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ALS-associated genes display CNS expression in the developing zebrafish

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is characterized by progressive muscle atrophy resulting from the deterioration of motor neurons in the central nervous system (CNS). Recent genome-wide association studies have revealed several genes linked to ALS, further demonstrating the complexity of the disease. The zebrafish (*Danio rerio*) is an attractive model organism to study the function of the rapidly expanding number of ALS-associated genes, in part, due to the development of genome editing techniques that have facilitated specific gene targeting. Before investing in the manipulation and phenotypic examination of these genes, however, it is important to ascertain the localization of expression in this organism. We performed an expression analysis of 29 total ALS-linked genes in the developing zebrafish, specifically focusing on those genes that displayed robust and reproducible expression at multiple different timepoints. First, we classified a subset of the most robustly expressed genes into three distinct groups: head-only expression, head and weak trunk expression, and head and robust trunk expression. Then, we defined the characteristic pattern of each gene at 2, 3, and 4 days post fertilization. This analysis will facilitate improved mutant phenotype assessment in zebrafish by focusing researchers on the areas of expression.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is the most prevalent neurodegenerative disease of midlife, with an occurrence of 3–5 cases per 100,000 people in the United States (Brown and Al-Chalabi, 2017). The disease is marked by progressive muscle weakness and eventual paralysis and the majority of ALS sufferers succumb to the disease within 3–5 years of diagnosis. Researchers have taken many approaches to understanding ALS, including the use of patient case studies, human-derived cell lines, and animal models. The use of animals to model the disease has been facilitated by the identification of genetic mutations associated with the disease. In 1993, the first genetic mutations related to ALS were discovered within the gene coding for superoxide dismutase 1 (SOD1) (Rosen et al., 1993). This information enabled researchers to begin studying this ailment in mice by developing the first transgenic mouse model of ALS: $SOD1^{G93A}$, which became the most studied animal model of the disease. Since then, mutations in more than 54 genes have been linked to ALS in humans, some with as many as 100 point mutations that may cause the disease (Banci et al., 2008). Furthermore, scientists may choose to employ different model organisms for the study of these genes. With the recent advancements in genome editing, the zebrafish (*Danio rerio*) has emerged as a favorable organism for studies related to known genetic mutations, as zebrafish embryos develop *ex utero* and can be easily manipulated and visualized. Furthermore, several other benefits of using zebrafish for such studies exist, including: high fecundity, sexual maturation within 2–3 months, and transparency during embryonic stages (Dooley and Zon, 2000; Babin et al., 2014).

Previous studies in zebrafish have demonstrated similarities of disease phenotypes with those documented in human cases (Woods et al., 2000), including studies of ALS (Kabashi et al., 2011; Babin et al., 2014; Kalueff et al., 2014). While some studies have reported observable motor phenotypes following mutation of an ALS-linked gene in the fish (Ciura et al., 2013; Kabashi et al., 2010; Sakowski et al., 2012), other have failed to identify motor deficits (Paulus and Link, 2014; Lebedeva et al., 2017). This discrepancy may be the result of compensation from similar genes in the fish, the zebrafish's ability to regenerate, or simply differences between the fish and human versions of the gene (Kizil

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et al., 2012; Ghosh and Hui, 2016). Thus, before specific mutations are generated within the zebrafish for the purpose of examining the role of a particular gene in motor neurons, it is important to know where thEe mRNA for the gene of interest is localized in the fish nervous system. Our study seeks to fulfill this goal using *in situ* hybridization (ISH). Importantly, this tool can be implemented in whole zebrafish to understand where these genes are expressed during development, leading to predictions about what roles their protein products may play in the organism.

One challenge associated with using zebrafish for genetic studies is the occurrence of a genome duplication, approximately 300 million years ago (Cresko et al., 2003). This radiation event resulted in the presence of two copies of each gene in the zebrafish genome, but over 80% of those duplicates were lost over time (Alsop and Vijayan, 2009). However, if these duplicated genes were still homologous to the original and had not resulted in a new function in the fish, one may predict that very few should remain expressed in this organism. With this logic, it appears likely that the remaining duplicated genes have either developed a novel function in zebrafish, or have become expressed in different tissues, causing them to be retained in the genome due to their overall benefit for the organism (Meyer and Schartl, 1999; Brunet et al., 2006). This complicates ALS research as several disease-linked human genes are duplicated in the zebrafish.

We describe here the expression patterns for 29 total ALS-linked genes within the developing zebrafish, with a particular focus on the CNS. This set of genes was chosen for two primary reasons. First, many of them have yet to be studied in the zebrafish, including a number that have only recently been identified. Second, multiple genes were chosen because paralogs exist for these genes and we were interested to examine whether these factors were localized to distinct populations of cells in the zebrafish and to note the potential for their paralogs to confound any loss of function phenotypic analysis. During the course of these studies, we became further focused on the characterization of 18 of these genes, 7 of which have duplicates. These genes were selected because they displayed robust and reproducible expression at three distinct developmental timepoints. The genes included in this analysis were: *peripherin* (*prph*), *matrin 3 (matr3l1.2), erb-b2 receptor tyrosine kinase 4a* (*erbb4a)* and 4b *(erbb4b), phosphoinositide 5-phosphatase a (fig4a), microtubule-associated protein tau a (mapta), dynactin* 1b (*dctn1b*), *T-cell restricted intracellular antigen-1 (tia1), valosin containing protein (vcp), heterogeneous nuclear ribonucleoprotein A1a (hnrnpa1a)* and *A1b (hnrnpa1b), charged multivesicular body protein 2Ba (chmp2ba)* and 2Bb *(chmp2bb), spastic paraplegia 11* (*spg11), Ewing sarcoma breakpoint region 1a (ewsr1a)* and 1b *(ewsr1b), fused in sarcoma (fus),* and *profilin 1 (pfn1)*. An additional 10 ALS-associated genes, including 1 duplicated gene, were also investigated for their expression, but lacked robust or reproducible detection at one or more of the 3 timepoints examined in this study. The expression for these genes is presented for the timepoint (s) where it was observed in multiple independent experiments. The expression of the 18 ALS-linked genes has been summarized in Table 1, based upon the intensity of the expression, on a scale of 0 (not detected) to 5 (ubiquitous expression) in the observed tissues at all three timepoints. A similar table for the 10 additional ALS-related genes and controls is summarized in Supplemental Table 1. This study will be useful for researchers seeking to perform genome editing-based mutagenesis studies of these genes within the zebrafish and will facilitate future studies of ALS within this organism.

2. Results

2.1. 2dpf expression of ALS-linked genes found in developing CNS

To begin our study of ALS-linked genes in zebrafish, we searched for a comprehensive list of these genes and their fish homologs. A number of different partial lists were found, but none with a thorough matching to their zebrafish counterparts. Therefore, we first generated a list of genes that have been implicated in ALS, either by direct causation through mutation, or simply association with the disease state. These genes, their zebrafish orthologs, and the publications demonstrating their link with ALS are listed in Table 2. To localize the mRNAs for the ALS-linked genes, we employed whole mount ISH using digoxigenin-labeled riboprobes to examine their expression beginning at 2 days post fertilization (dpf). This timepoint was chosen as a starting point because the motor neurons in the trunk of the zebrafish have become functional at this point (Myers et al., 1986; McKeown et al., 2009). The first observation we made was that 18 of these genes displayed robust and reproducible expression across multiple timepoints (Table 3). Given these expression patterns we chose to focus our study primarily on these 18 ALS-associated genes.

During our expression analysis of these genes we observed that a number were present only in the head of the zebrafish at 2dpf. *prph*, *matr3l1.2, erbb4a, fig4a, mapta,* and *dctn1b* were all detected throughout the head, but not the trunk of the zebrafish at 2dpf (Fig. 1A–F). Next, we found *tia1, vcp, ewsr1a, ewsr1b, hnrnpa1b, chmp2ba,* and their paralogs, *hnrnpa1a* and *chmp2bb,* expressed in subsets of cells in the head (Fig. 1G–N), with fainter expression in the trunk. Most noticeably, *chmp2ba* was localized to a population of cells in the midbrain that appeared to form a continuous line along the apical surface of the head (Fig. 1M). Finally, we observed similar expression patterns at 2dpf for *erbb4b, spg11,* and *fus,* all of which were detected in the head and faintly in the trunk at 2dpf (Fig. 1O–R). The final gene examined, *pfn1,* did not have an expression pattern that resembled any of the other genes examined in this study, as it failed to be detected in the nervous system, and was detected instead in clusters of cells along the trunk and the midbody of the fish, as well as within a small number of cells in the head at 2dpf (Fig. 1R).

2.2. Closer examination of genes expressed only in the head during development

After this initial examination at 2dpf, we separated the set of 18 genes expressed at all 3 timepoints into 3 groups based upon their gene expression pattern: head-only expression, head and weakly detected trunk expression, and head and robust trunk expression. We believe this is a logical classification scheme as ALS is a disease that heavily involves the motor neurons, cells which reside in the trunk and tail of the zebrafish (Babin et al., 2014). With this in mind, we were particularly interested in classifying those genes that may be present in motor neurons as opposed to those expressed only in other tissues. Although many previous studies of ALS-linked genes in this organism examined phenotypes related to swimming behavior and motor neuron development (Ramesh et al., 2010; Schmid et al., 2013), we find several of these genes are undetectable in the zebrafish trunk during development.

To more thoroughly understand the expression of these genes during development, we began a more in-depth examination of the head-only genes via whole mount ISH at 2dpf, 3dpf, and 4dpf in both dorsal and lateral views. At all three timepoints, we observed robust expression of *prph* in the forebrain and midbrain, with increased detection from 2dpf-4dpf (Fig. 2A–a”). By 3dpf and 4dpf, this gene was also detected in the hindbrain of the fish (Fig. 2A’-a”). *matr3l1.2* was robustly detected in the head at all 3 timepoints, with observed expression throughout the brain at 2dpf that became more localized at 3dpf and 4dpf (Fig. 2B–b”). Next, we investigated *erbb4a* and observed its expression in a population of cells in the brain, consistent with the location of the optic tectum (Fig. 2C and c). At 2dpf, the expression patterns of *fig4a* and *erbb4a* were similarly detected in a population of cells expanding between the two hemispheres of the brain (Fig. 2C and D). At 3dpf, *erbb4a* was detected in a subset of cells in the mid- and forebrain, with signal that persisted up to 4dpf (Fig. 2C’-c”). *fig4a* was found in the developing brain at 2dpf, while this expression became more widespread at 3dpf and 4dpf (Fig. 2D–d”).