Graduate School of Advanced Science and Engineering
Waseda University

博 士 論 文 概 要
Doctoral Thesis Synopsis

論 文 題 目
Thesis Theme

Study of the role of CRMP2 during the nervous system development in zebrafish

申 請 者
(Applicant Name)

Carolina	FIALLOS OLIVEROS
フィアヨス オリベロス	カロリナ

Department of Life Science & Medical Bioscience
Research on Molecular Brain Science

September 2019

Collapsin response mediator proteins (CRMPs) or dihydropyrimidinase-like (dpysls) family are proteins known for being involved in several processes during nervous system development such as axonal growth or cell migration. These proteins are evolutionarily conserved and studies in different species have shown their expression in several regions throughout the central and peripheral nervous systems. In zebrafish, CRMPs expression can be clearly observed already at 16 hours post fertilization (hpf) and it increases especially in the regions undergoing axonogenesis or neuronal differentiation. In addition, the high homology between zebrafish and human CRMPs together with the zebrafish features such as the transparency and fast development of the embryos makes it an organism of interest to better investigate how these proteins work.

The most studied CRMP family member is CRMP2, a protein that was originally identified as a mediator of the semaphorin signaling. In zebrafish, CRMP2 is expressed in several regions of the Central Nervous System, such as the telencephalon, clusters of hindbrain neurons, the retinal ganglion or the Rohon-Beard neurons in the spinal cord. Previous studies in Zebrafish have shown a requirement of CRMP2 for the proper positioning of neurons in the spinal cord and for the proper growth of the retinal axons. However, its functions need to be further analyzed.

This thesis focuses on the role of CRMP2 in the development of cranial motor neurons, a process that was not studied before. For this, the zebrafish mutant line Islet1-GFP was used. This transgenic line expresses Green Fluorescent Protein (GFP) in the cranial motor neurons, facilitating the direct visualization of the developmental process of these neurons. It is known that the neurons in the facial nucleus migrate caudally from rhombomers (r) 4 and 5 to 6 and 7 during early stages of development and that most of facial motor neurons are located at r6 by 36 hpf. It is also known that the trigeminal and facial axons start extending ventrally at 52 hpf, cross the midline at 58 hpf when they stop elongating for a few hours and complete their projections by 72 hpf. However, the mechanisms regulating these two processes still need to be elucidated. In this research, two well-known technologies such as Antisense Morpholino Oligonucleotides (AMO) and the CRISPR/Cas9 system were used with the aim of studying the involvement of CRMP2 in the migration and axonal elongation of the cranial motor neurons. In addition, by using these two methods, the possible differences between the knock-down and knock-out induced phenotypes were compared. This thesis consists of 5 chapters:

Chapter 1 describes the background, what is already known about CRMP family, the gene of interest (CRMP2) and some processes that take place in zebrafish such as facial neurons migration and trigeminal axon elongation. In Chapter 1, it is also explained how AMO and CRISPR/Cas9 technologies work and what their advantages are compared to other tools. In this chapter, the reasons

why this research is carried out and the objectives of this study are also indicated. As explained above, the main aim of this research was to study the role of CRMP2 in the development of the cranial motor neurons, more specifically, in the migration of the facial motor neurons and the elongation of the trigeminal axons. However, it was also intended to compare whether there were differences or not between the phenotypes induced by the knock-down and the knock-out of CRMP2.

Chapter 2 specifies all the materials and methods used in this project, including the zebrafish lines used and how a new CRMP2 KO mutant line was generated. To observe the KO phenotype, a CRMP2 KO mutant line had to be generated by CRISPR/Cas9 injection; only zebrafish carrying the same mutant sequence were selected to establish a specific mutant line and analyze the effects of the lack of CRMP2.

Chapter 3 shows the results obtained in this study using two different methods to analyze the role of CRMP2 during the development of zebrafish nervous system. AMOs were injected into zebrafish embryos to knock-down CRMP2 and the state of development of the cranial motor neurons was observed. For this, the transgenic zebrafish line Islet1-GFP was used. The cranial motor neurons of Islet1-GFP fish express GFP, enabling a direct visualization of their development. Knock-down induction of CRMP2 in this zebrafish line demonstrated a role of this protein in the migration of the facial motor neurons. The knock-down also indicated that this protein is required for the proper axon elongation of the posterior trigeminal motor neurons. At 50 hpf facial motor neurons should be located in the region corresponding to r 6 and 7; however, the facial neurons of the AMO injected embryos were still positioned along the facial nucleus with a greater density of neurons in r5. At 58 hpf, trigeminal axons are expected to be crossing the ventral midline; however, the morphants exhibited short axons, ending far from the crossing point. These observations indicate a role of CRMP2 in these processes.

On the other hand, the CRISPR/Cas9 technology was used to generate a CRMP2 KO ($CRMP2^{-/-}$) zebrafish mutant line to study the effects of the lack of CRMP2 in the development of the cranial motor neurons. At first, this line was used to confirm the results of previous studies that indicated a role of CRMP2 in the positioning of the caudal primary motor neurons in the spinal cord. Previous research showed that these neurons were ectopically located in the CRMP2 morphants; however, abnormalities were not clearly observed in $CRMP2^{-/-}$ embryos.

Following this analysis, the migration and axonal elongation of the cranial motor neurons in the mutants was also observed; again, taking advantage of the Islet1-GFP line. In this case, $CRMP2^{-/-}$ embryos exhibited defects in the facial motor neurons migration at 50 hpf and in the trigeminal axon

elongation at 58 hpf. The phenotypes found were different from the knock-down induced ones, more diverse and showing a different degree of penetrance. However, the KO phenotypes also supported the hypothesis that CRMP2 has a role in the proper migration and axon elongation of the cranial motor neurons. In addition, injection of CRMP2 mRNA in the mutant embryos rescued the phenotype in a good percentage of cases, supporting the idea that the absence of CRMP2 is causing the abnormalities in these two different processes.

Finally, DiI was injected into CRMP2 mutants to study the projection of retinal axons. Previous research indicated that CRMP2 morphants exhibited a clear reduction in the retinal axon growth. In this case, I observed thinner axons in many of the CRMP2$^{-/-}$ embryos; however, it was not a severe reduction as displayed in the previous research.

In Chapter 4 the results obtained are discussed. Although there is variability in the mutant phenotypes, the results support a role of CRMP2 in the facial motor neurons migration and trigeminal axons elongation. A percentage of CRMP2$^{+/+}$ embryos also exhibited some abnormalities, however, it is not likely that the phenotypes observed in the mutants are related to off-target effects or developmental defects. In addition, in a part of the mutant embryos, the mutant phenotype was rescued by CRMP2 mRNA injection, supporting the hypothesis. The differences between knock-down and knock-out induced phenotypes indicate the possible activation of the compensatory machinery or a maternal effect in the mutants; CRMP family members are expressed in similar regions and some carry out similar roles, therefore, it is likely that other CRMP family members could be taking the role of CRMP2 in the mutants. However, it is also possible that the mRNA from the heterozygous mother is mitigating the expression of the phenotype in the homozygous offspring.

At last, in Chapter 5 some suggestions about future research pathways are given, according to the observations during this study. Analyzing a different CRMP2 KO line to confirm in a different way that the phenotypes are due to the lack of CRMP2 and not to off-target effects or generating a CRMP double KO line to counteract possible compensation effects could help to clarify the function of CRMP2 in the development of cranial motor neurons and how this gene is working. In addition, during this study, some findings indicated a possible role of CRMP2 in the regeneration of the caudal fin in adult zebrafish. Many CRMP2 mutants exhibited abnormalities in the shape of their regenerated fins, while CRMP2$^{+/+}$ adults exhibited WT-like shapes. These observations are a good start point to perform further research about CRMP2 functions also outside the nervous system.

早稲田大学　博士（理学）　学位申請　研究業績書

(List of research achievements for application of doctorate (Dr. of Science), Waseda University)

氏 名　Carolina Fiallos Oliveros　　印

（As of June, 2020）

種 類 別 (By Type)	題名、　発表・発行掲載誌名、　発表・発行年月、　連名者（申請者含む） (theme, journal name, date & year of publication, name of authors inc. yourself)
Paper　○	"Dpysl2 (CRMP2) is required for the migration of facial branchiomotor neurons in developing zebrafish embryos". Carolina Fiallos Oliveros and Toshio Ohshima. The International Journal of Developmental Biology. *in press.* "Quantification of native mRNA dynamics in living neurons using fluorescence correlation spectroscopy and reduction-triggered fluorescent probes". Hirotaka Fujita, Ryota Oikawa, Mayu Hayakawa, Fumiaki Tomoike, Yasuaki Kimura, Hiroyuki Okuno, Yoshiki Hatashita, Carolina Fiallos Oliveros, Haruhiko Bito, Toshio Ohshima, Satoshi Tsuneda, Hiroshi Abe, and Takafumi Inoue. JBC. *in press.*
Poster presentation	Carolina Fiallos Oliveros and Toshio Ohshima "CRMP2 is required for the migration of facial branchiomotor neurons in developing zebrafish embryos". The 25th Japanese medaka and zebrafish meeting. Sept 4th, 2019