CHAPTER 5

General Discussion

In this PhD research I examined three aspects of the chemotaxis response to NaCl in the nematode *C. elegans*, which served as an ideal model for these studies. First, we investigate how the animal detects a cue (NaCl) from its environment. Second, we identify a mechanism that maintains stable cell identity and function of the neuron pair involved in detecting this NaCl cue. Finally, we attempt to identify differences in gene expression that can explain the variation in the animal's behavioral response.

In Chapter 2, we investigate how *C. elegans* uses a cGMP signaling compartment at the tip of the cilia of an amphid neuron pair to detect NaCl in its environment. We identify intraflagellar transport (IFT) as a core mechanism to form and maintain this compartment with a high concentration of guanylate cyclase receptor proteins. Insights gained from this study contribute to emerging insight in the regulation of similar sub-ciliary compartments in other systems, for example in hedgehog signaling in cells of higher vertebrates or cGMP signaling in photoreceptor cells.

In Chapter 3 we investigate the dynamics of a bistable genetic switch using the specification of the sensory neurons involved in detection of NaCl. When the function of a neuron depends on the continued expression of a terminal selector gene, stabilization of its mRNA expression is a major requirement to prevent loss of expression of its target genes. The transcription factor *che-1* is such a terminal selector gene. We find that CHE-1 forms a bistable genetic switch and uses a stabilization mechanism, named *target-reservoir buffering*, which involves preferential binding to its own promoter.

Finally, in Chapter 4 we correlate differences in gene expression between individuals with the outcome of our chemotaxis assay. Most animal behavior shows variation, allowing for flexibility in a dynamic, ever-changing environment. The consistency of the variation observed in the chemotaxis response of *C. elegans* suggests an underlying mechanism. Using *C. elegans* we can search for this mechanism in an isogenic population, eliminating genetic variability as a cause.

cGMP signaling compartment at the tip of a primary cilium

Signaling cascades that incorporate the cilium route can have their components localize to different sub-ciliary compartments. How these compartments are formed and maintained is not well understood (Blacque and Sanders, 2014). Given the importance of cilia in development and homeostasis and the implication of ciliary defects in a wide range of diseases (Nishimura et al., 2019; Reiter and Leroux, 2017), these compartments demand further investigation. In Chapter 2 we explore a cGMP signaling compartment at the tip of the primary cilium of a NaCl-sensing neuron in *C. elegans*.

The results obtained using *C. elegans* can help to better understand cilium biology. Primary cilia are highly conserved and their components like IFT, the microtubule-based axoneme, and the transition zone can be found throughout the tree of life (Carvalho-Santos et al., 2011; Mitchell, 2016). For example, proteins that are part of the BBSome (Nachury et al., 2007), named after the Bardet-Biedl Syndrome, are conserved between mammals and *C. elegans*, allowing us to study these proteins in animal models. Additionally, the family of MKS proteins, associated with the Meckel-Gruber Syndrome and involved in formation of the transition zone, perform a similar function in mammals and *C. elegans* (Garcia-Gonzalo et al., 2011; Williams et al., 2011). Moreover, sub-ciliary compartments can be found in cilia from other organisms than *C. elegans*. In mammals a cilium compartment at the tip is important in Hedgehog (Hh) signaling. Several members of this cascade concentrate there and it would be interesting to examine whether our results also apply to this Hh cilium tip compartment.

We identify IFT as the main driving force in the formation and maintenance of the tip compartment. We provide several lines of evidence: GCY-22 colocalizes with IFT particles moving along the cilium, chemical disruption of ATP production slows IFT and subsequently causes tip compartment collapse, and mutating IFT components interferes with tip compartment formation. Furthermore, mutating the BSSome subunit encoding *bbs-8* gene, involved in linking IFT cargo to the IFT-complexes, increases GCY-22 levels along the axoneme. Moreover, removal of both the transition zone component MKS-5 and the AKNMY2 homologue DAF-25 has a similar effect and prevents tip compartment formation. However, IFT seems to proceed normally, as visualized by tagging the kinesin OSM-3, showing that GCY-22 is no longer loaded as cargo. Taken together, when the link between GCY-22 and IFT is disrupted, its localization along the axoneme increases and tip formation is hindered.

IFT could also play an important role in sub-ciliary compartments of Hh signaling. Several studies identify the kinesin family member KIF7 as one of the regulators of Hh intracellular signaling in the cilium (He et al., 2017, 2014; Liem et al., 2009). However, it seems that the effects of *Kif7* mutations are indirect and that there might be other proteins that sequester the Hh signaling proteins SUFU of GLI2 at the tip. Mutations in *Kif7* alter the microtubule dynamics at the tip of the axoneme (Coles et al., 2015; Jiang et al., 2019), causing collapse of the cilium tip compartment. Likewise, in *C. elegans* as of yet unidentified proteins could facilitate docking of GCYs at the cilium tip. Our results from experiments with NaN_3, which show that an active process maintains the cilium tip compartment, could be seen in a similar light. When ATP production is stopped, IFT and other active processes in the cilium are affected, causing cilium shortening as well as tip compartment collapse.

To identify GCY-22 protein binding partners which might be involved in cilium tip compartment formation, two approaches can be taken. A genetic screen could be performed using high-throughput microcopy to identify candidate genes involved in the formation of the cilium tip. First, EMS could be used to randomly mutate *C. elegans* animals which subsequently can be screened, using a combination of microcopy and microfluidics, for animals with abnormal cilium tip localization of GCY-22::GFP. Only those with a promising mislocalization of GCY-22::GFP will then be allowed to produce progeny. Alternatively, immunoprecipitation of GCY-22::GFP, with or without crosslinking, can be performed to isolate the tip complex followed by mass spectrometry analysis to identify cilium tip components. So far, others have successfully isolated IFT complexes from the cilia of *C. elegans* showing that isolation of ciliary components is possible (Yi et al., 2017). However, these IFT complexes are present in all ciliated neurons whereas the GCY-22 tip compartment is only present in one neuron, ASER, potentially complicating this experiment. These approaches might identify possible binding partners of GCY-22 that allow it to accumulate at the cilium tip in high concentrations.

Similarly, immunoprecipitation experiments can be used to identify dimerization partners of GCY-22 protein. Preliminary results from an immunoprecipitation experiment on GCY-22::GFP, followed by mass-spectrometry, showed strong hits for three GCYs (Rademakers & Jansen, unpublished results), suggesting that GCY-22 forms heterodimers with other ASER-expressed GCYs. However, further biochemical experiments are required to identify GCY-22 partners and determine if these homo/hetero-complexes are the Cl^- receptor in ASER. It would be interesting to see where these and other ASER-expressed GCYs localize in the cilium, at the cilium tip (like GCY-22 and GCY-14; Ou et al., 2007), or along the length of the cilium (like DAF-11 in the ASI neurons; Jensen et al., 2010). Strong candidates to start with would be GCY-19 (expressed in ASER only) or GCY-4 (expressed in both ASEL/R). However, given the possibility to endogenously tag proteins easily using CRISPR/Cas9, each of the respective 7 ASER-expressed GCYs should be investigated.

When the GCY-22-filled cilium tip compartment is not properly formed or maintained, chemotaxis to NaCl is reduced, with the chemotaxis towards low concentrations affected most. We estimate that the density of GCY-22 at the tip is at least comparable to, and maybe even higher than, the density of rhodopsin in the photoreceptor cells in the mammalian eye (106,571 ±31,016 molecules/μm^2 and 48,300 molecules/μm^2, respectively). These results suggest that the cGMP signaling compartment at the tip of the ASER cilium is a sensitive Cl^- detector.

Still, whether GCY-22 is the direct Cl^- receptor of the ASER neuron remains an open question. So far, genetic studies indicate that GCY-14 and GCY-22 confer specificity for NaCl in the ASE neurons (Ortiz et al., 2009, 2006; Smith et al., 2013). Mutating GCY-22 affects chemotaxis to multiple compounds, although the strongest phenotype is in chemotaxis to NaCl (Ortiz et al., 2009). Others have suggested that a homodimer of GCY-22 is the receptor for Cl^- and that GCY-22 heterodimers with other GCYs function in chemotaxis to other salts (Ortiz et al. 2009; Smith et al. 2013). Furthermore, GCY-14 and GCY-22 contain all the necessary amino acids to function as homodimers (Murayama et al., 2013), making a functional GCY-22 homodimer possible.

We attempted to show that GCY-22 is capable of detecting NaCl and conferring NaCl the detecting capability to a ciliated neuron. For this, we ectopically expressed GCY-22 in the ASI neurons in animals lacking functional ASE neurons. These animals show no chemotaxis to NaCl, suggesting GCY-22 is not sufficient to detect NaCl. However, the downstream signaling pathway or neuronal circuits might not be able to facilitate such a response. Instead of using chemotaxis assays, cGMP or Ca^{2+} imaging of the ASI neurons can be used to determine whether ectopically expressed GCY-22 is sufficient to elicit a response to NaCl in these cells. Alternatively, ectopic expression of GCY-22 in *Xenopus* oocytes could show that the receptor produces cGMP in response to a NaCl stimulus. Finally, crystallography could be used to show whether the GCY-22 guanylate-cyclase domain changes to an active conformation in presence of NaCl.

Taken together, we identified a cGMP signaling compartment at the tip of a cilium that is formed and maintained by IFT. We found the guanylate cyclase GCY-22 to be present in the tip compartment at a high density and show that its localization and density are required for sensitive detection of NaCl.

Stabilizing mechanisms of the CHE-1 genetic switch

The tissues of a multicellular organism are continuously refreshed by removing old cells, e.g. by apoptosis, and replacing them with a steady supply of new cells from a few tissue-specific progenitor cells. This turnover rate varies between different tissues and cell types, with some tissues showing hardly any turnover at all. A study using ^{14}C dating to determine the age of a given cell compared to the birthdate of the individual, found that the average age of cells in the intercostal muscles, the muscles in between the ribs, is 15.1 years (Spalding et al., 2005). A slow turnover rate compared to rates of 2-10 days that have been reported for epithelial cells in the gastrointestinal tract (Darwich et al., 2014; Spalding et al., 2005). Most neuronal cell types in the undamaged central nervous system of humans do not turnover at all, i.e. an individual will use a particular neuron for its entire lifetime (Spalding et al., 2005). A consequence of this stability is that these cells need to maintain their proper function, or cell identity, for decades.

Terminal selector genes have been identified as the final step in neuronal cell type determination in various organisms (Gabilondo et al., 2016; Hirota and Mombaerts, 2004; Serrano-Saiz et al., 2018). Terminal selectors can form bistable switches, for example by autoinducing their own expression. In a bistable switch both the high expression and low/no expression states are stably maintained and an external factor or ‘trigger’ is required to switch between states. In the high expression state, a bistable switch formed by a terminal selector induces the expression of numerous target genes that give a cell its identity and function. One of the key weaknesses of a bistable switch is that it can spontaneously switch off due to molecular noise, such as variation in the expression of a core transcription factor

(Acar et al., 2005; Ozbudak et al., 2004). The transcription factor CHE-1 is a terminal selector that forms a bistable switch and induces the expression of the numerous target genes that give the ASE neurons their function.

Bistable switches can cope with molecular noise by increasing the levels and lifetimes of their components, i.e. increasing copy numbers and mRNA stability or protein concentration and stability of the factors involved (Mehta et al., 2007; Walczak et al., 2005). However, we found no indication that these strategies apply to the CHE-1 genetic switch. Instead, we found that the transcription factor CHE-1 uses a mechanism we call *target reservoir buffering* to stabilize its own expression and thereby stabilize the switch. This mechanism relies on the preferential binding of CHE-1 to its own promoter, ensuring that expression of the *che-1* gene can recover when the CHE-1 concentration drops due to molecular noise. CHE-1's target genes will be affected first when CHE-1 levels become insufficient to sustain transcription of both *che-1* and its target genes. However, *che-1* expression will be maintained due to the preferential binding of CHE-1 to its own promoter. In a way, the CHE-1 pool at the target promoters acts as a buffer for the *che-1* promoter. This mechanism ensures *che-1* expression for as long as possible, allowing *che-1* expression to recover and return to the high expression state.

When this mechanism is in effect, short-lived interruptions of the expression of the CHE-1 target genes might occur and affect ASE function. To bridge these interruptions, the stability of their gene products could maintain ASE function. Protein stability of chemoreceptors and downstream signaling proteins could allow the CHE-1 genetic switch to recover from short periods of low CHE-1 levels. Indeed, we found a delay between the depletion of CHE-1 and loss of chemotaxis, which can be attributed to the stability of the signaling proteins involved in ASE mediated chemotaxis. In Chapter 2 we showed that the pools of GCY-22 at the tip of the cilium and its base are very stable. Additionally, others have shown similar results for the cyclic nucleotide gated channel subunits TAX-2 and TAX-4 in the cilia of AWB and ASK neurons (Wojtyniak et al., 2013). If the products of the CHE-1 target genes are stable enough, ASE function can be maintained for short periods until the CHE-1 switch recovers.

We show that the stability of *che-1* expression depends on an *Otx*-related binding motif in the *che-1* promoter. Mutating this motif leads to stochastic loss of *che-1* expression, spontaneous transition to the OFF state of the genetic switch and loss of ASE function. However, a substantial chemotaxis response can be seen in populations of this mutant, suggesting that in some animals the CHE-1 switch can still maintain ASE function.

Otx-related genes are involved in development of the anterior nervous system in a wide range of species (Germot et al., 2001; Jin et al., 1994; Semina et al., 1996; Simeone et al., 2002). Three *Otx*-related genes (*ttx-1*, *ceh-36*, *ceh-37*) have been identified in *C. elegans* which are involved in the development of the AWC, AWB, AFD and the bilateral asymmetry of the ASE neurons (Chang et al., 2003; Lanjuin et al., 2003; Satterlee et al., 2001). Loss-of-function of *ceh-36*, the only *Otx*-related gene expressed in ASE, results in ASEL/R asymmetry defects and loss of chemotaxis to NaCl. However, *che-1* and *gcy-22* are still expressed, indicating that *ceh-36* is not essential in stabilizing the genetic switch (Traets & Van Zon, unpublished results).

A stabilizing function of a homeodomain-containing protein has been described in the differentiation of *C. elegans* touch receptor neurons (TRNs) (Topalidou et al., 2011; Zheng et al., 2015). Here, expression of the LIM-type homeodomain gene *mec-3* is stabilized by binding of the homeodomain protein ALR-1 to the *mec-3* promoter. To find the homeodomain-containing proteins that play a similar role in stabilization of *che-1* expression, proteomics of isolated chromatin segments (PICh) or chromatin immunoprecipitation with mass spectrometry (ChIP-MS) experiments can be performed (Déjardin and Kingston, 2009; Wierer and Mann, 2016). These methods can be used to isolate the *che-1* promoter region, containing the ASE and *Otx*-related motifs, and proteins bound there and identify any factors that stabilize *che-1* expression.

We rely on the loss of function of the ASER neuron to infer loss of cell identity. When the CHE-1 target genes are no longer expressed, the ASE neurons lose their capacity to detect salts. If these cells lose their function and ASE-specific gene expression, one can argue that they are no longer ASE neurons. However, we did not explore what kind of cell they become when *che-1* expression is lost.

In the lineage of the ASER neuron, the daughter cells of the precursor cell (cell ABpraaappp) differentiate into an ASER and ASJR neuron. Future studies could investigate if the CHE-1 depleted cells gain ASJR specific traits. Except for the ASE and ASG neurons, ciliated amphid neurons are capable of taking up a fluorescent dye through their cilia. However, in *che-1* loss-of-function and CHE-1 depleted animals, the 'ASE' neurons show dye-filling (Uchida et al., 2003), suggesting these cells gained neuronal features that are more broadly shared among the amphid neurons. Whether these cells also express early neuronal markers, such as the bHLH co-factor hlh-2, remains to be seen. Further experiments could try to steer CHE-1 depleted cells to an ASJ cell-type. The ASJ cell-type is determined by the *sptf-1* terminal selector gene and perhaps overexpression of this transcription factor could induce ASJ specification of the CHE-1 depleted cells (González-Barrios et al., 2015). If successful, this experiment would suggest that the CHE-1 depleted cell regained the ability to differentiate into different neuronal cell-types. Alternatively, the terminal selector *ttx-1* could be overexpressed to differentiate into an AFD cell fate (Satterlee et al., 2001; Serrano-Saiz et al., 2013a), the final cell type in a more distant lineage branch. It would be interesting to see if these cells acquired the thermosensory function of the AFD neurons, or even develop fingered cilia.

However, mechanisms exist that could prevent such a transition. In wild-type animals, when one terminal selector gene is expressed, cellular plasticity is restricted by chromatin remodeling, preventing other genetic switches from inducing their target genes (Patel and Hobert, 2017). Preventing a repressed chromatin state, by knock-down of the histone chaperone *lin-53* or other members of the Polycomb repressor complex 2 (PRC2), and subsequent ectopic expression of *che-1* allows conversion of germ cells into neurons (Patel et al., 2012; Tursun et al., 2011). Using a similar knock-down approach, reversing ASE cell fate and steering it to another ciliated amphid neuron might be possible.

We identified a minimal model for maintaining the expression of a terminal selector and neuronal cell fate. Several neuronal subtypes in mammals also depend on the continuous expression of specific transcription factors. Dopaminergic neurons in the olfactory bulb in mice depend on continuous presence of PAX6, a homeobox transcription factor, which induces crystallin αA expression (Ninkovic et al., 2010), and dopaminergic neurons in the central nervous system require continued expression of the LIM homeobox gene *Lhx1* (Serrano-Saiz et al., 2013b). It remains to be documented how the expression of these factors is stably maintained over the lifetime of these cells.

Behavioral variation

The behavioral response of an animal is subject to external factors, e.g. climate, time of day, danger from predators, and its internal state, determined by hormone levels or genetic makeup. Variation in these factors will change the behavioral response of an animal but in addition these responses can vary between individuals. Such differences can be attributed to genetic variation, but behavioral variation is observed even between isogenic individuals, for instance in cloned piglets (Archer et al., 2003; Søndergaard et al., 2012). In Chapter 4 we explored molecular sources of variation in the chemotaxis response between individuals of a *C. elegans* population.

Using single-worm RNA-sequencing on animals from our quadrant chemotaxis assay, we determined differences in gene expression between animals that moved to salt-containing and to no-salt-containing quadrants. Using unsupervised clustering analysis, we were unable to identify possible causative differences in gene expression profiles between the two groups. Supervised clustering did identify several differentially expressed genes, however, most of these were expressed at very low levels and only some could be validated using RT-qPCR.

We used a quadrant chemotaxis assay to analyze the response of *C. elegans* to NaCl (Jansen et al., 1999; Wicks et al., 2000). In this assay, animals are placed on a sectional petri dish with 4 quadrants, two of which contain an attractive concentration of NaCl. Animals are allowed to move over the plate for 10 minutes, after which most animals (~90%) can be found on NaCl containing quadrants. Among the animals that end on quadrants without NaCl, two responses can be identified (personal observation). Some move to these quadrants immediately after the start of the experiment, while others first move to the NaCl containing quadrants but move to the other quadrants during the assay. This indicates that there are multiple behaviors in our assay.

For the animals that initially move to the quadrants without salt various causes can be hypothesized. Variation in the presence or phosphorylation status of signaling molecules in the chemotaxis pathway might cause temporary detection inefficiencies. Variation in the function of the interneurons or downstream motor circuit might play a role as well. Animals that switch quadrants during the assay can do so for yet other reasons. For instance, desensitization could occur more quickly in some animals than in others. Alternatively, some animals might start to associate NaCl with the absence of food during the chemotaxis assay. Experiments on gustatory plasticity experiments show that *C. elegans* avoids NaCl when its presence is associated with the absence of food (Hukema et al., 2006; Jansen et al., 2002), and such an effect might also play a role here.

We were unable to identify differences in gene expression using unsupervised analysis. This can be attributed to several issues. First, the genes with causative variation in their expression might only be expressed in one or a few cells, for example in the ASE neurons or one of the interneurons involved in the chemotaxis response. Given that the adult hermaphrodite comprises precisely 959 somatic cells (making *C. elegans* an eutelic organism), this would result in an unfavorable signal-to-noise ratio. Several studies have shown that variation in behavior can indeed be caused by single or a few cells in a neuronal circuit (Gordus et al., 2015; Iwanir et al., 2019). Similarly, expression of a gene could vary only in a few cells and show steady expression in other neurons or even in most of the animal. Presumably, any differences in expression would be evened out and render our analysis ineffective in identifying any expression differences. Second, not every animal ended on a quadrant without NaCl for the same reason. Unsupervised clustering would be unable to identify clusters of animals if they did not share differentially expressed genes.

Supervised clustering, where the analysis takes into account to which quadrant the animal moved, did identify some genes that were differentially expressed between animals from NaCl or no NaCl-containing quadrants. However, with 20,470 protein coding genes and ~16,000 RNA genes, it is not unreasonable to expect that some genes by chance behaved in the way we set out to find, e.g. high expression in animals on salt quadrants and low expression in one or few animals on the quadrants without salt.

Two methods could have improved our search for a gene with causative variation in its expression. First, we could have narrowed down our search to a subset of the 959 somatic cells. For example, to identify neurons involved in the behavioral variation, cGMP or Ca^{2+} imaging in freely roaming animals on a chemotaxis assay can be used (Fang-Yen et al., 2015; Luo et al., 2014). Although whole brain imaging of *C. elegans* is possible (Nguyen et al., 2015; Yemini et al., 2019), doing so in freely moving animals might prove difficult when looking for variation in the cellular response. Instead, we can image chemosensory neurons, interneurons, or neurons from the downstream motor circuit separately and analyze their responses in animals on our quadrant assay. This approach could identify differences in the neuronal response between animals that moved to a quadrant with NaCl and those that did not. To improve the clustering analysis, we can subsequently analyze a subset of the genes from our single worm RNAseq data, focusing only on genes expressed in the differentially responding neurons.

Second, a gradient assay could be used in addition to the quadrant chemotaxis assay. In a gradient assay, a shallow gradient over a longer distance can be accomplished, e.g. a gradient of 0-50 mM NaCl over 25 cm, and used to test the chemotaxis behavior of *C. elegans*. Such an assay can be used to select animals from the extreme ends of the spectrum of the chemotaxis response. For instance, when started at the middle of a gradient, some animals migrate further up the gradient than others while some move down the gradient (Luo et al., 2014). By selecting animals from the far ends of the gradient, extremes in the variation can be selected and a possible shared mechanism could be identified.

In conclusion, I discussed three studies which explored various aspects of the chemotaxis response of *C. elegans*. We showed how the nematode maintains the function of its main NaCl detecting neuron pair through stabilizing the expression of a terminal selector gene. We showed how a cGMP sub-compartment at the tip of the cilia of this neuron pair is formed and functions as a sensitive detector of NaCl. Finally, we attempted to correlate variation in gene expression with differences between the chemotaxis response of individual animals.

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