Dpysl2 (CRMP2) is required for the migration of facial branchiomotor neurons in the developing zebrafish embryo

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ABSTRACT  Dihydropyrimidinase-like family proteins (Dpysls) are relevant in several processes during nervous system development; among others, they are involved in axonal growth and cell migration. Dpysl2 (CRMP2) is the most studied member of this family; however, its role in vivo is still being investigated. Our previous studies in zebrafish showed the requirement of Dpysl2 for the proper positioning of caudal primary motor neurons and Rohon-Beard neurons in the spinal cord. In the present study, we show that Dpysl2 is necessary for the proper migration of facial branchiomotor neurons during early development in zebrafish. We generated a dpysl2 knock-out (KO) zebrafish mutant line and used different types of antisense morpholino oligonucleotides (AMO) to analyze the role of Dpysl2 in this process. Both dpysl2 KO mutants and morphants exhibited abnormalities in the migration of these neurons from rhombomers (r) 4 and 5 to 6 and 7. The facial branchiomotor neurons that were expected to be at r6 were still located at r4 and r5 hours after the migration process should have been completed. In addition, mutant phenotypes were rescued by injecting dpysl2 mRNA into the KO embryos. These results indicate that Dpysl2 is involved in the proper migration of facial branchiomotor neurons in developing zebrafish embryos.

KEY WORDS: facial branchiomotor neuron, cell migration, Dpysl2

Introduction

Dihydropyrimidinase-like family proteins (Dpysls), also known as Collapsin response mediator proteins (CRMPs), are evolutionarily conserved cytosolic phosphoproteins expressed in several regions throughout the central and peripheral nervous systems during development (Wang and Strittmatter, 1996). They are known for being involved in axon growth, guidance and regeneration, neuronal polarity, apoptosis and cell migration in the nervous system (Schweitzer et al., 2005).

The most studied Dpysl family member is Dpysl2 (CRMP2), a protein that was originally identified as a mediator of semaphorin signaling and known for being involved in the collapse of the axon growth cones (Goshima et al., 1995). The features of zebrafish such as the transparency of the embryos and their rapid development (Kimmel et al., 1996) are known for being involved in axon growth, guidance and regeneration, neuronal polarity, apoptosis and cell migration in the nervous system (Schweitzer et al., 2005).

The most studied Dpysl family member is Dpysl2 (CRMP2), a protein that was originally identified as a mediator of semaphorin signaling and known for being involved in the collapse of the axon growth cones (Goshima et al., 1995). The features of zebrafish such as the transparency of the embryos and their rapid development (Kimmel et al., 1995) together with the high homology with the human DPYSL2 (Schweitzer et al., 2005) make it suitable to study this gene’s functions. In zebrafish, Dpysl2 is clearly expressed in different regions of the central nervous system throughout the development: by 16 hours post fertilization (hpf) its expression can be observed in areas such as the telencephalon, the trigeminal ganglion, clusters in the hindbrain and in the dorsally located Rohon-Beard neurons (Schweitzer et al., 2005); dpysl2 expression increases during the following hours of development and it is detected in more regions such as the retinal ganglion by 48 hpf (Christie et al., 2006). Dpysl2 expression can be observed in the brain at least up to 97 hpf (Christie et al., 2006). Previous studies in zebrafish have shown the requirement of Dpysl2 for the proper positioning of neurons in the spinal cord (Tanaka et al., 2012; Morimura et al., 2013) and also for proper retinal axon growth (Liu et al., 2018). However, its functions need to be further analyzed in brain development. Dpysl2 is involved in a variety of processes and it has been detected in many regions of the developing brain (Schweitzer et al., 2005; Christie et al., 2006); therefore, it could be an important piece for the proper establishment of the neural circuit in the brain.

In this study, we focused on the role of Dpysl2 in the development of cranial motor neurons.
We used the CRISPR/Cas9 system to generate a dpysl2 knock-out (KO) line to study Dpysl2, more specifically to study its involvement in the migration of facial branchiomotor (FBM) neurons during early stages of development, a process previously described by Higashijima et al., (2000). In addition, we used Antisense Morpholino Oligonucleotides (AMO) to knock down dpysl2 and corroborate our findings. We used two types of AMO to block either the translation or splicing of dpysl2 mRNA.

Higashijima et al., (2000) found that the facial branchiomotor neurons migrate caudally during the development; at 21 hpf most of these neurons are located at the region corresponding to the rhombomeres (r) 4 and 5 and they start migrating caudally until they reach r6 and r7. Most of them have reached this location by 36 hpf. We observed abnormalities in the location of these neurons in both dpysl2 KO mutants and morphants. Most of facial branchiomotor neurons were expected to be at r6 region, but were found between r4 and r5 at 50 hpf, indicating a role of Dpysl2 in this migration process.

**Results**

**Dpysl2 knock-out affects the migration of the facial branchiomotor neurons in early stages of nervous system development in zebrafish**

To generate a dpysl2 KO line, a fish carrying mutations induced by CRISPR/Cas9 injection was crossed with WT fish. In the F1, we identified a specific mutation consisting on a 1bp insertion (G->CA) at the target site, 3bp upstream to the PAM site (Fig. 1B). This mutation led to a frame shift mutation and a premature stop codon as consequence. Due to the premature stop codon, the length of Dpysl2 protein was shortened from 573 to 71 amino acids. We observed abnormalities in the location of these neurons in both dpysl2 KO mutants and morphants. Most of facial branchiomotor neurons were expected to be at r6 region, but were found between r4 and r5 at 50 hpf, indicating a role of Dpysl2 in this migration process.

![Fig. 1. Establishment of a dpysl2 knock-out (KO) line. (A) Scheme of the crossings performed to obtain the dpysl2 KO line. The fish carrying mutations induced by CRISPR/Cas9 injection was crossed with a WT fish. Posteriorly, the heterozygote descendants carrying the same specific mutation were crossed with Tg(isl1:GFP)rw0 to facilitate the observation of the cranial motor neurons in their offspring. The transgenic line Tg(isl1:GFP)rw0 expresses Green Fluorescent Protein (GFP) in the cranial motor neurons; therefore is a very useful line to study genes related to the motor neuron development (Higashijima et al., 2000). Dpysl2 KO (dpysl2−−) embryos expressing GFP in the cranial motor neurons were fixed at 50 hpf for dorsal observation of the facial branchiomotor neurons migration, at this time most of these neurons are expected to be located at r6. We observed the localization of the facial branchiomotor neurons in vivo at 28hpf and the general morphology of some embryos before fixation. We found no differences between dpysl2+/+ and dpysl2−− embryos in the positioning of the facial branchiomotor neurons, nor abnormalities in their morphology (data not shown). Defects were observed in the location of the facial branchiomotor neurons at 50 hpf in the 77.4% of the observed KO mutants (n= 31). In most of the embryos, a great number of neurons failed to migrate caudally and more neurons than expected were located between r4 and r5 (Fig. 2B). In the typical distribution of the facial branchiomotor neurons at this time point, there is a much larger number of them at r6 than between r4 and r5; however, in 48.4% of the cases we observed more cells between r4 and r5 than at r6 and in a 29% the amount of cells in these two regions was nearly the same. When we examined the siblings of the KO mutant embryos that did not carry the mutation (dpysl2−/) at 50 hpf we also found some abnormalities; however, it only affected the 36.4% of them (n=22). Most of them expressed WT-like patterns in the distribution of the facial branchiomotor neurons (Fig. 2A). These observations suggest a role of Dpysl2 in the migration of the facial branchiomotor neurons during development.
Dpysl2 mRNA injection into dpysl2 KO embryos can rescue the mutant phenotype

To prove that the dpysl2 loss-of-function was the cause of the abnormalities found in mutant embryos, we injected dpysl2 mRNA at different concentrations (20, 50 and 100 ng/ml) into 1 to 2 cell stage dpysl2-/- embryos. Dpysl2 mRNA injected and uninjected mutants were fixed at 50 hpf to observe dorsally the migration process of the facial branchiomotor neurons.

Among the embryos injected with 100 ng/ml of dpysl2 mRNA (n= 23), 56.5% expressed a WT-like pattern; most of facial neurons were located at r6 by 50 hpf (Fig. 2C). After injecting lower concentrations of dpysl2 mRNA the percentage of rescued embryos was also lower: 36.4% of the mutant embryos injected with 50 ng/ml of dpysl2 mRNA (n=11) and 31.3% of the ones injected with 20 ng/ml (n= 16) exhibited WT-like patterns, indicating a dose dependency. These results support a role of Dpysl2 in the migration of the facial branchiomotor neurons.

No effects were observed when dpysl2 mRNA was injected into WT embryos.

To confirm the relationship between the mutant phenotypes and the lack of dpysl2, we compared the frequency of embryos with abnormalities in the migration of the facial branchiomotor neurons and WT-like embryos observed in mutants (dpysl2-/-), dpysl2-/+ and dpysl2 mRNA injected dpysl2-/- mutants (Fig. 2D). For each group, we calculated the frequency of phenotypes that were WT-like, slightly affected or severely affected and tested whether the differences in these frequencies were significant using a chi-square test. The results of statistical analysis support that the increase in the rate of severe abnormalities observed in dpysl2-/- embryos is related to the mutation that we generated.

Dpysl2 knock-down causes abnormalities in the migration of the facial branchiomotor neurons

There is evidence of the low correlation between phenotypes induced by a knock-down and the ones that result from a knock-out (Kok et al., 2015), thus, the benefits of using knock-down techniques such as morpholino oligonucleotides has been questioned. However, previous research also showed that one of the causes of these discrepancies is the activation of a compensatory machinery; the knock-out of some genes leads to the upregulation of other genes, but this compensation does not take place after a knock-down (Rossi et al., 2015). Therefore, a knock-down study can be a good complement to the knock-out one. We observed an incomplete penetrance in the phenotype of dpysl2-/- mutant embryos that could be due to the activation of compensatory systems; therefore, to confirm the role of Dpysl2 in the migration process of the facial branchiomotor neurons, we also evaluated the effects of a dpysl2 knock-down. We injected different types of AMOs targeting dpysl2 into 1 to 2 cell stage embryos obtained by crossing WT and Tg(isl1:GFP) rw0 line zebrafish. We used either a translation blocking or a splicing blocking AMO to knock-down dpysl2 and a control AMO to confirm that the effects were due to the knock-down of dpysl2, not to the AMO injection itself. Another typical reason for the common discrepancies between knock-down and knock-out phenotypes can be the presence of maternal WT mRNA in the mutants (Zimmer et al., 2019). Translation blocking AMOs inhibit both maternal and zygotic mRNA, while splicing blocking AMOs affect only the zygotic mRNA (Eimon, 2014); for this reason, using both types of AMO in our research could help to understand better the machinery regulating dpysl2. The three different types of AMOs were injected at the same concentration.

AMO injected and uninjected samples were fixed at 50 hpf as well. Embryos were observed dorsally to confirm whether the migration from r4 and r5 to r6 and r7 was completed successfully. Results showed clear abnormalities in 83.9% of the facial branchiomotor neurons (VII) are located at r6. Abnormal regions are indicated with arrows. Va= Trigeminal anterior; Vp= Trigeminal posterior. Anterior is up. Scale bar, 50 μm. (D) Percentage of WT-like, slightly affected and severely affected phenotypes observed at 50 hpf in the facial branchiomotor neurons migration. dpysl2+/+ (n= 22), dpysl2-/- (n= 31) and dpysl2 mRNA injected (100 ng/μl) dpysl2-/- embryo exhibits a WT-like pattern; most of facial branchiomotor neurons are located at r6. Abnormal regions are indicated with arrows.

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In dpysl2-/- mutant embryos injected with 20 ng/ml of dpysl2 mRNA (n=11) and 31.3% of the ones injected with 20 ng/ml (n= 16) exhibited WT-like patterns, indicating a dose dependency. These results support a role of Dpysl2 in the migration of the facial branchiomotor neurons.

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Dpysl2 has a role in the migration of facial branchiomotor neurons during the development of zebrafish nervous system. During normal development, facial branchiomotor neurons migrate progressively from r4 and r5 to r6 and r7 and most of these neurons have reached their location in r6 and r7 by 36 hpf (Higashijima et al., 2000). We waited until 50 hpf to observe the dpysl2 KO mutant and dpysl2 AMO injected embryos, a time when most of facial branchiomotor neurons should be already at the caudal region of the facial nucleus. However, even at this time, we observed abnormalities in the positioning of these neurons.

When analyzing the migration process of the facial branchiomotor neurons some differences between mutants and morphants were observed. Previous reports expressed that a great number of morphant phenotypes were not observed when analyzing the corresponding mutants (Kok et al., 2015), but these discrepancies might be explained by different reasons. Dpysl2 morphant phenotypes were more severe and constant than the mutant ones.

Dpysl2KO mutants express variability in both the degree of expression of the phenotype and the pattern of the abnormalities observed. This variability could be dependent both on the level of penetrance of the mutation or on the degree of compensation by another genes. It has been reported previously that, in some cases, knocking out a gene will lead to the activation of a compensatory pathway and, as a result, other genes will be upregulated. The exact way this mechanism of adaptation to the cell environment works is not clear; however, it is not activated after an AMO induced knock-down and can be the cause of phenotypic differences between mutants and morphants (Rossi et al., 2015). Dpysl family members are expressed in similar regions throughout the nervous system and some of them accomplish similar roles. Previous studies have shown similar effects after knocking down dpysl2 (CRMP2) and dpysl3 (CRMP4) (Tanaka et al., 2012; Morimura et al., 2013) therefore, it is possible that dpysl3 or another dpysl family member is compensating the lack of dpysl2 in the KO mutants.

The phenotype induced by the injection of splicing blocking AMO was similar in all cases; most of cells were accumulated between r4 and r6 changing the usual distribution of the facial branchiomotor neurons (Fig. 3D). When the translation blocking AMO was injected, the phenotype observed was more severe (Fig. 3C), probably due to the higher efficiency inhibiting the production of Dpysl2 protein. Translation blocking AMOs bind to the post-spliced mRNA (both maternal and zygotic mRNA) and block the progression of the ribosomal initiation complex; splicing blocking AMOs block the proper processing of pre-mRNA (Eimon, 2014), they do not affect the maternal mRNA. The presence of maternal mRNA could mitigate the effects of the knock-down and this could explain why the phenotype induced by the splicing blocking AMO is less severe than the induced by the translation blocking AMO. The lack of a morphant phenotype after controlAMO injection supports the specificity of the effect of knocking down dpysl2; it is unlikely that the severe phenotype induced by the translation blocking AMO is due to an off-target effect. In the same way, the protein produced by the maternal WT mRNA could explain the WT-like phenotypes in the KO mutants generated by crossing heterozygous parents (Zimmer et al., 2019).

The phenotypes observed in dpysl2 KO mutants were more diverse than the morphant phenotypes; however, in both cases, we found abnormalities in the migration of the facial branchiomotor neurons. Therefore, our analysis let us assess that Dpysl2 has a role in the migration of the facial branchiomotor neurons during the

Discussion

The role of Dpysl family proteins in axonal growth and cell migration in the nervous system has been studied in different species. Dpysl2 role has also been studied in zebrafish to understand better its functions in vivo. However, here we used different means to show for the first time the involvement of this protein in the migration of the cranial branchiomotor neurons in zebrafish. We generated a dpysl2 KO line by using the CRISPR/Cas9 technology and also knocked it down using different types of morpholino oligonucleotides.

Our studies using both CRISPR/Cas9 system and AMOs show that...
Dpysl2 is required for FBM neuron migration

Materials and Methods

Animals
Zebrafish (Danio rerio) were maintained according to standard procedures (Westerdijk, 2000). Zebrafish embryos were incubated at 28.5 °C as indicated by Kimmel et al. (1995). To prevent pigmentation, 0.003%
1-phenyl-2-thiourea (PTU) was added to the fish water before 24 hours after the fertilization (Westerfield, 2000). RIKEN Wako (RW) wild type and Tg(isl1:GFP)rw0 transgenic zebrafish strains were obtained from the Zebrafish National BioResource Center of Japan (https://shigen.nig.ac.jp/zebra/). All experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at Waseda University (2017-A029, 2018-A005 and 2019-A027).

Generation of a dpysl2 KO mutant line by CRISPR/Cas9
To generate a dpysl2KO by using CRISPR/Cas9 system, we selected our target sequence as 5'-GGAGAAATCTAATGTGCC-3' and selected the oligonucleotides sequences, partial sequence of sgRNA, as follows. Oligo 1: 5'-TAGGAGAAAATCTAATGTGCC-3'; Oligo2: 5'-AAACGGCACTATTAGATTCT-3' (eurofins, Tokyo, Japan). These oligonucleotides were subcloned into DR247 plasmid, which was a gift from Keith Joung (Addgene plasmid # 42250; http://n2t.net/addgene:42250; RRID:Addgene.42250) (Hwang et al., 2013), and the sequence was confirmed. pT3TS-nCas9n was a gift from Wenbiao Chen (Addgene plasmid # 46757; http://n2t.net/addgene:46757; RRID:Addgene.46757) (Jao et al., 2013). sgRNA and Cas9 mRNA were synthesized using the mMessage mMachine Kit (Invitrogen by Thermo Fisher Scientific, Vilnius, Lithuania). This mRNA was injected at different concentrations into 1 to 2 cell stage mutant embryos as specified in the results.

Microscopy and imaging
For fluorescent microscopy a FV1000 confocal laser scanning microscope (Olympus) with UNPlanFL 20x (NA= 0.50) and LUMPlanFLN 40x (NA= 0.80) water immersion objectives was used. The images were processed using Adobe Photoshop and Adobe Illustrator.

Data analysis
We used ImageJ to measure the area of facial branchiomotor neurons occupying the regions from r4 to r5 and from r5 to r6. The sample was considered WT-like when the r5-r6 area was considerably bigger than r4-r5 (2.5 times or more); slightly affected when the region from r5 to r6 was between 1.5 and 2.4 times bigger than r4 to r5; and severely affected if r5-r6 had similar or smaller size than r4-r5 (less than 1.4 times bigger).

IBM SPSS Statistics 25 was used for statistical analysis. Chi-squared test was performed to determine whether the differences in frequencies between groups were statistically significant. When we compared the frequencies of slightly affected, severely affected and WT-like phenotypes between three groups, a P-value< 0.05 indicated significant differences were present. A post-hoc analysis was performed after the chi-squared test (Beasley and Schumacker, 1995); the z-scores were analyzed to determine a new specific P-value and determine which group was significantly different compared to the others. The new P-value is indicated in the corresponding figure.

Conflict of interest
The authors declare no conflict of interest.

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