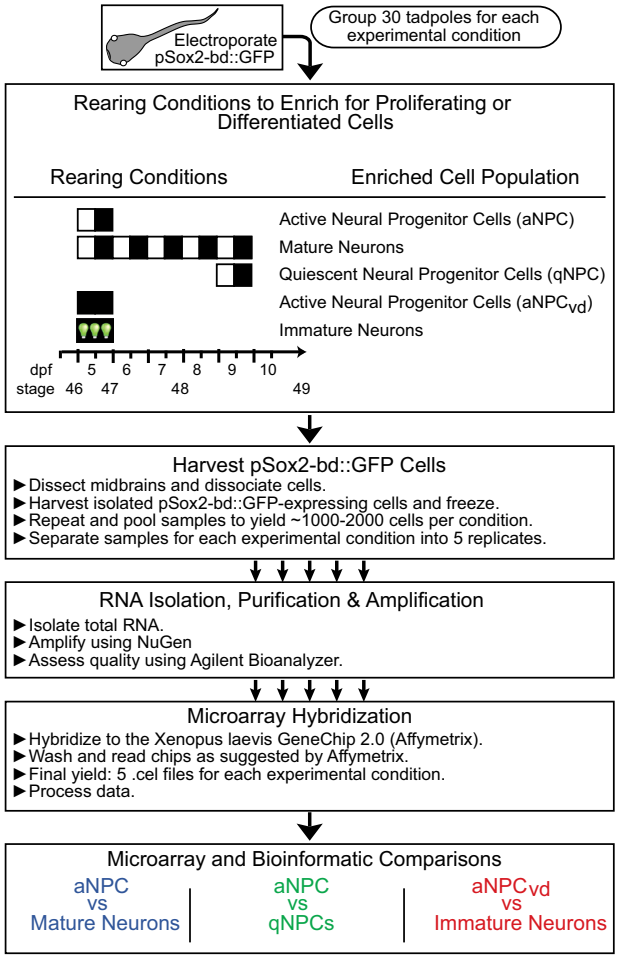

Fig. 1. Flow diagram of the protocols for animal rearing, cell isolation, RNA preparation and microarray hybridization. At stage 46 or 48, tadpoles were electroporated with a GFP-expression construct and placed in one of three visual experience conditions: normal 12 h light:12 h dark conditions; visual deprivation (vd), or enhanced visual experience. These rearing conditions produced 5 cell groups: active NPCs (aNPCs), Mature Neurons, Immature Neurons, Active NPCs isolated from visually-deprived tadpoles ($aNPC_{vd}$), and quiescent NPCs (qNPCs). See text for details. GFP+ cells were harvested from dissociated midbrains and RNA was isolated and prepared for microarrays. The bottom panel shows which samples were compared by microarray analysis to identify differentially expressed genes that might be involved in cell proliferation and neurogenesis.

We used multiple microarray analyses to identify transcripts that were differentially expressed in cell populations enriched for aNPCs relative to neurons or qNPCs. To do this, the expression values of the transcripts for five replicates of each of the five experimental conditions were normalized, outliers representing hybridization artifacts were removed and the averages across the probe replicates were calculated. We then made three comparisons of the transcript expression data from the cDNA microarrays between the different experimental conditions described above (Fig. 1). In one comparison, microarray data from aNPCs and Mature Neurons were compared. The second comparison

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